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DISSERTATION

In vitro Analysis of the Functional Differences between Proteins of the ADF/Cofilin Family

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2001

COLORADO STATE UNIVERSITY

April 11, 2001

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HUI CHEN ENTITLED *IN VITRO* ANALYSIS OF THE FUNCTIONAL DIFFERENCES BETWEEN PROTEINS OF THE ADF/COFILIN FAMILY BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Advisor

Department Head

ABSTRACT OF DISSERTATION

In vitro Analysis of the Functional Differences between Proteins of the ADF/Cofilin Family

The actin depolymerizing factor (ADF)/cofilins (AC) are an essential group of actin filament depolymerizing and severing proteins that regulate actin filament turnover *in vivo*. In metazoans, two or more members are often expressed in the same cell. Each member has qualitatively similar interactions with actin, but differences have been reported in the regulation of their expression that suggests they may have different functions. We compared the interaction of chick ADF and chick cofilin with chick actin. The critical concentration of ADF-actin (6 μM) is much higher than that of cofilin-actin (0.8 μM) and actin alone (0.13 μM). The apparent dissociation constant of MgATP-actin and ADF measured by non-denaturing polyacrylamide gel electrophoresis is 1 μM, 10-fold lower than for cofilin. Cofilin promotes filament assembly whereas ADF does not. These data suggest that ADF is likely to be a sequestering protein for ATP-G-actin *in vivo*. Long filaments predominate at steady state in samples containing cofilin, whereas short filaments predominate in samples containing ADF, demonstrating that ADF severs and/or depolymerizes filaments to a greater extent. ADF severs and depolymerizes filaments from the pointed end faster than cofilin and ADF activity is pH-dependent.

Severing is favored at pH 7.8 whereas enhancement of off-rate is favored at pH 6.8. ACs from other species behaved either like ADF or cofilin based on their interactions with G-actin and F-actin. The chick ADF-like group includes *Xenopus* XAC, and starfish depactin. The chick cofilin-like group includes *Arabidopsis* ADF1, *Caenorhabditis* UNC-60B, *Drosophila* twinstar, *Dictyostelium* cofilin, and *Acanthamoeba* actophorin. Therefore, ADF and cofilin differ quantitatively in their interaction with actin. When actin monomer concentration is between the critical concentration of ADF-actin and cofilin-actin, ADF will depolymerize filaments whereas cofilin will promote filament assembly. These findings explain the different effects observed when ADF and cofilin are overexpressed in cells.

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ACKNOWLEDGEMENTS

First, I'd like to thank my mentor, Dr. James R. Bamburg, for his inspiration and consistent support from various aspects not just limited to technical problems or my language barrier. His enthusiasm for science has deeply influenced me. He has allowed me to work independently, but always has an open door when guidance is needed. Laurie Minamide, our lab mom and my first lab technique teacher, has taught me many basic biochemical techniques. Judy Sneider purified many AC proteins for these studies. Dr. Barbara Bernstein has inspired my thoughts and helped me with light microscopy. Dr. Judy Boyle taught me negative staining and help me with electron microscopy. Dr. Kyoko Okada had many helpful discussions with me. I thank them for their patience and friendship. I also thank Dr. Peter Meberg, Dr. William Painter, Dr. Mike Brown, Todd Verastro, Dr. Tom Kuhn, Dr. Brian Agnew, Elizabeth Soda, and Dr. Ravine Gungabissoon. Working in a laboratory full of nice, generous, and humorous people has made my graduate years very rewarding and enjoyable. I also thank the members of my graduate committee: Drs. Douglas Ishii, Jennifer Nyborg, Kevin Lumb, Paul Laybourn, and Christine Wilcox.

I'd like to thank my family. My father, Yongnan Chen, inspired my love for biomedical sciences in my youth. My mother, Shuchun Wang, always encouraged me and gave me a hand when help was needed. My husband, Xin Lin, has given his support through the years for my second doctor's degree.

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Abp1	actin binding protein 1
AC	actin depolymerizing factor/cofilin
ADF	actin depolymerizing factor
ADP	adenosine diphosphate
ADP·Pi	hydrolyzed adenosine triphosphate
Aip1	actin interacting protein 1
Arp2/3	actin related protein 2/3
ATP	adenosine triphosphate
Cc	critical concentration
Cs	steady state concentration
DBP	vitamin D binding protein, also called group specific component
EM	electron microscopy
F-actin	filamentous actin
G-actin	globular actin (monomeric actin)
JAS	jasplakinolide
K_D	dissociation constant
$K_{D\ app}$	apparent dissociation constant
Lat A	latrunculin A
PAGE	polyacrylamide gel electrophoresis
Pi	phosphate
PtdIns(4,5)P_2	phosphotidlyinositol-4,5-bisphosphate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WASp	Wiscott-Aldrich syndrome protein

Chapter One

Introduction

Regulating actin filament dynamics *in vivo*

Preface and Acknowledgement:

The introduction presented in this chapter was revised and updated from a review published in Trends in Biochemical Sciences (Chen *et al.*, 2000). The order and list of authors is as follows: Hui Chen, Barbara W. Bernstein, and James R. Bamburg. Recent reviews have been cited in place of many original references. We would like to thank Drs. Tom Pollard, Laurent Blanchoin and Kyoko Okada for many helpful discussions, and the NIH (grants GM35126 and GM54004 to JRB) and the March of Dimes Birth Defects Foundation (6-FY98-92 to BWB) for their support.

Summary

The assembly and disassembly (*i.e.*, turnover) of actin filaments in response to extracellular signals underlie a wide variety of basic cellular processes such as cell division, endocytosis and motility. The bulk turnover of subunits is 100-200 times faster in cells than it is with pure actin, suggesting a complex regulation *in vivo*. Significant progress has been made recently in identifying and clarifying the roles of several cellular proteins that coordinately regulate this turnover.

The dynamic nature of actin filaments allows cells to respond to extracellular signals by moving, changing shape, and translocating intracellular organelles. Such processes are critical to the development and function of a multicellular organism, but are also important in the disease state, for example the ability of a tumor cell to become metastatic. A number of proteins are involved in modulating actin dynamics, among them being proteins in the actin depolymerizing factor (ADF)/cofilin (AC) family. The regulation and function of AC proteins must be integrated with the activity of actin interacting protein-1 (Aip1), profilin, β–thymosin, gelsolin, tropomyosin, and capping proteins (including the actin-related protein, Arp2/3 complex) to control processes such as lamellipodial extension.

Actin dynamics

Actin is a major constituent of the cytoskeleton of almost all eukaryotic cells. Actin exists either in a monomeric form, G-actin, or in a filamentous form, F-actin. Each actin subunit binds to either ATP, ADP·Pi, or ADP. The minimal concentration of actin required for assembly (called the critical concentration, Cc) is lower for ATP-actin than for ADP-actin (Carlier & Pantaloni, 1997). Each filament end has a Cc. At the Cc, the rate of subunit addition to the end of filament equals the rate of subunit dissociation from the same end. Net polymerization occurs when the G-actin concentration is higher than Cc, and net depolymerization occurs when the G-actin concentration is lower than Cc. The major difference between ATP-actin and ADP-actin is in the behavior of the polymer. In the absence of nucleotide hydrolysis, polymers behave as equilibrium polymers with identical Cc's for both ends.

However, actin filaments are non-equilibrium polymers since nucleotide hydrolysis occurs in the filament. This hydrolysis lags behind the assembly. Filaments have a pointed end (an arrowhead structure seen in filaments decorated with myosin fragments), which is the slow growing (minus) end with higher Cc (Cc^-). The barbed-end of the filament is the fast growing (plus) end with lower Cc (Cc^+). At steady state (Fig. 1.1a), the G-actin concentration (also called the steady state concentration, Cs) is such that a net assembly of subunits at the plus end equals the net disassembly at the minus end, a process called treadmilling (Wegner, 1982). Filament length and number are relatively constant. The filament turnover cycle is thought to consist of the addition of ATP-monomer to the barbed end, hydrolysis of ATP within the incorporated subunit, release of P_i into solution, dissociation of the ADP-monomer from the pointed end, and exchange of ATP for ADP on the monomer (Fig. 1.1b). *In vivo*, ATP-actin is the predominant form in the monomer pool (Rosenblatt *et al.*, 1995), whereas ADP-actin, and under conditions of rapid assembly perhaps ADP-P_i-actin (Maciver *et al.*, 1991), are the major internal subunits of F-actin.

The motile events of non-muscle cells involve active, precisely controlled reorganization of the actin filament network. Filament turnover is 100-200 fold faster in cells than for pure actin *in vitro* (Zigmond, 1993). Since actin filaments gain and lose their subunits only at their ends, this enhanced turnover could arise from an alteration in subunit on/off rates, increase in number of free filament ends, or both. The rapid turnover cannot be accomplished without proteins that regulate actin assembly. These proteins have a variety of activities. Some sequester actin monomers to prevent spontaneous nucleation of filaments (β-thymosin) or interact with actin monomers to enhance

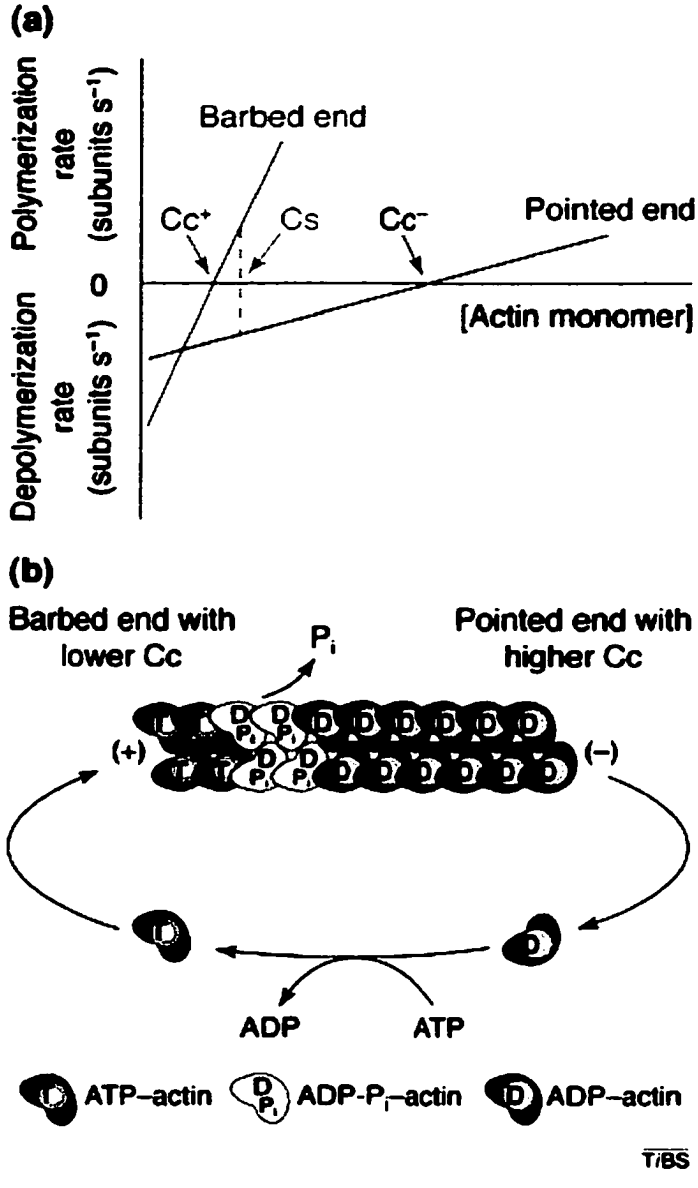


Figure 1.1
The turnover of an actin filament. **A.** The actin filament is a polar structure with two different ends. The slow growing pointed end has a higher critical concentration (Cc) than the fast growing barbed end. At steady state, the net assembly at the barbed end equals the net disassembly at the pointed end (treadmilling). The G-actin concentration under these conditions is called steady state concentration (Cs). **B.** The filament turnover at steady state involves a sequence of actin assembly, ATP hydrolysis, P_i release, filament disassembly, and nucleotide exchange. For more detailed discussion, refer to section on **Actin Dynamics** on page 2. (For a more detailed kinetic analysis see Carlier and Pantaloni, 1997).

nucleotide exchange (profilin). Some sever F-actin to generate more filament ends for assembly and/or disassembly (e.g., AC proteins, gelsolin). Others cap filament ends to regulate addition or loss of actin subunits (e.g., capping protein, gelsolin, Arp2/3 complex), to nucleate filament growth (e.g., Arp2/3 complex, spectrin:actin:protein 4.1 complex), or to enhance subunit dissociation by AC proteins.

How ADF/cofilin proteins modulate actin filament dynamics

AC proteins are an essential group of actin-binding proteins ubiquitous among eukaryotes. Their highly complex regulation allows them to modulate with spatial and temporal precision the filament turnover needed for many actin-based processes in non-muscle cells. A growing body of evidence supports the importance of AC in actin turnover in cells: 1) AC proteins are localized to cell regions with highly dynamic actin filaments (Moon *et al.*, 1993; Nagaoka *et al.*, 1995a); 2) AC proteins increase the turnover rate of actin in the comet tail of *Listeria monocytogenes* (Carlier *et al.*, 1997; Rosenblatt *et al.*, 1997); and 3) unlike higher eukaryotes, which express two or three different AC proteins, yeast express only one; mutations that reduce its activity cause defects in filament disassembly (Lappalainen & Drubin, 1997).

Many AC proteins bind to ADP-G-actin with about 30 to 80 fold higher affinity than they bind to ATP-G-actin at physiological ionic strength (Blanchoin & Pollard, 1998; Carlier *et al.*, 1997; Ressad *et al.*, 1998). AC proteins bind monomeric actin in a 1:1 complex and inhibit nucleotide exchange (Hayden *et al.*, 1993; Nishida, 1985). With the exception of AC from yeast and *Dictyostelium*, the activity of AC is inhibited by phosphorylation at Ser3 (Agnew *et al.*, 1995) or its equivalent Ser2 in amoeba (Blanchoin

et al., 2000b) or Ser6 in plants (Smertenko *et al.*, 1998). In metazoans, ACs are the only known substrates for LIM kinases (Arber *et al.*, 1998; Yang *et al.*, 1998), two forms of which are differentially regulated by members of the rho family of GTPases (Sumi *et al.*, 1999). Both ubiquitous and specific phosphatases are involved in dephosphorylation of AC (Meberg *et al.*, 1998). AC proteins are also inhibited by PtdIns(4,5)P_2 binding (Yonezawa *et al.*, 1990), pH < 7.0 (Hayden *et al.*, 1993; Hawkins *et al.*, 1993), and tropomyosin-saturation of F-actin (Bernstein & Bamburg, 1982).

With the exception of the AC protein from amoeba, actophorin, binding to amoeba F-actin (Blanchoin & Pollard, 1999), AC proteins bind F-actin in a cooperative manner (Hawkins *et al.*, 1993; Hayden *et al.*, 1993). Binding of AC to ADP-actin is of higher affinity than binding to ADP-Pi- or ATP-actin (Carlier *et al.*, 1997; Maciver *et al.*, 1998), suggesting that the change in conformation of F-actin, which follows P_i release (Belmont *et al.*, 1999), enhances AC binding. F-actin has a major helical twisted conformation with a crossover every 35 nm. F-actin also has a minor unstable twisted conformation with a crossover every 27 nm. The binding of AC to the high affinity site on F-actin stabilizes the minor twisted conformation (Galkin *et al.*, 2001). The binding of AC to a second low affinity site on actin might weaken the actin-actin contacts in the filament and thus might cause filament severing. The AC-stabilized twist also eliminates the phalloidin-binding site on F-actin so that AC-saturated F-actin does not stain with fluorescent phalloidin.

The mechanism by which AC proteins enhance filament dynamics has been somewhat controversial. In early studies of AC action, a weak (non-stoichiometric) filament severing activity was detected (Hayden *et al.*, 1993; Nishida *et al.*, 1984;

Maciver *et al.*, 1991) suggesting that one mechanism for enhanced depolymerization was the generation of additional filament ends. The concentration of filament ends reached a plateau and the number of filaments eventually declined (Hayden *et al.*, 1993), suggesting reannealing or redistribution of subunits has occurred. However, Carlier *et al* (1997) reported that recombinant AC proteins enhanced both the association rate (up to 12 fold) at the barbed ends of filaments and the dissociation rate (up to 22 fold) at the pointed ends in the apparent absence of any filament severing activity. Other workers reported less of an effect on subunit off-rates and more severing activity with different AC proteins (Maciver *et al.*, 1998; Blanchoin & Pollard, 1999; Moriyama & Yahara, 1999). Du and Frieden (1998) interpreted their actin assembly kinetics in the presence of yeast cofilin completely in terms of severing, although other models could not be excluded. Some of these differences in AC activity could be due to a weaker severing activity of recombinant proteins compared to tissue-derived proteins (Ichetovkin *et al.*, 2000). Moriyama and Yahara (1999) developed an assay that looked at both filament numbers (by trapping with a barbed-end capping complex) and rates of subunit loss. They found that porcine cofilin increased filament numbers by severing filaments with a maximum of about one severing event per 290 actin subunits (about 0.8 μm in length). Subunit off-rates were also enhanced to a maximum of about 6.4 fold. Mutant forms of cofilin were identified that differentially affected severing or enhanced the off-rate at the pointed end. Yeast cells expressing mutant cofilin with defects in severing are more impaired in their growth than those expressing mutant cofilins with defects in depolymerization, suggesting that the ability of AC to sever is an essential process and independent of depolymerization. Taken together, a current model for generic AC

activity (Fig. 1.2) suggests that severing occurs, probably requiring saturation of regions of an actin filament with AC, and that subunit loss at the pointed end of the filament is also enhanced. Severing would occur most likely at the junction between AC-saturated and naked F-actin and might only occur on relatively long filaments (>1 μm) which may tend to fragment from thermal motion.

Another important consideration for the regulation of filament turnover in cells is the possible tight coupling of cycles of AC phosphorylation and dephosphorylation (phosphocycling) to filament turnover. Under *in vivo* conditions thought to enhance filament turnover, levels of phosphorylated and dephosphorylated AC often do not change, but the half-life of the phosphate group on AC decreases significantly (Meberg *et al.*, 1998). Filament turnover could be linked to AC-phosphocycling by the mechanism shown in Fig. 1.2. Nucleotide exchange can be a rate-limiting step in reassembly. AC inhibits nucleotide exchange on the ADP-actin that dissociates from pointed ends. Transient phosphorylation of AC that dissociates from this complex will allow the free ADP-actin to exchange nucleotide. Removal of the phosphate from AC then reactivates it for another round of subunit removal. Thus, phosphocycling of AC could help drive filament dynamics, especially if the kinase and phosphatase are spatially separated. Within cells, other proteins, particularly profilin, contribute to these regulatory activities (Blanchoin & Pollard, 1998).

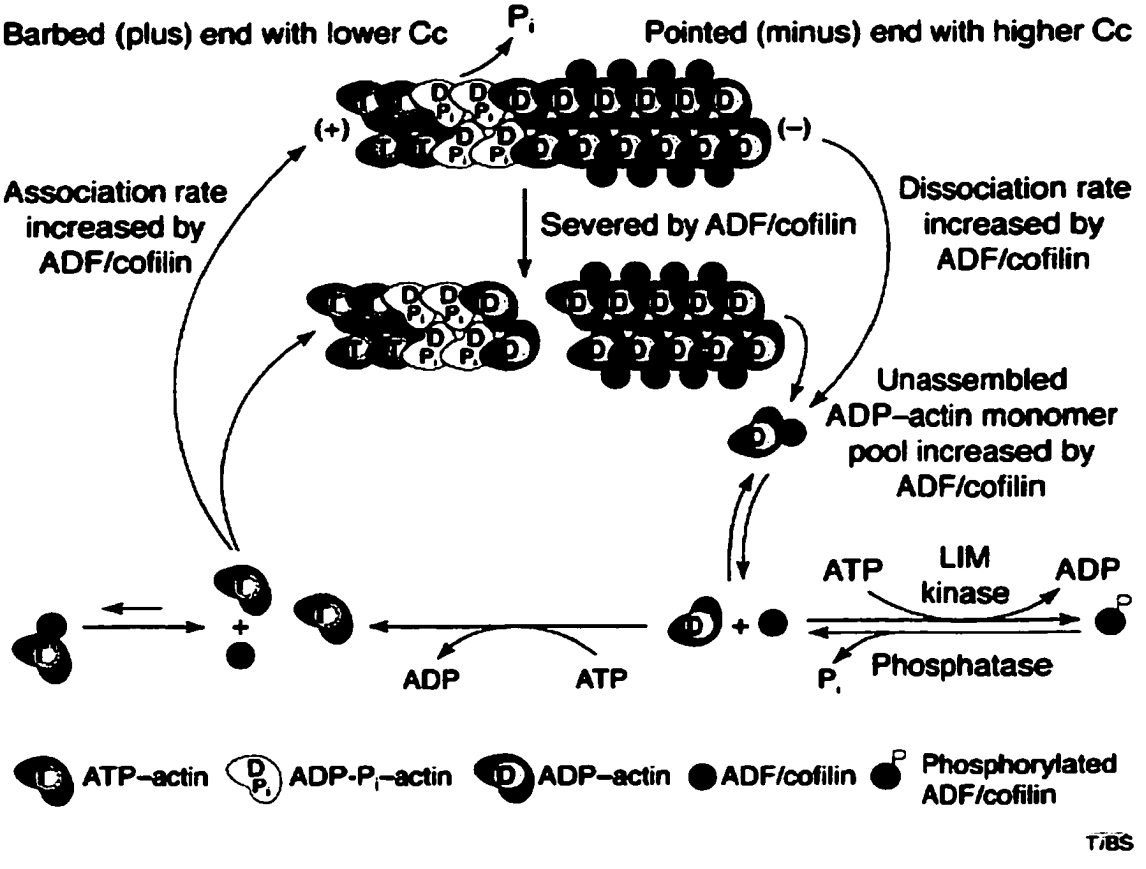


Figure 1.2
ADF/Cofilin enhances the turnover of actin filaments. AC increases the dissociation rate at the pointed (minus) end and may enhance the association rate at the barbed (plus) end. The dissociation of phosphate from ADP-P_i-actin filaments promotes AC binding to the filament (Carlier *et al.*, 1997; Blanchoin & Pollard, 1999; Maciver *et al.*, 1991), and the binding of AC to the filament may also promote P_i dissociation (Blanchoin & Pollard, 1999). Filament severing by AC creates short filaments with additional free pointed and barbed ends that can contribute to enhanced turnover. The nucleotide exchange on the monomer can be a rate-limiting step. The binding of AC to ADP-actin monomer inhibits nucleotide exchange. The phosphorylation of AC prevents its association with ADP-actin allowing the binding of profilin to enhance the exchange of ATP for ADP on actin monomer. The dephosphorylation of AC reactivates it to bind and depolymerize actin filaments.

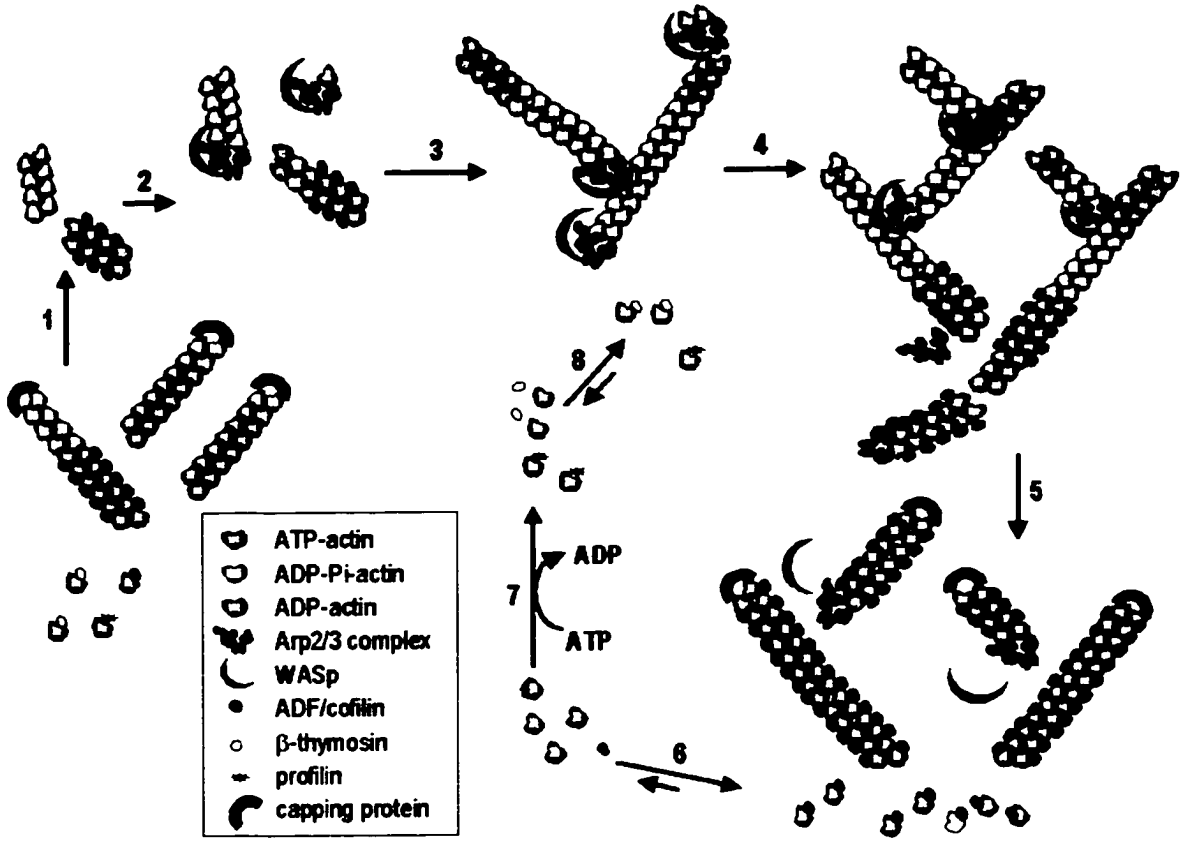
The complex regulation of actin dynamics in lamellipodial extension

Whether considering neurosecretion, lamellipodial extension or any other cellular process dependent upon actin filament turnover, eventually the precise temporal and spatial coordination of assembly and disassembly needs to be understood. Currently, lamellipodial movement seems to be the best understood process (Svitkina & Borisy, 1999b). We suggest that the following three features can effectively integrate the activity of numerous actin-binding proteins required for coordinated cell movement; protein localization, signaling that modulates multiple proteins, and a rate-limiting step that changes under different cellular conditions.

Molecules involved in actin filament turnover are highly localized or spatially regulated in the leading edge. This allows actin to undergo cycles of assembly at the front and disassembly in the rear of the lamellipodium that generate force for extension (Welch *et al.*, 1997). Drugs that inhibit polymerization of actin block forward movement of the cell (Schafer *et al.*, 1998). The barbed ends of the filaments are facing toward the leading edge where actin assembles predominately. Electron microscopic examination of the lamellipodia of keratocytes and fibroblasts (Svitkina & Borisy, 1999a), showed an extensively branched array of actin filaments (called dendritic brush) at the leading edge. Arp2/3 complex, consisting of 7 subunits, is localized at the Y-junction of branches (Svitkina & Borisy, 1999a). As an alternative to individual filament treadmilling, a treadmilling model for the entire actin filament array has been proposed (Machesky & Way, 1998) (Fig. 1.3). The model involves steps of nucleation with pointed-end capping, elongation of free barbed ends at the leading edge, ATP-hydrolysis and P_i release,

Figure 1.3
Model for the turnover of the actin filament array at the leading edge of lamellipodia. The cytoskeletal network at the leading edge of motile cells is characterized by an extensively branched array of actin filaments with barbed ends facing forward and pointed ends making Y-junctions (about 70° angles) with other filaments. Y-junctions are formed by the Arp2/3 complex (Svitkina & Borisy, 1999a). The treadmilling of the dendritic array is regulated by several actin-binding proteins as described below. Numbers in parentheses refer to the numbered steps in the diagram. (1) The barbed ends of actin filaments are usually capped by capping protein when the cell is in the resting state. When cells are stimulated, free barbed ends are generated in the leading edge (shaded area) to nucleate filament growth and to drive membrane protrusion and cell movement. There are three possible mechanisms for the generation of free barbed ends (reviewed in Condeelis, 2001): dissociation of capping proteins in presence of the elevated level of PtdIns(4,5)P_2 , filament severing by ADF/cofilin, and actin nucleation dependent on Arp2/3. The Arp2/3complex is activated upon binding to WASp, one form of which is activated by the small GTPase cdc42. (2) The active Arp2/3 complex nucleates actin filament assembly and/or caps the free pointed end of the ATP- or ADP·P_i-actin filaments (Machesky *et al.*, 1999b; Blanchoin *et al.*, 2000a; Pollard *et al.*, 2001). (3) The activated Arp2/3 complex binds to the side of a filament and then nucleates filament growth or captures the pointed ends of a preexisting filament (Bailly *et al.*, 1999; Blanchoin *et al.*, 2000a). An alternative model proposes that the activated Arp2/3 complex binds to and branches the barbed end of actin filaments (Pantaloni *et al.*, 2000). Growth of filaments is rapid and the lag in P_i dissociation leads to filaments in the leading edge that are composed predominantly of ATP and ADP-Pi-actin and do not bind ADF/cofilin. (4) At the rear of lamellipodia, two mechanisms could contribute to the rapid depolymerization: filament severing or uncapping of pointed ends by removal of Arp2/3 complex. Severing by AC likely occurs at junctions between regions of filaments that are saturated with AC and naked F-actin. The activated Arp2/3 complex dissociates from the pointed end of ADP-actin because it has weaker affinity for ADP-actin than ATP- or ADP·P_i-actin (Blanchoin *et al.*, 2000a). (5) Capping of the barbed ends by capping proteins prevents their further elongation. AC enhances depolymerization of ADP-actin from free filament ends in the rear of lamellipodia. (6) The complex of AC and ADP-actin that dissociates from the filament ends is in equilibrium with AC and ADP-actin monomer. (7) The nucleotide exchange on actin monomer is a slow process, further inhibited by AC. Profilin enhances nucleotide exchange (Blanchoin & Pollard, 1998; Nishida, 1985; Didry *et al.*, 1998). (8) ATP-actin monomers are sequestered by β-thymosin (Sun *et al.*, 1995) to prevent spontaneous nucleation, but provide a pool of ATP-actin for assembly.

Figure 1.3



capping of barbed ends as the filament array moves away from the leading edge, pointed-end uncapping, and disassembly, presumably from the pointed end. In Fig. 1.3 we incorporate other actin regulating proteins into a model for array treadmilling that diagrams the spatial control of filament dynamics.

Actin assembles predominately at the leading edge because the concentration of uncapped barbed-ends is high in this region and is usually rate-limiting for polymerization. The uncapped barbed ends can be generated by *de novo* nucleation, severing of existing filaments, removal of barbed-end capping proteins, or a combination of the three. The Arp2/3 complex might serve to generate new barbed ends through *de novo* nucleation (Mullins *et al.*, 1998; Machesky *et al.*, 1999b) or it might capture and stabilize pointed ends of actin filaments generated through severing (Bailly *et al.*, 1999). The free barbed ends can be generated through filament severing by AC upon stimulation (Zebda *et al.*, 2000). Inactivation of AC by Lim kinase blocks the creation of barbed ends in EGF stimulated cells (Zebda *et al.*, 2000). The availability of barbed ends is reduced by the barbed-end capping proteins, which bind filaments with high affinity (K_D ~1 nM) (Schafer & Cooper, 1995). These capping proteins are modulated by Ca^{2+} and/or PtdIns(4,5)P_2 levels (Schafer & Cooper, 1995). The effect of severing will greatly amplify barbed-end assembly in the presence of pointed-end capping proteins such as the Arp2/3 complex (Machesky & Gould, 1999a), simply because the number of free barbed ends relative to pointed ends will increase. The localization of Arp2/3 complex to the leading edge helps to provide the spatial organization necessary for locomotion. The Arp2/3 complex is activated for actin nucleation by binding to proteins in the Wiscott-Aldrich Syndrome protein (WASp) family some of which are activated by the rho family

GTPase, cdc42 (Svitkina & Borisy, 1999b; Machesky & Gould, 1999a). Currently, there are two possibly mechanism for the formation of dendritic brush implied from *in vitro* studies. WASp-activated Arp2/3 complex binds laterally along pre-existing filaments on ATP-actin or ADP-Pi-actin subunits and nucleates filament growth (Marchand *et al.*, 2001; Blanchoin *et al.*, 2000; Pollard *et al.*, 2001), thus promoting actin assembly and the formation of the Y-junctions in the dendritic brush. Another mechanism is barbed end branching through the interaction between activated Arp2/3 complex and the barbed end of a filament (Pantaloni *et al.*, 2000). The filaments adjacent to the membrane are apparently resistant to AC depolymerization (Svitkina & Borisy, 1999a), possibly because they are composed predominantly of ATP- and ADP-P_i actin. If actin assembly rates are rapid and P_i release is slow, AC proteins would be restricted in binding to regions of filaments somewhat removed from the leading edge (Maciver *et al.*, 1991). Another possible mode of AC regulation in the leading edge involves Arp2/3 complex capping of pointed ends. Arp2/3 complex has higher affinity (K_D ~40 nM) for the pointed ends of ATP-actin filaments than those of ADP-actin filaments (K_D ~1 μM) (Blanchoin *et al.*, 2000a), and the binding of AC to F-actin is inhibited on ATP-actin or ADP-Pi-actin subunits (Blanchoin & Pollard, 1999). Thus, Arp2/3 capping of ATP-actin or ADP-Pi-actin filaments prevents AC binding and filament depolymerization, explaining the stable branched filaments *in vivo* when filaments are capped by Arp2/3 (Svitkina & Borisy, 1999a). AC proteins can rapidly depolymerize filaments capped with Arp2/3 complex *in vitro* (Ressad *et al.*, 1999) probably due to the loss of P_i from actin subunits on the pointed ends of these filaments.

The temporal coordination of assembly may be generated by signals such as PtdIns(4,5)P_2 that could modulate the activity of several proteins simultaneously. Extracellular signaling that reduces phospholipase C activity or activates PtdIns-4 kinase and PtdIns-5 kinase, could increase PtdIns(4,5)P_2 and stimulate barbed-end assembly via several coordinated processes. These include PtdIns(4,5)P_2 inhibition of AC depolymerizing and severing activities (Yonezawa *et al.*, 1990), prevention of capping protein from binding to barbed-ends, and uncapping of gelsolin-bound filaments (McGrath *et al.*, 1998; Schafer & Cooper, 1995). However, other signals such as Ca^{2+} and pH might activate both assembly- and disassembly-enhancing proteins. The integrated effect will depend upon the relative concentrations and availability of each protein and signaling species in the modulated microdomain.

At the rear of lamellipodia, two likely mechanisms contribute to the fast depolymerization and supply of G-actin needed for assembly at the front. One mechanism is the removal of the Arp2/3 complex, facilitating AC-dependent depolymerization from the pointed end. Another mechanism is a Ca^{2+}-independent filament severing by AC (Bamburg, 1999), perhaps aided by active Aip1 (Okada *et al.*, 1999; Rodal *et al.*, 1999), and/or a Ca^{2+}-dependent severing by gelsolin (Schafer & Cooper, 1995). Aip1 is localized to dynamic regions of the cell cortex including lamellipodia in *Dictyostelium* (Konzok *et al.*, 1999). It binds with a maximum stoichiometry of 1:2:2 with AC and actin (Okada *et al.*, 1999). Aip1 itself has no significant F-actin severing or depolymerization activity, but it greatly enhances filament depolymerization promoted by yeast cofilin or *Xenopus* AC (Okada *et al.*, 1999;Rodal *et al.*, 1999). In motile adenocarcinoma cells, a rapid increase in filament number at the

leading edge occurs in a calcium-independent fashion (Chan *et al.*, 1998), suggesting that the severing activity of AC proteins might be rapidly modulated.

Not all actin filaments lacking Arp2/3 complex can be depolymerized or severed by AC. The binding of AC and tropomyosin to actin filaments is mutually exclusive. Tropomyosin inhibition of AC activity (Bernstein & Bamburg, 1982) provides another level of control for coordinating both the spatial and temporal turnover of actin filaments. Specific isoforms of tropomyosin distribute differentially during the development of some cells (Weinberger *et al.*, 1996). How different filament populations arise remains an open question.

Although AC proteins are essential for enhanced actin dynamics *in vivo* (Carlier *et al.*, 1997; Lappalainen & Drubin, 1997; Rosenblatt *et al.*, 1997), clearly they do not work alone. In addition to the multiple species controlling their behavior directly, their effectiveness in disassembly and severing is greatly augmented by other monomer sequestering proteins. These proteins, profilin and thymosin β4, may lack depolymerizing or severing activity themselves (Sun *et al.*, 1995), but they are present in high enough concentration to contribute significantly to the maintenance of the ATP-G-actin pool. AC can then rebind to filaments, removing more monomers and/or severing. In contrast to ACs, which have higher affinity for binding ADP-actin over ATP-actin, β-thymosins have a 100-fold higher affinity for ATP-actin than for ADP-actin and sequester the ATP-actin monomers in a 1:1 complex (Goldschmidt-Clermont *et al.*, 1992). Under normal intracellular conditions, profilin binding to G-actin accelerates nucleotide exchange (Nishida, 1985), generating more of the preferred ATP-G-actin for filament addition. By enhancing nucleotide exchange, sometimes the rate limiting step in

actin cycling, profilin increases the subunit turnover in the presence of AC up to 125 fold over actin alone (Blanchoin & Pollard, 1998; Didry *et al.*, 1998).

Differential regulation of ADF and cofilin

Multiple homologues of ADF/cofilin proteins are often expressed in multi-cellular organisms, although a single member is expressed in single-cell organisms. The multiplicity of ADF/cofilin in *Caenorhabditis elegans* is achieved by alternative splicing of one gene to produce two products, UNC-60A and UNC-60B. In vertebrates, ADF and cofilin are products of different genes. In single-cell organisms, one AC protein can function well enough to fulfill its need, but multiple homologues in multi-cellular organisms are required for tissue specificity and to differentially regulate the temporal/spatial expression. In maize, three ADF/cofilin homologues, ZmADF1-3, have been found. ZmADF1 and ZmADF2 are expressed in pollen, whereas ZmADF3 is expressed tissues other than pollen (Lopez *et al.*, 1996). The expression of ADF and cofilin also show a temporal and tissue specific regulation in animals. In chick muscle cells, ADF and non-muscle cofilin decrease as muscle develops while muscle cofilin levels increase (Nagaoka *et al.*, 1996). In chick brain the ADF level is much higher than that of cofilin throughout development (Devineni *et al.*, 1999). The tissue- and temporal-specific expression of each ADF/cofilin homologue indicates there is differential gene regulation. Expression of ADF and cofilin in a single cell is differentially regulated in response to the monomer actin pool (Minamide *et al.*, 1997). Over-expression in mouse myoblasts (C2 cells) of β-sm actin, a mutant human actin that does not polymerize normally, selectively down-regulates expression of mouse ADF and actin at both the

protein and mRNA level, but not the expression of cofilin or several other actin binding proteins. In degenerating denervated and dystrophic muscles, cofilin, but not ADF, is significantly increased in amount (Shinagawa *et al.*, 1993). Thus, one reason for the multiple isoforms of these proteins appears to be in the regulation of their expression.

The activity of ADF/cofilin is regulated by pH shift and the extent varies from one ADF/cofilin homologue to another. Human ADF shows a greater pH-dependent F-actin depolymerizing activity than *Arabidopsis* ADF1, as determined by sedimentation assay (Ressad *et al.*, 1998), whereas actophorin shows no pH sensitivity whatsoever (Maciver *et al.*, 1998). The critical concentration of human ADF-actin varies from 2 μM at pH 6.5 to 7 μM at pH 8.2 (Ressad *et al.*, 1998). The distributions of ADF and cofilin in Swiss 3T3 cells differ when cells are subjected to intracellular pH shift. ADF co-localizes more with G-actin at higher pH and more with F-actin at lower pH, whereas cofilin/actin co-localization appears less pH-dependent in the same cells (Bernstein *et al.*, 2000). Thus ADF shows more dramatic pH-induce alterations in cellular distribution than cofilin.

Some quantitative differences have been reported among ADF/cofilin homologues in their biochemical properties. Human ADF has higher binding affinity to ATP-G-actin than does actophorin (Maciver *et al.*, 1998) suggesting human ADF, but not actophorin, might be one of the sequestering protein to increase the predominant MgATP-actin monomer pool *in vivo*. The extent of enhanced barbed end assembly by different ADF/cofilins varies. *Arabidopsis* ADF1 and yeast cofilin, but not actophorin, promote filament assembly at barbed ends by increasing assembly rate and/or nucleation (Carlier *et al.*, 1997).

Conclusions and focus of dissertation

Recent biochemical and structural studies have provided important insights into how AC proteins work with other actin binding proteins to enhance the turnover of actin filaments, but a number of questions remain to be answered. How do the signal transduction pathways controlling AC phosphocycling impact the activities of the other proteins discussed here? How are the bifurcating signaling pathways regulating AC phosphocycling kept in balance? Is the competitive binding of tropomyosin and AC used to distinguish different filament populations and if so how are these selected? Do different AC homologues vary qualitatively or quantitatively in their effects on filament turnover? What are the intracellular implications based on the differences, especially in cells that contain more than a single member of the AC family? A combination of molecular, biochemical and ultrastructural approaches will help answer these questions in the near future. The data presented in this dissertation are aimed to answer the last two questions.

The main focus of my dissertation is to determine whether different AC homologues interact differently with actin. This chapter has presented background information regarding the actin filament dynamics and the role ADF/cofilin in its regulation. Particular attention has been paid to the differential regulation of ADF and cofilin reported in literatures. In chapter two, ADF and cofilin from one species, chicken, are compared in their interaction with chicken muscle actin. Quantitative differences between ADF and cofilin are presented for G-actin binding, filament depolymerizing and severing, pH sensitivity, timed sedimentation, fluorescence kinetics, and electron

microscopy. ADF has a higher affinity for MgATP-G-actin than cofilin, suggesting ADF might be a major monomer sequestering protein. ADF is more effective in filament severing and enhancing off-rate from pointed ends than cofilin. In chapter three, AC proteins from many organisms across phylogeny are assayed for G-actin binding and filament severing by non-denaturing polyacrylamide gel electrophoresis, timed sedimentation, and fluorescence kinetics. Most of them fall into two categories, ADF-like or cofilin-like groups. In chapter four, the effects of actin binding drugs, latrunculin A and jasplakinolide, on the interaction of actin with the actin binding proteins, ADF and thymosin β4, are studied. Latrunculin A competes in its binding to G-actin with ADF and thymosin β4. Jasplakinolide protects filaments from depolymerization by ADF and latrunculin A. In chapter five, the significance of each major finding presented in this dissertation is discussed. In addition, unanswered questions arising from this work are explored and future experiments are proposed.

Chapter Two

Differential Regulation of Actin Dynamics by Actin Depolymerizing Factor and Cofilin

Preface and Acknowledgement

The work presented in this chapter will be submitted to the Journal of Biological Chemistry. The order and list of the authors will be Hui Chen, Judith M. Sneider, Judith A. Boyle, Barbara W. Bernstein, Aimin Tang, Sharon Kelly, and James R. Bamburg. The data presented in this chapter were acquired and analyzed by Hui Chen. Judith M. Sneider purified recombinant chick ADF and recombinant chick cofilin. Dr. Judith A. Boyle performed the electron microscopy. Sharon Kelly and Aimin Tang established the methods I used for the non-denaturing gel electrophoresis. Dr. Barbara W. Bernstein assisted with fluorescence assays. I would like to thank Dr. Alan Weeds for providing recombinant gelsolin expression plasmid, and Drs. James Casella and Susan Craig for providing spectrin:actin:protein 4.1 complex. I thank Dr. Kyoko Okada for many helpful discussions.

Summary

The ADF/cofilins are an essential group of actin filament depolymerizing and severing proteins that regulate actin filament turnover *in vivo*. In vertebrates, ADF and

cofilin are products of different genes but are co-expressed in many cell types. Each has qualitatively similar interactions with actin, but differences have been reported in the regulation of their expression. We examined the quantitative differences between chick ADF and chick cofilin using several assays. The steady state critical concentration of ADF-actin is about 6 μM compared to 0.8 μM for cofilin-actin. The apparent K_Ds for G-actin binding to ADF or cofilin were estimated from binding curves obtained by non-denaturing polyacrylamide gel electrophoresis performed at different ionic strength. The K_{Dapp} of ADF to MgATP-actin was 10-fold lower than for cofilin, suggesting that ADF, but not cofilin, is likely to be a sequestering protein for the predominantly MgATP-actin monomeric pool found in cells. Cofilin promoted filament assembly by enhancing nucleation, but ADF did not. Furthermore, ADF severed and depolymerized filaments into a heterogeneous mixture of various sizes at pH 8.1 as assayed by timed sedimentation and electron microscopy, whereas long filaments predominated in cofilin-actin mixtures. A fluorescence severing assay was developed in which filaments were capped at both ends, severed by ADF or cofilin, and the depolymerization induced by addition of vitamin D binding protein to sequester monomer. Chick ADF had a faster depolymerization rate at pH 7.8 than pH 6.8, but at both pHs it depolymerized filaments faster than chick cofilin. To further compare their function, we measured their filament depolymerizing and severing activities at pH 6.8 and 7.8 using the assay developed by Moriyama and Yahara (1999) with slight modification. At pH 6.8, cofilin increased the minus end off-rate from actin by 5-fold whereas ADF increased the minus end off-rate 40-fold. Both had little severing activity at pH 6.8. Of particular interest, at pH 7.8, cofilin did not increase the minus end off-rate but it severed filaments to create an 8-fold

increase in filament number whereas ADF increased the minus end off-rate 4-fold and increased filament number 20 fold. These data demonstrate that the filament depolymerizing and severing activities of ADF and cofilin are sensitive to pH shift, and that ADF is the more pH-sensitive protein. At pH 7.8 cofilin had little effect on the critical concentration at steady state whereas ADF substantially increased the unassembled actin pool. This finding suggests that cofilin enhances filament nucleation at pH 7.8 by severing without depolymerizing whereas ADF rapidly depolymerizes filaments.

Introduction

The ADF/cofilin family proteins are actin-binding proteins ubiquitous among eukaryotes. They are essential to cell division (Gunsalus *et al.*, 1995; Abe *et al.*, 1996). Single cell eukaryotes express only one member of this family whereas metazoans express two or more members. ADF was initially isolated from embryonic chick brain, and identified as an F-actin depolymerizing factor (Bamburg *et al.*, 1980). Cofilin was originally identified as an F-actin co-filamentous protein from porcine brain (Nishida *et al.*, 1984). Homologues of ADF/cofilin have been independently identified in many eukaryotes and some have been named by sequence comparison either as ADF or as cofilin (Bamburg, 1999).

Within any vertebrate species, ADF and cofilin have ~ 70% amino acid sequence identity. In addition, they share many qualitative similarities in biochemical properties. They bind monomeric actin in a 1:1 complex and inhibit nucleotide exchange (Hayden *et al.*, 1993; Nishida *et al.*, 1984; Hawkins *et al.*, 1993). Many ADF/cofilins are reported to

bind to ADP-G-actin with about 30- to 80-fold higher affinity than they bind to ATP-G-actin at physiological ionic strength (Carlier *et al.*, 1997; Blanchoin & Pollard, 1998; Ressad *et al.*, 1998). With the exception of ADF/cofilin from yeast and *Dictyostelium*, the activity of ADF/cofilin is inhibited by phosphorylation at ser3 (Agnew *et al.*, 1995) or its equivalent ser6 in plants (Smertenko *et al.*, 1998) or ser2 in amoeba actophorin (Blanchoin *et al.*, 2000b). Lim kinases phosphorylate cofilin (Arber *et al.*, 1998; Yang *et al.*, 1998), whereas both ubiquitous and specific phosphatases are involved in dephosphorylation of ADF/cofilin (Meberg *et al.*, 1998). The actin depolymerizing activity of ADF/cofilins is also inhibited by PtdIns(4,5)P_2 binding (Yonezawa *et al.*, 1990), pH below 7.0 (Hayden *et al.*, 1993; Hawkins *et al.*, 1993), and tropomyosin-saturation of F-actin (Bernstein & Bamburg, 1982).

ADF/cofilin plays an essential role in regulating actin filament turnover needed for many actin-based processes in non-muscle cells. (1) ADF/cofilins typically localize to regions of cells characterized by high actin dynamics, including neuronal growth cones, ruffling membranes, the cleavage furrow of cells undergoing cytokinesis, and yeast cortical actin patches (Bamburg & Bray, 1987; Yonezawa *et al.*, 1987; Moon *et al.*, 1993; Nagaoka *et al.*, 1995b). (2) Mutations in yeast cofilin that reduced its activity caused defects in filament disassembly (Lappalainen & Drubin, 1997). (3) *Xenopus* XAC and *Arabidopsis* ADF1 increase the turnover rate of the actin comet tail of *Listeria monocytogenes* (Rosenblatt *et al.*, 1997; Carlier *et al.*, 1997). (4) *Arabidopsis* ADF1 increases the dissociation rate at the pointed (minus) end and may enhance the association rate at the barbed (plus) end (Carlier *et al.*, 1997). The structural basis for the enhancement of filament turnover by ADF/cofilin has been proposed by Galkin *et al.*,

(2001). The binding of two ADF/cofilins on each actin subunit induces a 12° tilt of actin subunits and thus disrupts the subunit interactions along the filament axis and causes severing.

Differences in expression and localization in cells among ADF/cofilin homologues have been reported. In muscle cells, ADF, but not cofilin, decreases as muscle develops (Shinagawa *et al.*, 1993). In degenerating denervated and dystrophic muscles, cofilin, but not ADF, is significantly increased in amount (Nagaoka *et al.*, 1996). Over-expression in mouse myoblasts (C2 cells) of β-sm actin, a mutant human actin that does not polymerize normally, selectively down-regulates expression of mouse ADF and actin at both the protein and mRNA level, but not the expression of cofilin or other actin binding proteins (Minamide *et al.*, 1997). The pH dependence is different from one ADF/cofilin homologue to another. Human ADF has a pH-sensitive destruction of actin filaments but actophorin does not (Maciver *et al.*, 1998). Human ADF showed greater pH-dependent depolymerizing activity than *Arabidopsis* ADF1 (Ressad *et al.*, 1998). Shifting of intracellular pH in mammalian cells resulted in a greater co-localization of ADF with G-actin at higher pH and with F-actin at lower pH ($p<0.001$), but cofilin/actin co-localization was less pH-dependent (Bernstein *et al.*, 2000).

Binding differences among ADF/cofilin homologues to actin also have been reported. Human ADF has higher binding affinity to ATP-G-actin than does actophorin (Maciver *et al.*, 1998). *Caenorhabditis elegans* UNC-60A depolymerizes but does not co-sediment with rabbit muscle actin, whereas UNC-60B co-sediments with but does not depolymerize rabbit muscle actin (Ono & Benian, 1998). The extent for enhancing plus end assembly by ADF/cofilin homologues is different. *Arabidopsis* ADF1 and yeast

cofilin but not actophorin may nucleate and/or enhance filament assembly at the barbed end (Carlier *et al.*, 1997) although those results could be explained in terms of severing of filaments without an effect on barbed-end assembly.

Phylogenetic studies reveal three protein classes containing the actin-depolymerizing factor homology domain: the ADF/cofilins, the twinfilins, and the drebrin/Abp1s (Lappalainen *et al.*, 1998). All members of the ADF/cofilin class appear to have evolved from a single ancestral protein. The three distinct subclasses (ADF, muscle cofilin, and non-muscle cofilin) emerged before the divergence of birds and mammals. Yeast mutants defective in cofilin are lethal, but can be rescued with either mammalian ADF or cofilin (Iida *et al.*, 1993), suggesting that at least in simple single-cell eukaryotes the protein has sufficient functional overlap to provide the regulation needed for actin dynamics. However, based upon their sequence, structure and biochemical properties, it is unclear why two or more proteins are co-expressed in the cells of metazoans.

This study investigates the interaction of ADF and cofilin with G- and F-actin using assays that allow us to determine quantitative differences in their interactions. Here, we demonstrate that there are differences between chick ADF and chick cofilin in MgATP-G-actin binding, filament severing, filament depolymerizing, and pH sensitivity. ADF is more effective in filament severing and depolymerizing than cofilin. Cofilin tends to promote filament assembly. These findings help explain behavioral differences that have been observed in cells overexpressing either an ADF or a cofilin.

Materials and Methods

Proteins

CaATP-G-actin was extracted from chick muscle acetone powder and purified according to Pardee & Spudich (1982). G-actin was gel filtered over Sephadex G-150 in G-buffer (2 mM Tris, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.01% NaN_3, pH 8.0), and the final ATP was adjusted to 0.5 mM prior to freezing in liquid nitrogen. MgATP-G-actin was prepared from CaATP-G-actin by incubation with 0.1 mM $MgCl_2$ and 0.2 mM EGTA for 10 min on ice (Kinosian *et al.*, 1993). The MgATP-G-actin was converted to MgADP-actin by the addition of 1 mM glucose and 20 u/ml hexokinase (Gershman *et al.*, 1989). Chick brain ADF was purified from frozen 14-17 day old embryonic chick brain (Giuliano *et al.*, 1988). Recombinant chick ADF and chick cofilin were prepared as described by Adams *et al.* (1990) and Abe *et al.* (1990), respectively. Both ADF and cofilin were bound to and eluted from a Green A column (Millipore) as the final step of purification. ADF was bound to a Green A column in 10 mM Tris, pH7.5 and 5 mM DTT, cofilin was bound to a Green A column in 10 mM PIPES, 10 mM Tris, pH 6.5, and 5 mM DTT. Both ADF and cofilin were eluted with salt gradients up to 200 mM NaCl in the respective buffer. ADF or cofilin was dialyzed against 2 mM Tris, pH 8.4 with 0.5 mM DTT prior to studies using non-denaturing polyacrylamide gel electrophoresis (PAGE). The purity of proteins was determined using SDS-PAGE. The concentration of actin was determined spectrophotometrically using the extinction coefficient of 0.63 l/g at 290nm (Houk & Ue, 1974). The extinction coefficients for ADF and cofilin at 280 nm are 11650 $M^{-1}cm^{-1}$ and 18020 $M^{-1}cm^{-1}$, respectively, calculated by the method of Gill & von Hippel (1989), which was developed for denatured proteins. The concentrations of

chick ADF and chick cofilin were first calculated by spectrophotometric measurement in G-buffer, then checked by quantification on Coomassie Blue R-250 stained SDS-polyacrylamide gels using actin for generating a standard curve. ADF and cofilin concentrations by this method were always within 20% and usually within 10% of the spectrophotometrically determined value.

Recombinant human gelsolin was prepared as described by Pope *et al.* (1997). The extinction coefficient for gelsolin at 280 nm is 114230 $M^{-1}cm^{-1}$ (Pope *et al.*, 1997). Gelsolin-actin 1:1 complex was prepared by mixing an equal molar ratio of gelsolin and G-actin in the presence of 10 mM $CaCl_2$, and incubating for 10 minutes prior to adding 30 mM EGTA. The complex was gel filtered over Sephadex G-150 in 10 mM HEPES, pH 7, 50 μM $MgCl_2$, 0.1 mM EGTA, 1 mM NaN_3, and 0.2 mM DTT. Human Vitamin D-binding protein (DBP), also called Gc-globulin, was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA). Spectrin:actin:protein 4.1 complex was kindly provided by Drs. James Casella and Susan Craig (Johns Hopkins University).

Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was used to study the effect of actin bound nucleotide and divalent cation on the interaction between ADF or cofilin and G-actin. ADF or cofilin at different concentrations was mixed with G-actin in 2 mM Tris, pH 8.4 and 0.5 mM DTT containing either 0.2 mM ATP or ADP. The mixtures were incubated for 10 minutes at room temperature, the free actin and the complex of actin-ADF/cofilin were then separated on a 7.5% non-denaturing polyacrylamide gel at 4°C. The running buffer with 1X salt contained 50 mM bicine, 40 mM triethanolamine, pH

8.4, 0.2 mM ATP or ADP, and 5 mM EGTA (if assaying for the interaction with Mg-actin) at an ionic strength of 34 μ. Salt in the running buffer was varied from 0.5X to 3X salt to adjust the ionic strength from 20μ to 90μ. Gels were stained with Coomassie Blue R-250 when aliquots from samples containing 5 μM G-actin were used, and stained with SYPRO red (Molecular Probes) when aliquots from samples containing 1.5 μM G-actin were used. An internal actin standard was run on each gel to quantify the concentration of free actin and ADF/cofilin-actin complex. Digitized images were obtained with a Photometrics chilled CCD camera and band densities were quantified using 1D Phoretix software (Non Linear Dynamics Ltd, England), and analyzed by Kaleidagraph analysis/graphics software (Synergy Software, Reading, PA). An estimate of the apparent dissociation constant (K_D) defined in equation (1) was obtained by curve fitting to the transformed form of equation (1) shown as equation (2).

$$K_{Dapp} = \frac{[Actin]_f [AC]_f}{[AC\text{-}actin]} \qquad (1)$$

$$[AC\text{-}actin] = ([AC]_t+K_D+A)/2-([AC]_t*[AC]_t+2*(K_D-A)*[AC]+(K_D+A)*(K_D+A))\text{^}0.5/2 \qquad (2)$$

$[Actin]_f$ is the concentration of free actin measured directly from the gel; $[AC]_f$ is the concentration of free ADF or cofilin and was estimated as the difference between the total AC and the amount of AC in the AC-actin complex; [AC-actin] is the concentration of ADF/cofilin-actin complex measured directly from the gel; $[AC]_t$ is the total concentration of ADF/cofilin. A is the total concentration of actin, 5 μM for Coomassie

stained gel and 1.5 μM for SYPRO red stained gel. In entering equation (2) into Kaleidagraph, the following substitutions are made: m0 = $[AC]_t$; m1 = K_D. Since this is not a true equilibrium system, measurements are reported only as K_{Dapp}.

The amount of actin displaced by DBP from ADF-actin 1:1 complex was also measured by non-denaturing gel electrophoresis. To form this complex, 5 μM MgATP-actin and 15 μM ADF were incubated in 2 mM Tris, pH 8.4, 0.5 mM DTT, 0.2 mM ATP, 0.21 mM $MgCl_2$, and 0.2 mM EGTA for 10 min on ice. A 3:1 ADF to actin ratio was used to drive most of the actin into complex. Increasing concentrations of DBP were incubated with the ADF-actin complex for 15 minutes at room temperature. The proteins were separated on a 7.5% non-denaturing polyacrylamide gel at 4°C using 50 mM bicine, 40 mM triethanolamine, 0.2mM ATP, pH 8.4 as the running buffer. The gel was stained with Coomassie Blue R250. Digitized gel images were obtained and analyzed as above.

Job Plot

The method of continuous variation, also known as a Job plot, is a useful method to determine the binding stoichiometry of two components (Huang, 1982). Since ADF or cofilin binds to G-actin at 1:1 molar ratio as determined by gel filtration and chemical cross-linking (Giuliano *et al.*, 1988; Nishida *et al.*, 1984), a Job plot was used to determine if recombinant ADF and cofilin and actin were homogenous in actin binding affinity. In this method, the total molar concentration of ADF and CaATP-actin was held constant at 6 μM and the total molar concentration of cofilin and CaADP-actin was held constant at 2 μM, but the mole fraction of each was varied. The mixtures were then subjected to non-denaturing polyacrylamide gel electrophoresis to separate free actin and

ADF/cofilin-actin complex. The concentration of complex was plotted against the molar ratio of ADF or cofilin to actin. If the recombinant protein preparations contain > 20% of inactive protein, the peak will show a substantial deviation from its position at a 1:1 molar ratio. No such deviations were observed.

Timed sedimentation assay

The steady state filament length distribution in the presence of ADF and cofilin was studied by a timed-sedimentation assay. Actin 20 μM was polymerized alone or in the presence of 20 μM ADF or 20 μM cofilin in F-buffer at pH 8.1 (0.1 M KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM ATP, 0.2 mM EGTA, 15 mM Tris, 15 mM PIPES) with an ATP regenerating system (10 u/ml creatine phosphokinase and 10 mM creatine phosphate). The samples were incubated at 4 °C overnight and then centrifuged at 4°C from 5 minutes to 60 minutes at 436,000g in the Beckman TLA100 rotor. The total supernatant was removed, mixed, and the pellet was resuspended in SDS-containing buffer (0.25 M Tris, pH6.8, 10% glycerol, 10% β-mercaptoethanol, and 1% SDS). Fractions from supernatant and pellet were subjected to SDS-PAGE (15% T, 2.67% C).

SDS-PAGE

Samples with unknown protein concentration and 5 actin standards (0.1 μg to1.6 μg) were loaded on an 18-well SDS-polyacrylamide mini gel (15% T, 2.67% C) (Laemmli, 1970). After electrophoresis, the gel was stained with Coomassie Blue R-250. The gel image was digitized using a CCD camera and bands were quantified using the 1D

Phoretix program (Synergy Software, Reading, PA). The amount of protein was obtained from the actin standard curve.

Electron microscopy

Aliquots from samples used for non-denaturing PAGE were also used for electron microscopy. G-actin and ADF or cofilin were mixed in 2 mM Tris, 0.5 mM DTT, 0.2 mM ATP, 50 mM Bicine, 40 mM triethanolamine, and 5 mM EGTA, pH 8.4 at room temperature for 30 minutes. Aliquots (10 μL) of the mixtures were applied to 0.5 % formvar-coated copper grids. After 15 seconds, 5 drops of 1 % uranyl acetate were used to negatively stain the sample. The drop of remaining uranyl acetate was removed from the grid by blotting with the ragged edge of a torn piece of filter paper. Grids were examined in a JEOL electron microscope at 100 kV (Huxley, 1957).

Aliquots of the total sample and supernatants from a timed-sedimentation assay were fixed with 0.1% glutaraldehyde and diluted with water to give a concentration of actin of about 1 μM. A 2 μl aliquot of the diluted sample was applied to a formvar-coated copper grid, allowed to dry, and negatively stained as described above.

Measurement of filament severing

Filament severing by ADF or cofilin was measured by the depolymerization from newly generated pointed ends. In this assay, 60 nM spectrin:actin:protein 4.1 complex and 3.3 μM actin (5% pyrene-labeled) were mixed in F-buffer (100 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM EGTA, 0.5 mM DTT, and 10 mM PIPES at pH 6.8 or 10 mM Tris at pH 7.8) to nucleate filament growth from the barbed ends. Changes in

fluorescence intensity were monitored at 18°C with an AVIV ATF 105 spectrofluorometer set for excitation at 365 nm and emission at 404 nm. The bandwidth was 2 nm for excitation and 4 nm for emission. The steady state, as determined by the fluorescence signal reaching a plateau, was reached in 6 minutes (followed for up to 60 min in some experiments). Upon reaching steady state, 200 nM gelsolin-actin 1:1 complex and 0.6 μM ADF or cofilin were added and the sample was incubated for 10 minutes. Gelsolin-actin 1:1 complex will cap free barbed ends including initial free barbed ends and those generated by the severing induced by ADF or cofilin. DBP, a potent actin monomer sequestering protein (K_D = 1 nM; McLeod *et al.*, 1989), was added in excess to sequester monomers and thus induce depolymerization from the pointed ends of the filaments by maintaining the actin concentration below the critical concentration.

Measurement of filament severing and depolymerization rates at pointed filament ends

The rate of depolymerization from pointed ends was measured using a modification of the method of Moriyama and Yahara (1999). Ca-G-actin (5% pyrenyl-labeled) was incubated with $MgCl_2$ (1 mol equivalent and 50 μM in excess) on ice for 10 minutes, then mixed with various concentrations of gelsolin. Polymerization was initiated by adding 2 mM $MgCl_2$, 100 mM KCl, 0.2 mM EGTA, and either 10 mM PIPES at pH 6.8 or 10 mM Tris at pH 7.8. After 4 hours of polymerization at room temperature, ADF or cofilin and gelsolin-actin 1:1 complex (77 nM at pH 6.8 or 154 nM at pH 7.8) were added and allowed to incubate at room temperature for 10 minutes. The gelsolin-actin 1:1 complex caps the free barbed ends generated by the severing action of ADF or cofilin.

DBP was then added to induce depolymerization from the pointed ends of the filaments. Changes in fluorescence intensity were monitored at 18°C with an AVIV ATF 105 spectrofluorometer. The dead time was about 10 seconds and the final concentrations for actin and DBP were 3.34 μM and 3.5 μM, respectively. The wavelength was 365 nm for excitation and 404 nm for emission. The bandwidth was 2 nm for excitation and 4 nm for emission. The same assay was also done without pyrene labeled actin using light scattering at 400 nm to follow filament depolymerization.

The initial off-rate from pointed ends (V_i) is proportional to the total number of free pointed ends (N_p) and the pointed end off-rate constant (k_{p-}). The total number of free pointed ends, N_p can be expressed as the summation of the initial number of filaments (N_o), taken to be identical to the gelsolin concentration used to nucleate filament growth, and the number of filaments created by ADF or cofilin severing (n).

$$V_i = k_{p-} N_p = k_{p-} (N_o + n)$$

Measurement of critical concentration

Actin at 16 μM was polymerized in F-buffer at pH 7.8 in the absence or in the presence of ADF or cofilin. ADF and cofilin were in 2.5 molar ratio to actin. The samples were then diluted with F-buffer at pH 7.8 to the final actin concentration varied from 2 μM to 14 μM. The light scattering signals of the steady state F-actin were monitored at 17°C with an AVIV ATF 105 spectrofluorometer at 400 nm. The signal is plotted against actin concentration and the critical concentration is derived from the intercept of the linear regression line.

Results

The effect of actin bound nucleotide on the interaction between G-actin and ADF or cofilin.

Proteins used in our assay were more than 98% pure as determined by densitometric scanning of Coomassie Blue R-250 stained SDS-polyacrylamide gels (Fig. 2.1). Job plots of G-actin binding to ADF or cofilin were used before each experiment to determine if each protein was equally active. The maximum amount of the ADF-actin complex (Fig.2.2a, 2.2c) and cofilin-actin complex (Fig. 2.2b, 2.2d) formed at 1:1 molar ratio of ADF/cofilin to actin. Since ADF and cofilin bind to G-actin at 1:1 molar ratio (Giuliano *et al.*, 1988; Nishida *et al.*, 1984), our preparations of ADF, cofilin, and actin do not contain a significant fraction of inactive protein.

Non-denaturing PAGE was used to determine the apparent dissociation constants of G-actin-ADF and G-actin-cofilin. Under the normal low ionic strength (34 μ) conditions used for electrophoresis, ADF had slightly weaker affinity for MgATP-G-actin than MgADP-G-actin (less than 2-fold difference in K_{Dapp}), and the binding is not substantially affected by actin-bound cation (Ca vs. Mg) (Fig. 2.3; Table 2.1). For experiments performed using 5 μM Mg-ATP-actin, an increasing amount of cofilin did not form an increasing amount of complex in the gel, whereas the free actin disappeared

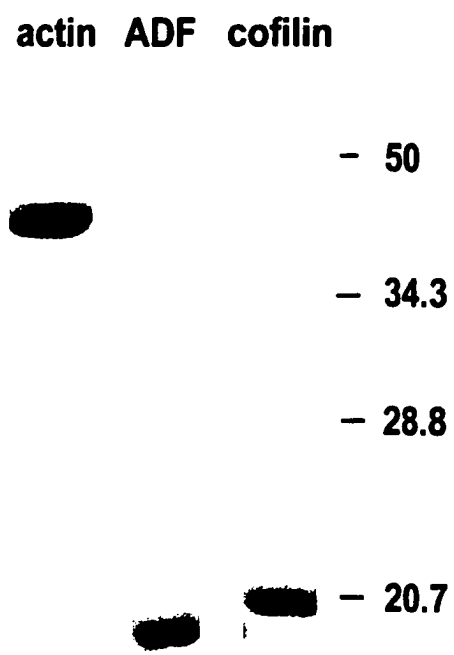


Figure 2.1
SDS-PAGE of actin, ADF, and cofilin. The amounts of protein loaded are 1.6 μg of actin, 1 μg of ADF, and 1 μg of cofilin. The 15% gel was stained with Coomassie Blue R-250. Positions of molecular weight standards (kD) are shown.

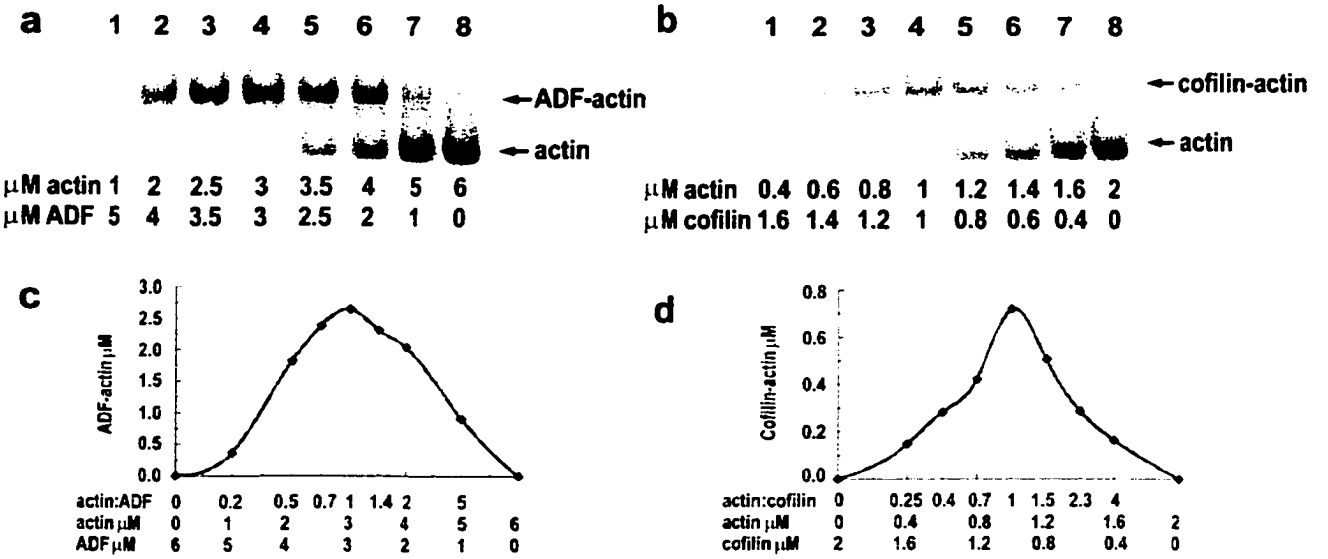


Figure 2.2

Preparations of ADF and cofilin are homogeneous in activity. Job plots were used to determine the binding stoichiometry of G-actin to ADF or cofilin. Increasing amount of actin was incubated with decreasing amount of chick ADF (a), or cofilin (b). The complexes of actin with ADF or cofilin were separated from free actin by non-denaturing PAGE and stained with Coomassie blue R-250. The amount of complex was determined by quantitative densitometry of the bands from (a) and (b) and was plotted against the protein concentration (c) and (d). Maximum complex formation occurred at a 1:1 molar ratio of the actin:ADF or actin:cofilin mixtures.

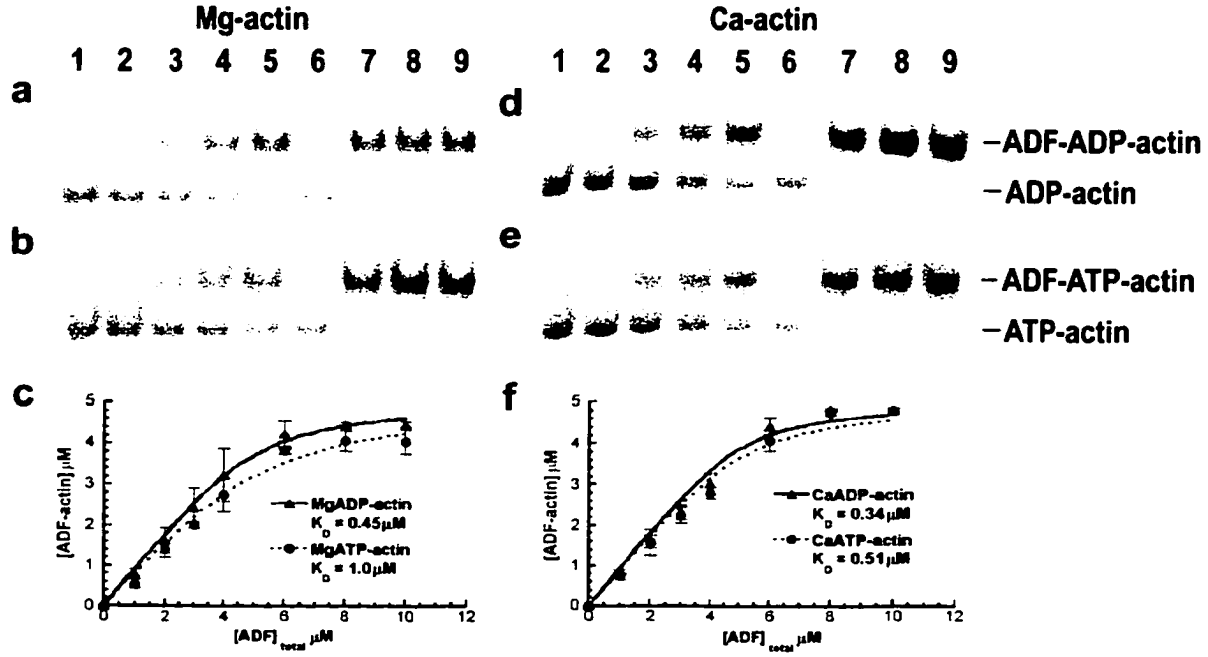


Figure 2.3

The interaction of ADF with G-actin is not greatly affected by the nucleotide or divalent cation bound to actin. Chick ADF at 0, 1, 2, 3, 4, 6, 8, 10 μM (lanes 1, 2, 3, 4, 5, 7, 8, 9, respectively) were incubated with: (a) 5 μM MgADP-actin; (b) 5 μM MgATP-actin; (d) 5 μM CaADP-actin; (e) 5 μM CaATP -actin. An internal standard of 2.5 μM actin was used in lane 6. Each lane was loaded with 12 μl of sample. The complex was separated from free actin by non-denaturing PAGE and stained with Coomassie Blue R. The binding curves were obtained by quantitative densitometry of gels. The amount of complex was plotted against the amount of total ADF as shown in (c) for the interaction with Mg-actin and in (f) for the interaction with Ca-actin. The curves were fitted to the equation (see Materials and Methods) to give apparent dissociation constants.

Table 2.1: The apparent dissociation constants of ADF and cofilin for G-actin at ionic strength $\mu = 34$:

	MgATP-actin	MgADP-actin	CaATP-actin	CaADP-actin
chick ADF	1.0 μM (4)	0.5 μM (4)	0.5 μM (3)	0.4 μM (3)
chick cofilin	10.7 μM* (3)	0.5 μM* (3)	0.9 μM (3)	0.7 μM (3)

The number in the parentheses indicates the number of experiments.
* SYPRO red staining at 1.5 μM actin concentration.

from the gel (Fig. 2.4b). This suggested that cofilin-actin had lower critical concentration and/or nucleated assembly faster, and thus induced filament formation even at low ionic strength. Therefore, we repeated the binding study between cofilin and Mg-actin using a lower concentration of actin (1.5 μM) and a more sensitive protein stain (SYPRO red) to detect the lower amounts of protein. The total actin (including the free actin and the complex of actin and cofilin) was fully recovered in the gel and the results are shown in Fig. 2.4g and 2.4h, Table 2.1. Thus, ADF and cofilin show similar high affinity binding for CaATP-actin, CaADP-actin, and MgADP-actin, but only cofilin shows a marked decrease (K_D = 10.7 μM, 20 fold) in affinity for MgATP-actin.

The effect of ionic strength on the interaction between ADF/cofilin and G-actin

Carlier *et al.* (1997) reported a significant ionic-strength effect on the affiniity of ADF/cofilin for actin. Therefore, we varied the ionic strength of the running buffer from 20 μ to 90 μ (0.5X to 3X running buffer). Consistent with previous observations (Carlier *et al.*, 1997), apparent affinities of ADF for MgATP-actin and MgADP-actin decreased about 40 fold with increasing ionic strength (Table 2.2). However, the affinity of ADF for MgATP-actin was only 2-4.5 fold weaker than for MgADP-actin over this entire range of ionic strengths. Cofilin bound to MgADP-actin with a similar apparent affinity as ADF at ionic strength 34 μ, but in the highest ionic strength at which we could run these gels (90 μ), cofilin bound to MgADP-actin with about a 3-fold higher apparent affinity than ADF. At the highest ionic strength (90μ), no cofilin-actin complex could be

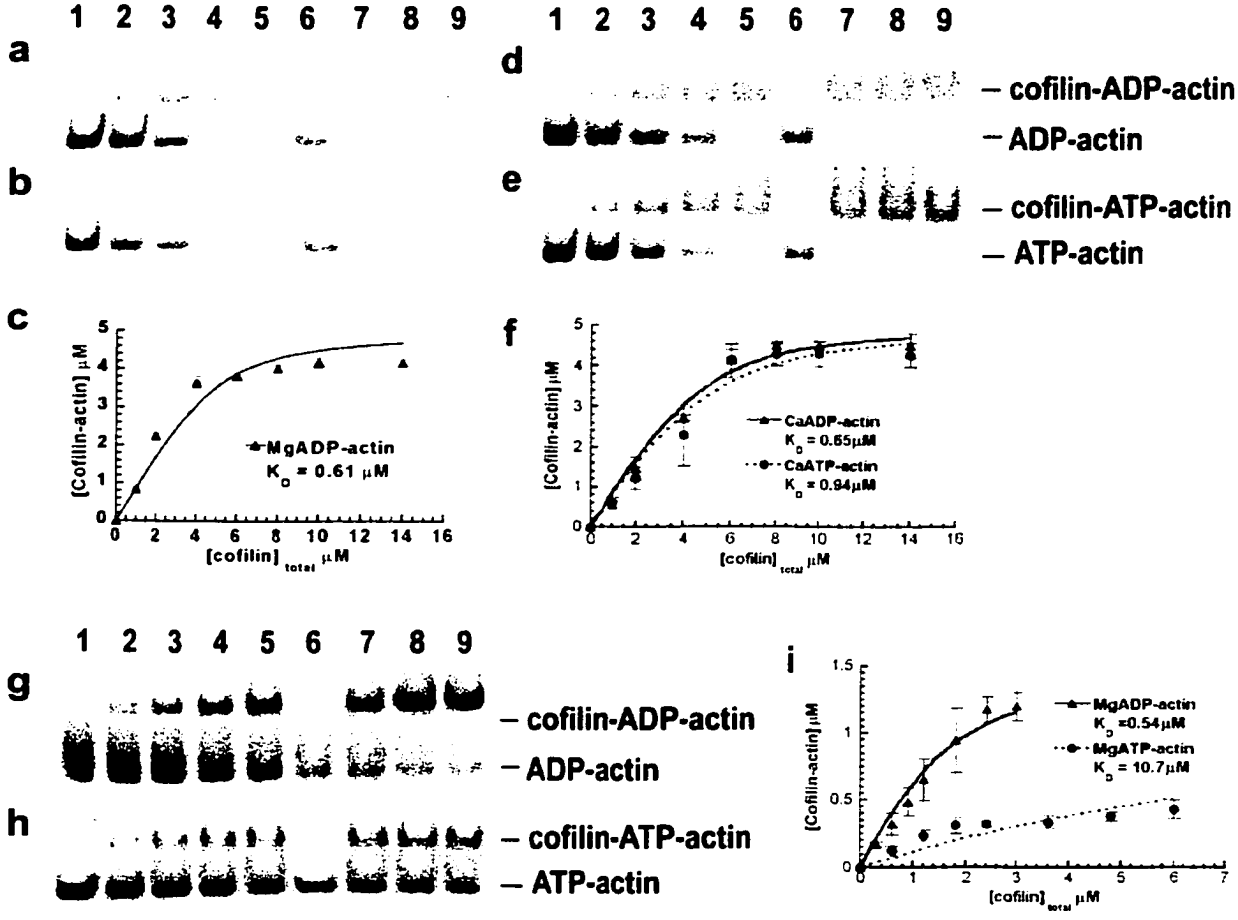


Figure 2.4

The interaction of cofilin with G-actin is affected by the nucleotide and divalent cation bound to the actin.

Chick cofilin at 0, 1, 2, 4, 6, 8, 10, 14 µM (lane 1, 2, 3, 4, 5, 7, 8, 9) was incubated with: (a) 5 µM MgADP-actin; (b) 5 µM MgATP-actin; (d) 5 µM CaADP-actin; or (e) 5 µM CaATP-actin. (g) Chick cofilin at 0, 0.3, 0.6, 0.9, 1.2, 1.8, 2.4, 3 µM (lanes 1, 2, 3, 4, 5, 7, 8, 9, respectively) was incubated with 1.5 µM MgADP-actin. (h) Chick cofilin at 0, 0.6, 1.2, 1.8, 2.4, 3.6, 4.8, 6 µM was incubated with 1.5 µM MgATP-actin. An internal standard of 0.75 µM actin (g and h) or 2.5 µM actin (a, b, d, and e) was used in lane 6. Each lane was loaded with 12 µl of sample. The complex was separated from free actin by non-denaturing PAGE. Gels in (g and h) were stained with SYPRO red, and gels in (a, b, d, and e) were stained with Coomassie blue R-250. Binding curves obtained by quantitative densitometry of gels are shown in (c and i) for Mg-actin and in (f) for Ca-actin. The curves were fitted to the equation (see Materials and Methods) to give apparent dissociation constants.

Table 2.2: The apparent dissociation constants of ADF and cofilin for G-actin at ionic strength 20μ to 90μ:

Ionic	Chick ADF		Chick cofilin	
strength	MgATP-actin	MgADP-actin	MgATP-actin	MgADP-actin
20 μ	0.28 μM (4)	0.08 μM (4)		
34 μ	1.0 μM (4)	0.5 μM (4)	10.7 μM* (3)	0.5 μM* (3)
62 μ	1.3 μM (3)	0.29 μM (3)		
90 μ	12.4 μM (3)	3.2 μM (3)	>40 (3)	1.1 μM (3)

* SYPRO red staining.
Number in parentheses indicates number of replicate experiments.

detected by Coomassie blue staining when 5 μM MgATP-actin and increasing amounts of cofilin were used, whereas the free actin again disappeared from the gel. This finding suggested that cofilin-MgATP-actin nucleated assembly faster and/or had a much lower critical concentration than ADF-MgATP-actin such that cofilin induced actin filament formation (and thus the loss of actin from the gel) while ADF did not. Direct electron microscopic observation of negative stained preparations was used to verify that filaments formed in cofilin-MgATP-actin mixture as described below.

Cofilin promotes F-actin assembly

To further identify the nature of actin in the non-denaturing PAGE samples, electron microscopy of negatively stained samples was used. In the standard gel electrophoresis buffer (34μ and pH 8.4), no filaments were observed when 5 μM actin alone, or 5 μM actin and 2 μM ADF were incubated for 30 min (Fig. 2.5a, and 2.5b), whereas filaments were observed when 5 μM actin and 2 μM cofilin were used (Fig. 2.5c). Thus the actin in the cofilin samples used for the gels polymerized into filaments that could not enter the gel. These data suggest that cofilin, but not ADF, promotes actin filament assembly.

The critical concentration (Cc) of monomer for filament assembly was determined using a light scattering assay to quantify relative polymer mass. At near physiological ionic strength (100 mM KCl, 2 mM $MgCl_2$, 10 mM Tris, 0.2 mM ATP, 0.5 mM DTT, and 0.2 mM EGTA; calculated ionic strength 122 μ) and pH 7.8 (Fig. 2.6), the Cc of actin alone is 0.1 μM, and 0.8 μM in presence of 2.5 molar excess of cofilin and 6.2 μM in presence of 2.5 molar excess of ADF. The critical concentration of cofilin-actin is

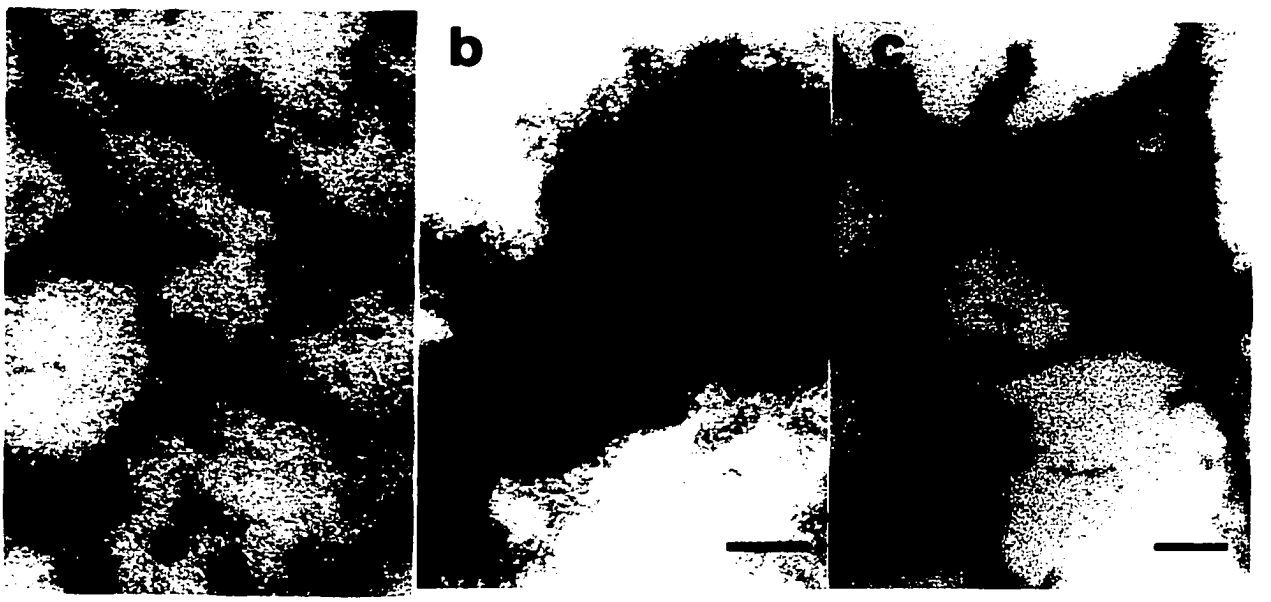


Figure 2.5
Cofilin promotes filament assembly. Samples prepared for electrophoresis on the non-denaturing gels were incubated for 30 min and aliquots were viewed by electron microscopy after negative staining. No filaments were found in the samples containing 5 μM actin either alone (a) or in the presence of 2 μM ADF (b). However, numerous actin filaments were observed in samples containing 5 μM actin and 2 μM cofilin in the same buffer (c). Bar: 200nm.

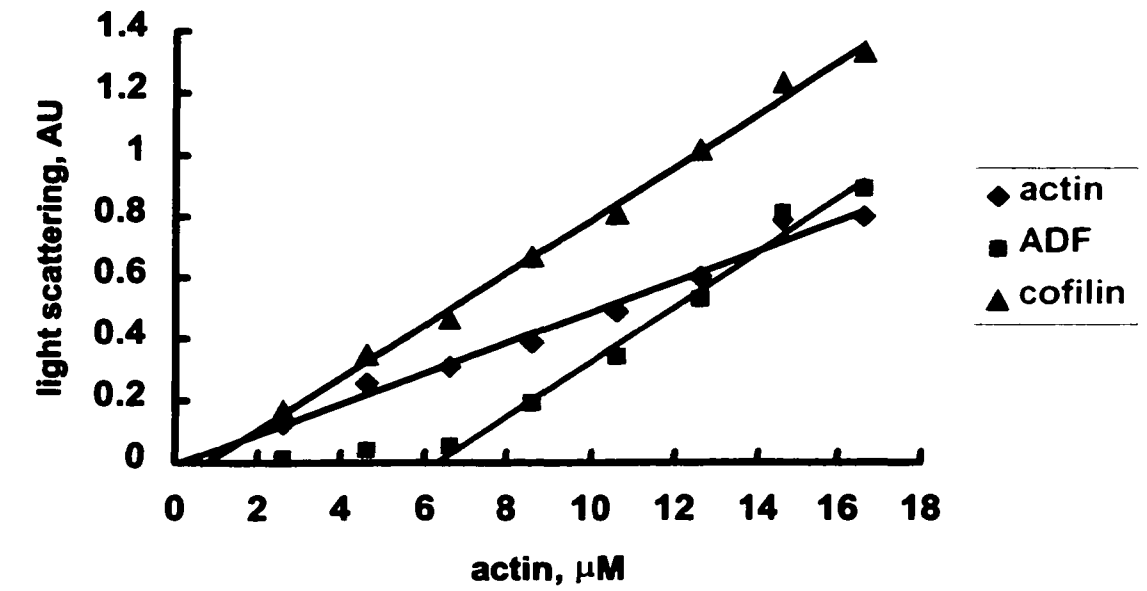


Figure 2.6
Cofilin-actin has lower critical concentration than ADF-actin. Actin from 2 to 16 μM was polymerized in F-buffer at pH 7.8 either in the absence or in the presence of 2.5 molar excess of cofilin or ADF. The light scattering signals of the sample at steady state were plotted against actin concentration. The intercepts reflect the critical concentration.

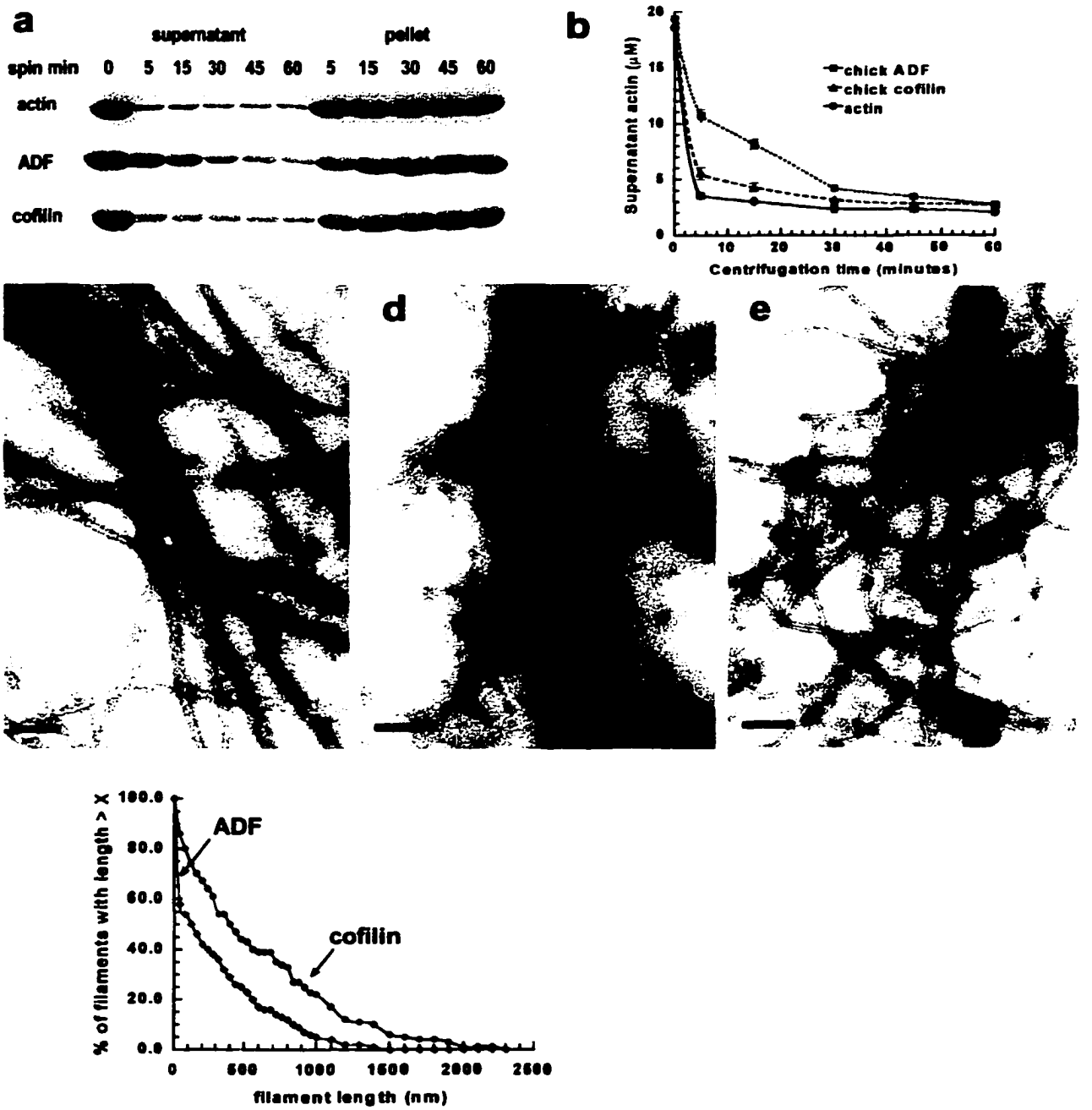
much lower than ADF-actin, but still higher than actin. Thus cofilin might promote filament assembly at the actin concentrations used for gel binding (5 μM) by severing newly formed actin filaments to serve as nucleation sites without depolymerizing the filament.

ADF enhances filament numbers compared to cofilin

A timed-sedimentation assay was performed in F-buffer at pH 8.1 containing ATP regenerating system with 20 μM actin to estimate the steady state distribution of filament lengths in mixtures of actin incubated with either ADF or cofilin in the presence of an ATP regenerating system. After centrifugation for 5 minutes at 436,000 g, the sedimented actin was 85% of total actin in samples containing F-actin alone and 75% in samples containing F-actin and cofilin in a 1:1 molar ratio (Fig. 2.7a, 2.7b). In the presence of ADF, less than 50% of the actin was sedimented within 5 minutes, whereas it took 35 minutes to sediment 75% of the actin (Fig. 2.7a, 2.7b). To determine the approximate sizes of filaments sedimented at the different times we calculated the clearance times from the sedimentation coefficient and the k factor. The sedimentation coefficients (s) were calculated assuming the actin filaments are rigid rods (Ralston, 1993; Cantor & Schimmel, 1980), though this is not the case with long filaments. The run time (in hours) required to pellet molecules equals the k factor (k = 7 for Beckman TLA 100 rotor at 436,000g) divided by the sedimentation coefficient of the molecule. Actin filaments containing more than 35 subunits (96 nm in length) would sediment within 5 minutes, whereas a tetrameric globular complex of two actin subunits and two ADF/cofilins would

Figure 2.7

ADF severs and depolymerizes filaments to a greater extent than cofilin. (a) 20 μM F-actin in the absence or in the presence of ADF or cofilin at 1:1 molar ratio was polymerized to steady state overnight at 4°C in an ATP regenerating buffer, pH 8.1 and centrifuged in a Beckman TLA100 rotor at 436,000g for times from 0 to 60 minutes. The supernatant and the solubilized pellet were subjected to SDS-PAGE and stained with Coomassie blue R-250. (b) The concentration of supernatant actin was plotted against the spin time. Within 5 minutes, greater than 80% of the actin pelleted in samples containing F-actin alone, and 75% of the actin pelleted in samples containing F-actin and cofilin. In the presence of ADF and actin, less than 50% of actin sedimented within 5 min and centrifugation for 35 min was required to pellet more than 75% of the actin. The error bars show the standard deviation from triplicate experiments. (c-e) Electron microscopy was used to observe the filament distribution in the starting mixtures. Short filaments predominated in samples containing actin and ADF (d), whereas long filaments predominated in samples containing actin alone (c) and actin and cofilin (e). (f) The filament length distribution of actin filaments in the presence of ADF (diamond) or cofilin (square). The total number of filaments measured is 191 in the presence of ADF and 105 in the presence of cofilin. Bar: 100 nm.



require 35 minutes to sediment, and actin dimer would require 55 minutes to sediment under the conditions used for these studies. Our data suggest that long filaments predominate in solutions containing either F-actin alone or F-actin with cofilin and that shorter but a more heterogeneous filament population predominates in F-actin solutions containing ADF.

Aliquots of samples prepared for the timed sedimentation experiment were applied to EM grids before sedimentation, negatively stained and observed with the electron microscope to examine the length distribution of the filaments (Fig. 2.7c-2.7e). Long and straight filaments were seen in F-actin (Fig. 2.7c), long and curved filaments predominate in cofilin-actin mixtures (Fig. 2.7e, 2.7f), whereas short filaments predominate in ADF-actin mixtures (Fig. 2.7d, 2.7f). The mean filament length is 575 nm in cofilin-actin mixture and 291 nm in ADF-actin mixture. The median length is 391 nm in cofilin-actin mixture and 89 nm in ADF-actin mixture. The short filaments could arise from the severing activity of ADF, but they could also arise from depolymerization and/or nucleation of new filaments. To address this question, we utilized a severing assay to measure the depolymerization from pointed ends following severing.

ADF is more effective in pH dependent filament severing/depolymerization than cofilin

A severing assay was performed with 3.3 μM F-Actin (5% pyrene label). The pointed end was capped by spectrin:actin:protein 4.1 complex and the barbed end was

capped by gelsolin:actin 1:1 complex which was maintained in excess. The depolymerization rate upon addition of DBP following treatment with ADF or cofilin was measured at pH 7.8 and pH 6.8 (Fig. 2.8). F-actin untreated with ADF or cofilin gave almost no change in assembly as measured by loss of pyrene fluorescence. What changes do occur are probably due to mechanical severing in transferring the sample to the cuvette. The initial depolymerization rate in the presence of ADF relative to actin alone is 22 at pH 7.8 and 11 at pH 6.8, much greater than that of cofilin (3 at pH 7.8 and 4 at pH 6.8). These findings demonstrate that both ADF and cofilin sever filament, but that there is either more severing in the presence of ADF than cofilin, or that ADF enhances subunit dissociation from the pointed ends of filaments better than cofilin, or both. The rate of depolymerization in the presence of ADF is also sensitive to pH, being more rapid at more alkaline pH. Since the enhancement of depolymerization following filament severing is proportional to number of severing events and the enhancement of off rate from pointed ends, we utilized a method developed by Moriyam & Yahara (1999) that permits measurements of severing and changes in pointed end off-rate in the same experiment.

Vitamin D binding protein and ADF compete for binding G-actin

An *in vitro* binding assay was used to determine the effect of DBP on the binding between ADF and G-actin. DBP binds actin monomer with nM affinity (Pollard & Cooper, 1986), about 100 to 1000-fold tighter binding than ADF, and displaces ADF from ADF-actin complex (Fig. 2.9). In the following filament depolymerization assay,

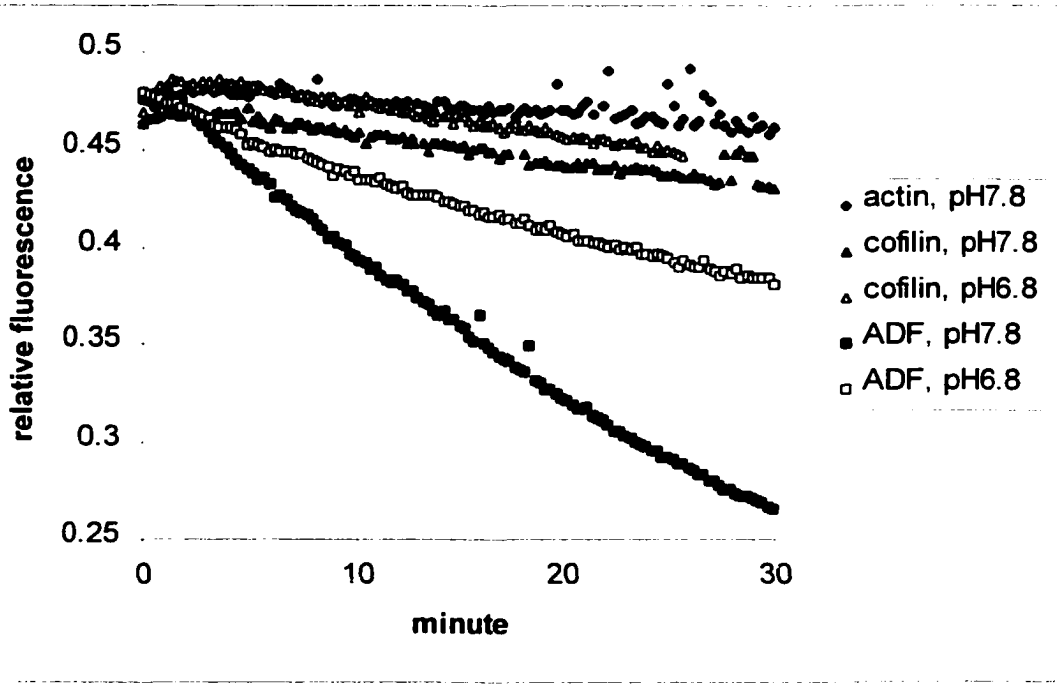


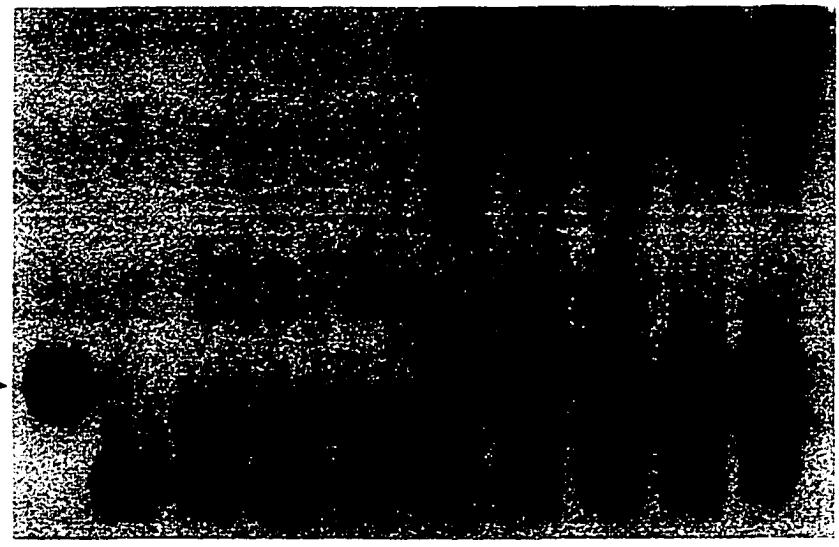
Figure 2.8
ADF is more effective than cofilin in depolymerizing filaments at physiological ionic strength. Spectrin:actin:protein 4.1 complex at 60 nM was used to nucleate 3.3 μM 5% pyrene-actin polymerization from barbed ends at pH 6.8 (open) or pH 7.8 (filled) to reach steady state. Then 200 nM gelsolin-actin 1:1 complex and 0.6 μM ADF (square) or 0.6 μM cofilin (triangle) were added to cap pointed ends and to sever filaments, respectively. The mixtures were incubated for 10 minutes, and DBP at 3.5 μM was added to induce depolymerization from pointed ends.

Figure 2.9

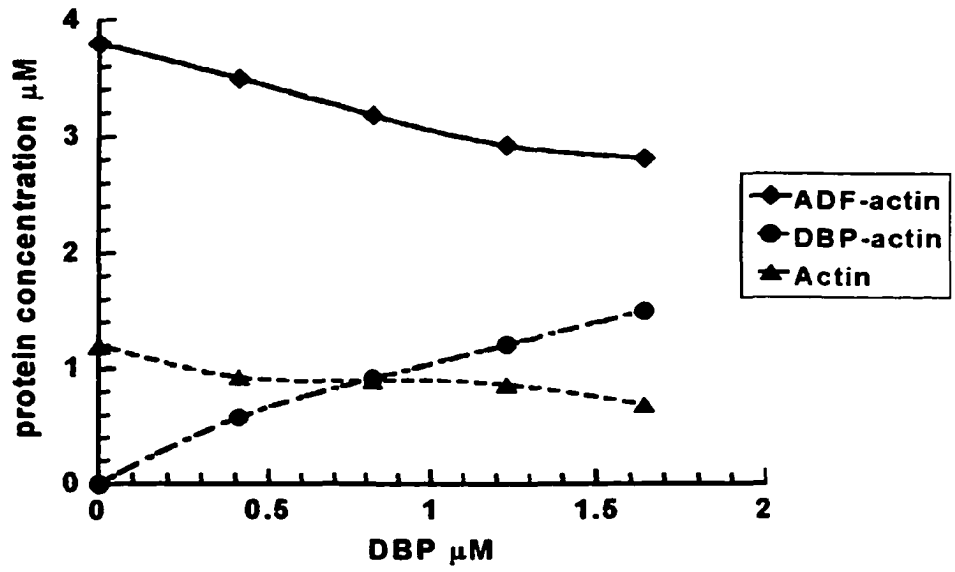
DBP quantitatively displaces actin from ADF-actin complex. (a) DBP at 0 to 4 μM was incubated with 5 μM MgATP-actin alone (lane 2 to 5) or DBP at 0 to 1.6 μM was incubated with 5 μM MgATP-actin and 15 μM ADF (lane 6 to 10). The free actin was well separated from ADF-actin complex and DBP-actin complex by non-denaturing PAGE. ADF-actin complex ran slightly slower than DBP-actin complex. The gel was stained with Coomassie blue R. (b) The amount of ADF-actin complex and DBP-actin complex were determined by quantitative densitometry and plotted against increasing concentration of DBP. The amount of DBP complex was stoichiometric with the amount of DBP added indicating a very tight complex.

a

Lane	1	2	3	4	5	6	7	8	9	10
DBP μM	4	0	0.8	1.6	4	0	0.4	0.8	1.2	1.6
actin μM	0	5	5	5	5	5	5	5	5	5
ADF μM	0	0	0	0	0	15	15	15	15	15



b



the addition of DBP would displace ADF/cofilin from G-actin, and allow it to recycle in filament depolymerization, thus increasing both depolymerization and quenching of pyrene-actin. However, neither of these would affect the initial depolymerization rates as is demonstrated below.

ADF is more effective in pH dependent filament severing and depolymerizing than cofilin

The severing/depolymerizing assay developed by Moriyama & Yahara (1999) is based upon the change in fluorescence of a small amount of pyrene actin used as a label for assembly. However, the fluorescence enhancement of pyrene actin upon assembly is partially quenched by the binding of ADF/cofilin (Carlier *et al.*, 1997). Since the amount of pyrene actin is only 5% of the total actin and since ratios of ADF/cofilin to total actin do not exceed 1:4, the maximum effect due to quenching should never exceed 25% of the total signal. In most cases it should be much less since in the pre-incubation step, before addition of DBP, quenching is allowed to reach a steady state. However, to be certain that our fluorescence measurements were tracking filament depolymerization and not changes in quenching, we first we compared the depolymerization as measured by light scattering and by fluorescence. The fluorescence quenching is less than 10% of the total signal when 5% of the total 3.3 μM actin is pyrene labeled and ADF is at 0.8 μM or less. Under these conditions, light scattering and fluorescence assays gave similar results (Fig. 2.10a-f). Since light scattering is noisier than fluorescence, we chose to use fluorescence for the depolymerization assay. With increasing concentrations of ADF or cofilin, the overall

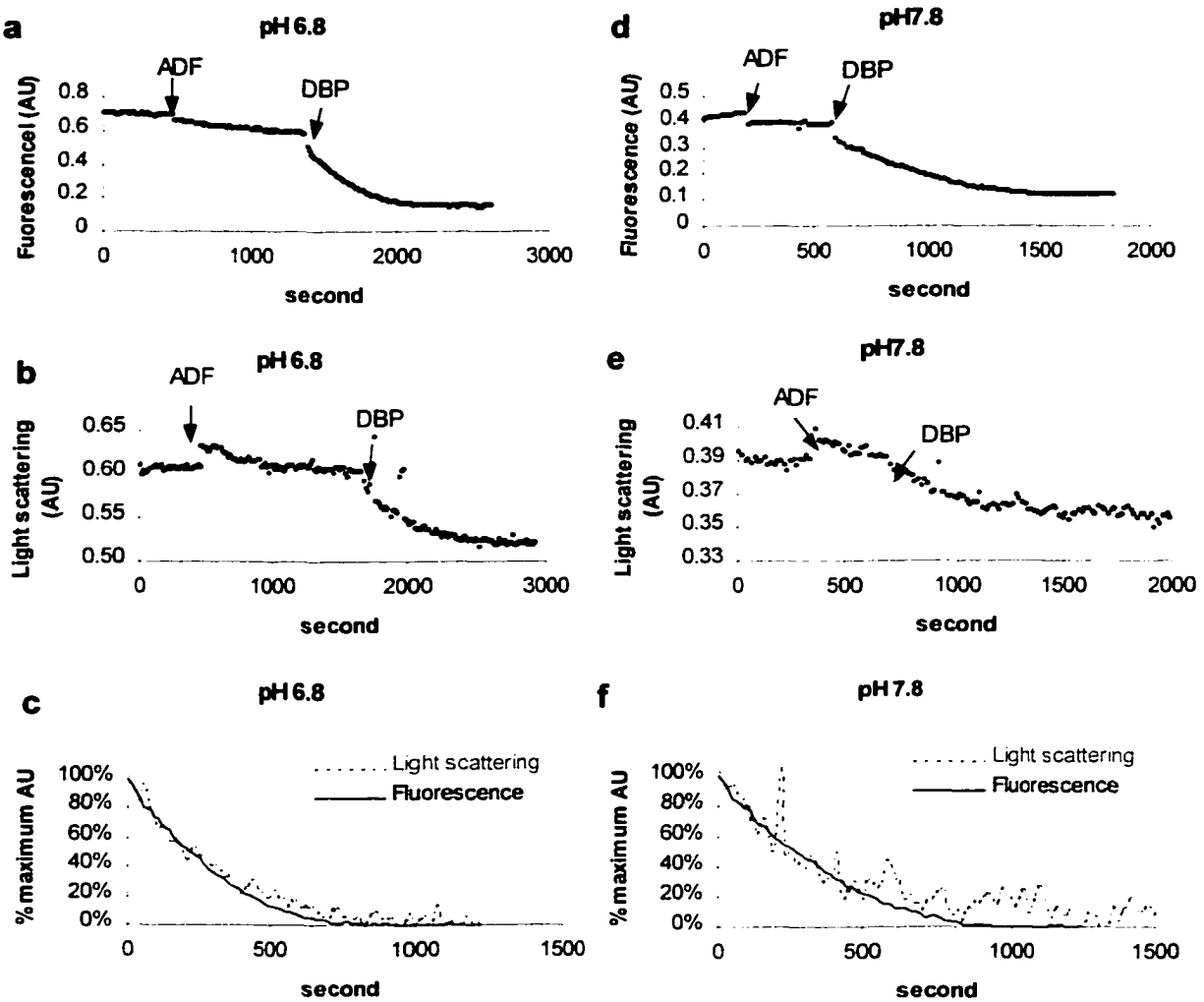


Figure 2.10.

Light scattering and fluorescence gave comparable results in determining the rate of filament depolymerization at pH 6.8 (a-c) and pH 7.8 (d-f). Gelsolin at 1:267 (a-c) or 1:200 (d-f) molar ratio to actin was used to nucleate 3.3 μM 5% pyrene-actin polymerization. At steady state, 0.6 μM ADF and gelsolin-actin 1:1 complex at 77 nM (a and b) or 154 nM (d and e) were added. The fluorescence (a and d) and light scattering (b and e) signals were traced until a new steady state was reached. Then DBP at 3.5 μM was added to induce further depolymerization. The traces of depolymerization induced by DBP via light scattering and fluorescence were overlaid in (c and f).

depolymerization was enhanced (Fig. 2.11e-f). At pH 6.8, cofilin (0.8 μM) increased the minus end off-rate from actin by 5 fold whereas ADF (0.8 μM) increased the minus end off-rate 40-fold (Fig. 2.11g-h). Both ADF and cofilin had little effect on filament severing at pH 6.8. Of particular interest, at pH 7.8, cofilin (0.8 μM) did not increase the minus end off-rate but it severed filaments to create an 8 fold increase in filament number whereas ADF (0.8 μM) increased the minus end off-rate 4-fold and increased filament number 20 fold. These results indicate that the filament depolymerizing and severing activities of both ADF and cofilin are sensitive to pH, that ADF is more pH sensitive than cofilin, and that ADF enhances overall depolymerization (including severing and depolymerizing) more effectively than cofilin.

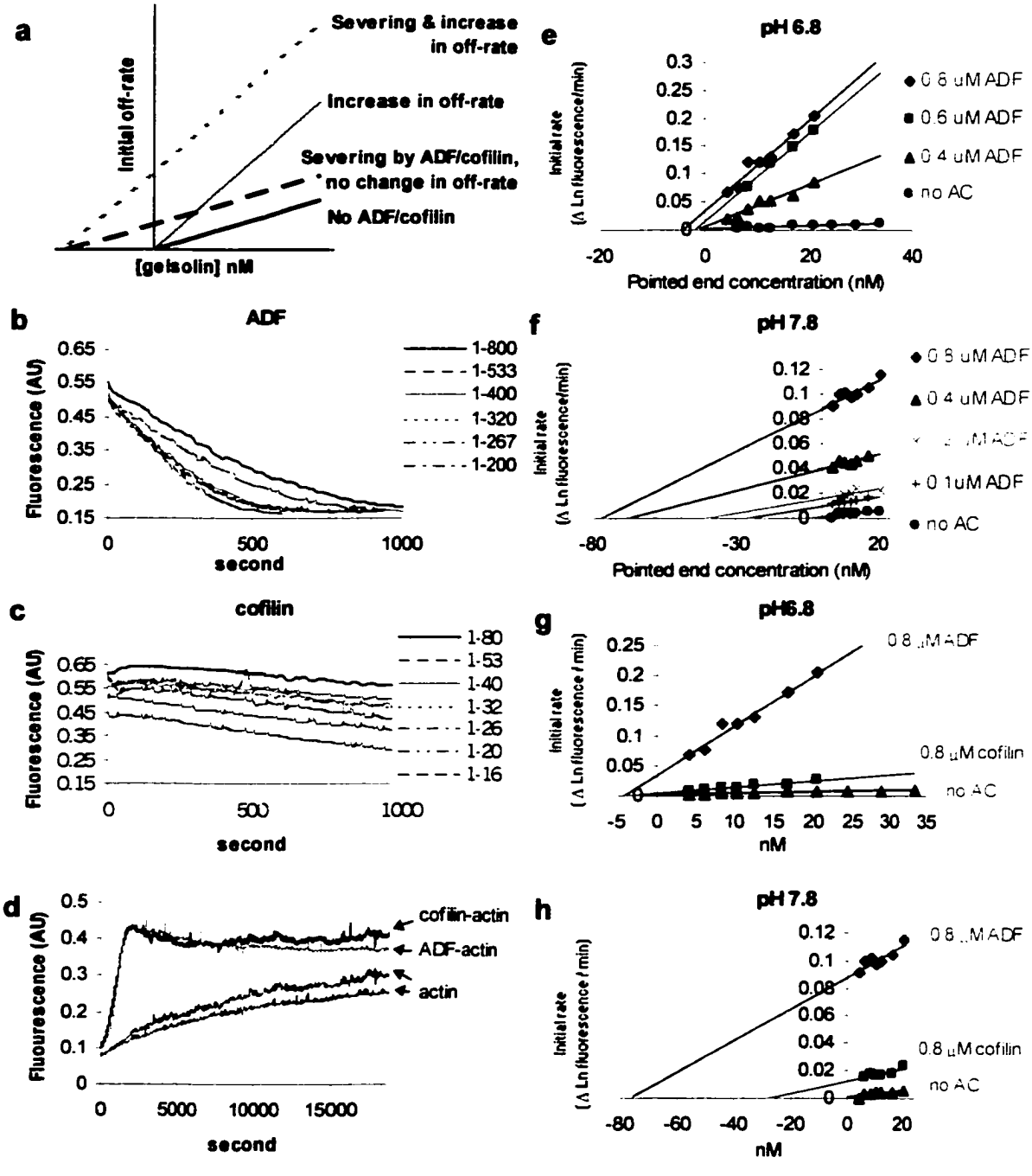
Discussion

Recombinant ADF and cofilin are homogenous

During ADF purification from chicken embryonic brain and cultured cells, a Green A-Sepharose column was introduced as a final step to bind active ADF (Giuliano *et al.*, 1988; Bamburg *et al.*, 1991). Immnoreactive forms of ADF with little or no activity did not bind to the Green A column and were separated from the active ADF at this step (Bamburg *et al.*, 1991). Recently, it was reported that *Dictyostelium* cofilin purified from vegetative cells has more effective filament severing and depolymerizing activity than recombinant *Dictyostelium* cofilin (Ichetovkin *et al.*, 2000). However, recombinant *Dictyostelium* cofilin purified utilizing binding and elution from a Green A column had activities more similar to the native protein, whereas the *Dictyostelium* cofilin that failed to bind to the Green A column had much reduced filament binding and severing.

Figure 2.11

ADF is more effective than cofilin in depolymerizing and severing filaments at physiological ionic strength. (a) A schematic plot of theoretical curves for initial off-rate vs. initial gelsolin concentration to illustrate how the curves would change if the ADF/cofilin effects were limited to severing (bold dashed line), enhancing off-rate (solid gray line) or both (dashed gray line). (b-c) Gelsolin nucleated F-actin (5% pyrene labeled) was pre-incubated with 77 nM gelsolin-actin 1:1 complex and 0.8 μM ADF (b) or 0.8 μM cofilin (c) for 10 minutes. The molar ratios of gelsolin to actin are shown on the panel. The fluorescence decrease upon the addition of DBP was plotted verse time. The drop in fluorescence is faster in samples pre-incubated with ADF than with cofilin. (d) The polymerization of 3.3 μM actin (5% pyrene labeled) in the absence or in the presence of 0.8 μM ADF or 0.8 μM cofilin in F-buffer, pH 6.8 was traced at 18°C. ADF and cofilin enhance the rate of actin polymerization to a similar extent. (e-h) The initial depolymerization rate of 3.3 μM F-actin pretreated with various concentrations of ADF or cofilin is plotted against gelsolin concentration. At pH 6.8 (e), ADF enhances the off-rate constant up to 40-fold over actin alone, but ADF had little effect on filament severing. At pH 7.8 (f), ADF severs filaments and increases filament number up to 20 fold. At both pH 6.8 (g) and pH 7.8 (h), ADF is more effective than cofilin in enhancing the overall depolymerization.



This finding suggests that the Green A resin is selective for binding the more active conformation of ADF. The recombinant ADF and cofilin used in our assay were bound to and eluted from Green A column as the final step in their purification. They have similar activities to those reported for the tissue derived proteins (Adams *et al.*, 1990). Job plots were also used to verify that actin and recombinant ADF or cofilin are homogenous for each preparation.

Chick ADF, but not chick cofilin, may act as a G-actin sequestering protein

In vivo the monomeric actin pool is about 100-fold higher than the critical concentration for actin under physiological ionic conditions *in vitro* (Zigmond, 1993). The increased monomer pool is attributed to the presence of many actin monomer binding proteins. Previous studies suggested ADF/cofilins do not act as major G-actin sequestering proteins *in vivo* because the monmeric actin pool *in vivo* is predominantly ATP-actin (Rosenblatt *et al.*, 1995) and several ADF/cofilins have 30 to 80-fold weaker affinities for MgATP-actin than for MgADP-actin under physiological ionic conditions (Carlier *et al.*, 1997; Blanchoin & Pollard, 1998; Ressad *et al.*, 1998). Here, using non-denaturing PAGE where no protein modification was used, we find that chick ADF has only a 2-4 fold difference in affinity for MgADP-actin and MgATP-actin at ionic strengths from 20 μ to 90 μ. Chick cofilin had much weaker affinity for MgATP-actin than MgADP-actin, which is consistent with previous studies examining plant ADF and amoeba actophorin (Carlier *et al.*, 1997; Maciver & Weeds, 1994; Blanchoin & Pollard, 1998). These results suggest that chick ADF might act as a major actin monomer sequestering protein whereas cofilin would not, given their affinities for ATP-actin and

their intracellular concentrations in the 10-25 μM range (Koffer *et al.*, 1988; reviewed in Bamburg, 1999). In embryonic chick brain, ADF is isolated bound to actin monomer on DNase affinity column whereas cofilin is not, even though both are present in significant amounts (Devineni *et al.*, 1999). Indeed, ADF is the second major actin monomer binding protein in embryonic brain and is present in amounts sufficient to sequester close to 20% of the actin monomer pool (Devineni *et al.*, 1999). Thus, chick ADF and chick cofilin, though they share 70% sequence identity, function differently in cells.

It is interesting to note that the interaction between cofilin and ATP vs ADP-G-actin is also affected by the nature of the divalent cation, Mg^{2+} or Ca^{2+}, bound to the actin. When Mg is bound to actin there is a 20-fold decrease in the apparent affinity of cofilin for ADP-actin, which does not occur if Ca is bound. The actin monomer has about a 4-5 fold higher affinity for Ca^{2+} over Mg^{2+} (Estes *et al.*, 1992), but in resting hepatocytes and polymorphonuclear leucocytes (PMNs), intracellular Mg^{2+} (~0.5 mM) is more than 1000-fold higher than intracellular Ca^{2+} (40~130 nM) (Gasbarrini *et al.*, 1992; Zaffran *et al.*, 1993). Thus the actin-bound divalent cation is thought be Mg^{2+} inside resting cells. Indeed, in skinned frog skeletal muscle, Mg^{2+} was found to be the divalent cation bound to F-actin in the thin filaments in situ (Kitazawa *et al.*, 1982). When hepatocytes are under anoxia or PMNs are chemoattractant-stimulated, Ca^{2+} influx significantly increases and average levels get up to 1.5 μM, about 10- to 40-fold higher than the resting level, whereas the intracellular Mg^{2+} level may double to 1.1 mM (Gasbarrini *et al.*, 1992). Thus, even when the concentration of intracellular Ca^{2+} reaches its highest average value, it is likely to be 1000 fold less than that of intracellular Mg^{2+}, and the actin-bound cation would still be Mg^{2+}. However, calcium concentration in

microdomains of the cell may transiently rise considerably above the 1.5 μM average up to the order of 300 μM (Llinas *et al.*, 1992). Within these domains, especially in cortical areas underlying calcium channels, a significant portion of the actin could possible exist in the Ca^{2+} form and thus show much less of a nucleotide dependence for ADF and cofilin binding.

Chick ADF is more effective in pH dependent filament depolymerization and severing and chick cofilin is more effective in promoting filament assembly

Whether ADF/cofilins sever actin filaments has been somewhat controversial. In early studies of chick ADF and porcine cofilin a weak filament severing activity was detected (Hayden *et al.*, 1993; Nishida *et al.*, 1984) suggesting that one mechanism for enhanced depolymerization was the generation of additional filament ends. The number of filaments eventually declined (Hayden *et al.*, 1993) suggesting re-annealing or subunit redistribution had occurred. However, Carlier *et al.* (1997) reported that recombinant *Arabidopsis* ADF1 enhanced both the association rate (up to 12 fold) at the barbed ends of filaments and the dissociation rate (up to 22 fold) at the pointed ends at pH 7.8 in the apparent absence of filament severing. Other workers reported less of an effect on subunit off-rates and more severing activity with human ADF, *Acanthamoeba* actophorin, *Dictyostelium* cofilin, and porcine cofilin (Maciver *et al.*, 1998; Moriyama & Yahara, 1999; Ichetovkin *et al.*, 2000). Some of these differences in ADF/cofilin activity could be due to a weaker severing activity of recombinant proteins compared to tissue derived proteins (Ichetovkin *et al.*, 2000), but as demonstrated here it is most likely due to inherent differences in the proteins themselves.

Moriyama and Yahara (1999) developed a novel assay that looked at both filament numbers (by trapping with a barbed end capping complex) and rates of subunit loss from the uncapped pointed ends. They found that porcine cofilin increased filament numbers by severing filaments with a maximum of about one severing event per 290 actin subunits at pH 7.1. Subunit off-rates were also enhanced to a maximum of about 6.4 fold. Mutant forms of cofilins were identified that differentially affected severing or the off-rate at the pointed end. Yeast cells expressing mutant cofilin with defects in severing are more impaired in their growth than those with defects in depolymerization, suggesting that the ability of ADF/cofilin to sever is an essential process and independent of depolymerization. Taken together, severing and depolymerization are two separable processes that account for the enhanced filament turnover rate by ADF/cofilins.

Here, we developed a new assay to measure the overall depolymerization rate following filament severing by ADF or cofilin. Since both ends of actin filaments are capped at the start of this assay, filament ends become available only if severing occurs. Results confirm the fact that both ADF and cofilin can sever F-actin. Chick ADF is more effective in depolymerizing filaments than chick cofilin and depolymerization is enhanced at alkaline pH. By applying the assay of Moriyama & Yahara (1999), we confirm the results of our other assays showing that chick ADF is more effective in filament severing and depolymerization than chick cofilin. Filament severing is favored at alkaline pH and an enhancement of off-rate from the pointed end is favored at acidic pH. Thus, ADF is more effective at increasing the overall filament depolymerization than cofilin. In addition, chick cofilin enhances filament assembly through nucleation.

These data help explain the intracellular changes observed upon over-expressing either of these proteins. Overexpression of *Dictyostelium* cofilin by seven-fold induced a 4-fold increase in F-actin and little change in G-actin (Aizawa *et al.*, 1996). The overexpression of cofilin also stimulated cell motility and membrane ruffling. In contrast, cells expressing the active and non-phosphorylatable XAC(A3)-GFP gradually round up and lose their ability to adhere to the substratum as expression increases, suggesting the assembled actin pool is decreased (Bamburg, 1999). Wounding a monolayer of cells transiently increases intracellular pH and induce a translocation of ADF, cofilin, and actin to the edge of newly expanded lamellipodia (Bernstein et al., 2000). Blockage of the transient alkaline shift blocks lamellipodial expansion at the leading edge. Lamellipodial expansion is an actin assembly driven process that requires the exposure of barbed ends. The transient alkaline shift could activate the severing activity of ADF and induce more free barbed ends for filament assembly. Blocking the alkaline shift might prevent the severing by ADF and cofilin, and prevent the formation of newly formed plus ends, thus inhibiting filament assembly. Although the pH-induced changes in lamellipodial expansion could be due in part or in toto to other pH-sensitive proteins, the inhibition of barbed-end generation in adenocarcinoma cells, stimulated to extend a lamellipod by EGF treatment by microinjection of antibody to ADF/cofilin (Chan *et al.*, 2000) or by overexpression of Lim kinase to inactivate ADF/cofilin (Zebda *et al.*, 2000), strongly suggests ADF/cofilin proteins play a major role in barbed-end generation.

ADF and cofilin enhance actin assembly from filament severing

Arabidopsis ADF1 and yeast cofilin have been reported to promote filament assembly at barbed ends (Carlier *et al.*, 1997). The enhancement could arise from increased assembly rate, nucleation, and/or increased number of ends through severing. Our data shows an equal enhancement of actin polymerization by ADF and cofilin at pH 6.8, which is consistent with the weak but equal severing activity of ADF and cofilin at pH 6.8. These data indicate the promotion of filament assembly by ADF/cofilin is likely due to the increased number of ends through filament severing. The critical concentration of cofilin-actin is much lower than that of ADF-actin at pH 7.8. Thus when the actin monomer concentration is between these two values, ADF will sever and depolymerize filaments that may nucleate spontaneously whereas cofilin will sever these and provide more nucleation sites for filament growth. This is consistent with our EM observations that show cofilin, but not ADF, causing actin filament formation in our non-denaturing PAGE buffer when actin was at 5 μM.

The steady state supernatant actin concentration from the timed sedimentation assay should be identical to the actin critical concentration. However, the values from our timed sedimentation assay for actin alone, ADF-actin, and cofilin-actin differ from the values from our light scattering assay but are very close to each other. The difference between the results of the two assays could arise in part from the different experimental conditions including pH and buffer system (containing ATP regenerating system in the timed sedimentation assay). However, it is more likely that the higher values from the timed sedimentation assays arose because we removed the entire supernatant and mixed it prior to gel analysis. In that case, if small actin oligomers were loosely "pellet" at the

bottom of the tube, they could easily have been disturbed in sampling and contribute to the actin in the supernatant.

In conclusion, chick ADF severs and depolymerizes filaments and sequesters monomers more effectively than chick cofilin. The severing by ADF is favored at pH 7.8 and enhancement of pointed end off-rates is favored at pH 6.8 (Fig. 2.12). Chick cofilin promotes cofilin-actin filament assembly by nucleation. Thus, ADF and cofilin function differently in regulating actin filament dynamics.

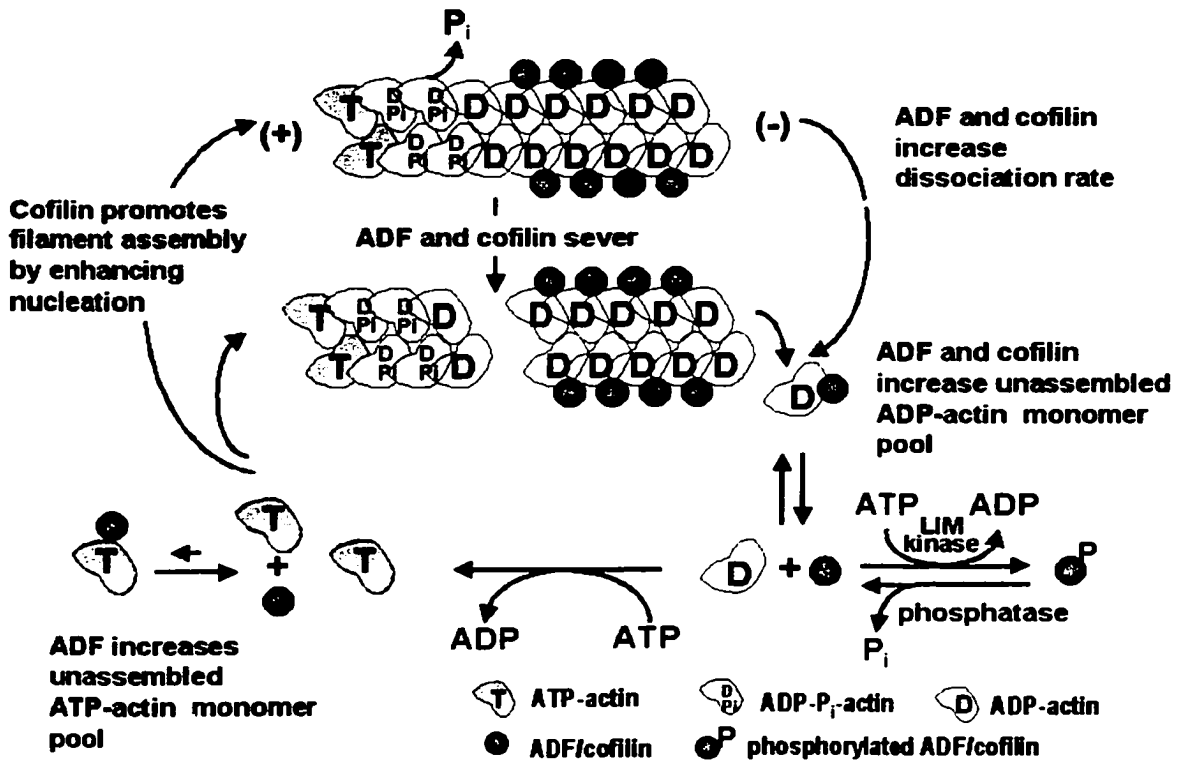


Figure 2.12. ADF and cofilin enhance the turnover of actin filaments. ADF and cofilin increase the overall depolymerization by filament severing and enhancement of off-rate constant. The enhancement of off-rate by ADF and cofilin is favored at acidic pH. Filament severing is favored at alkaline pH. ADF is more effective than cofilin in both processes. Cofilin promotes filament assembly by enhancing nucleation through severing of newly formed filaments. The critical concentration of cofilin-actin is close to that of actin alone but much lower than that of ADF-actin. Both ADF and cofilin increase the unassembled ADP-actin monomer pool but this increase is very small for cofilin and large for ADF. ADF, but not cofilin, can also increase the predominant ATP-actin monomer pool *in vivo*.

Chapter Three

Classification of ADF/cofilin family proteins based on their biochemical properties

Preface and Acknowledgement

The research presented in this chapter will be submitted for publication. The order and list of the authors is as follows: Hui Chen, Judy Sneider, Laurie Minamide, and James R. Bamburg. The data presented in this chapter were acquired and analyzed solely by Hui Chen. Dr. Kris Gunsalus provided the expression plasmid for *Drosophila* twinstar and Laurie Minamide and Kris Gunsalus purified the recombinant twinstar. Dr. Sharon Ashworth provided the expression plasmid for *Arabidopsis* ADF1 and some of the recombinant ADF1 protein. Dr. Hiroshi Abe provided the expression plasmids for *Xenopus* XAC1 and XAC2. Drs. Laurent Blanchoin, Shoichio Ono, John Condeelis, David Drubin, and Issei Mabuchi provided recombinant *Acanthamoeba* actophorin, recombinant *Caenorhabditis* UNC-60A and UNC-60B, recombinant *Dictyostelium* cofilin, recombinant yeast cofilin, and endogenous starfish depactin, respectively.

Summary

The ADF/cofilins are an essential group of actin filament depolymerizing and severing proteins that regulate actin filament turnover *in vivo*. In most multi-cellular organisms, two or more members are co-expressed in many cell types. Each member has qualitatively similar interactions with actin, but differences have been reported in their pH sensitivity and *in vitro* activities. We previously showed that chick ADF and chick cofilin have quantitative differences in their interactions with G- and F-actin suggesting different roles in regulation of actin filament dynamics. Chick ADF has higher affinity for MgATP-actin and causes a higher critical concentration for assembly of actin than cofilin, suggesting chick ADF might function as an actin monomer sequestering protein. Chick ADF severs and depolymerizes filaments more effectively than chick cofilin and shows a greater pH-dependence for both activities. To further classify other members of the ADF/cofilin family based on their biochemical properties, we compared their binding affinities to G-actin and filament severing and depolymerizing activities using the same assays we previously used to compare chick ADF and chick cofilin (see Chapter Two). Non-denaturing polyacrylamide gel electrophoresis was used to measure the apparent affinity for G-actin, timed sedimentation was used to compare steady state filament length distribution, and a fluorescence severing assay was used to compare their effectiveness in filament depolymerization (combination of severing and off-rate enhancement). Most ADF/cofilins from different species fall into one of two groups based upon their behavior. The ADF-like group includes Chick ADF, *Xenopus* XAC1, and starfish depactin. This group has higher affinities for MgATP-G-actin than the cofilin-like group, and has greater severing and/or depolymerizing activity that is pH

dependent. The cofilin-like group includes chick cofilin, *Drosophila* twinstar, *Acanthamoeba* actophorin, *Arabidopsis* ADF1, *Caenorhabditis* UNC60B, and *Dictyostelium* cofilin. Proteins in this group have a significantly (>10X) weaker affinity for MgATP-G-actin than for MgADP-actin, and/or have less severing, and/or depolymerizing activity than the ADF-like group.

Introduction

The actin-binding proteins in the ADF/cofilin family are ubiquitous among eukaryotes. They are essential to cell division (Gunsalus *et al.*, 1995; Abe *et al.*, 1996). ADF/cofilins play an essential role in accelerating filament turnover needed for many actin-based processes in non-muscle cells (reviewed in Chen *et al.*, 2000). Actin depolymerizing factor (ADF), the first member of this family, was initially isolated from embryonic chick brain (Bamburg *et al.*, 1980). Cofilin was subsequently identified as an F-actin co-filamentous protein from porcine brain (Nishida *et al.*, 1984). Chick ADF and chick cofilin are 70% identical in amino acid sequence (Abe *et al.*, 1990). Homologues of ADF/cofilin have been independently identified in many eukaryotes and some have been named by sequence comparison either as ADF or as cofilin. Representatives of this family include: *Acanthamoeba castellanii* actophorin (Cooper *et al.*, 1986; Quirk *et al.*, 1993), *Dictyostelium discoideum* cofilin (Aizawa *et al.*, 1995), *Saccharomyces cerevisiae* cofilin (Moon *et al.*, 1993; Iida *et al.*, 1993), *Toxoplasma gondii* ADF (Allen *et al.*, 1997), *Arabidopsis thaliana* ADF (Carlier *et al.*, 1997), *Zea mays* ADF 1-3(Rozycka *et al.*, 1995), *Caenorhabditis elegans* UNC-60A and UNC-60B (McKim *et al.*, 1994; Ono & Benian, 1998), *Drosophila melanogaster* Twinstar or D61 (Gunsalus *et al.*, 1995;

Edwards *et al.*, 1994), *Asterias amurensis* depactin (Mabuchi, 1983), *Xenopus laevis* XAC (Abe *et al.*, 1996), *Gallus gallus* ADF (Bamburg *et al.*, 1980), *Gallus gallus* cofilin (Abe *et al.*, 1990), *Homo sapiens* and *Sus scrofa* ADF or destrin (Berl *et al.*, 1983; Nishida *et al.*, 1985; Hawkins *et al.*, 1993), and *Homo sapiens* and *Sus scrofa* cofilin (Nishida *et al.*, 1984; Ogawa *et al.*, 1990; Gillett *et al.*, 1996). All of these proteins share 25-90% identity in amino acid sequence (Lappalainen *et al.*, 1998).

ADF/cofilins have qualitative similarities interacting with actin (Abe *et al.*, 1990; Adams *et al.*, 1990). They bind to monomeric actin in a 1:1 complex and inhibit nucleotide exchange (Hayden *et al.*, 1993; Nishida *et al.*, 1984; Hawkins *et al.*, 1993). *Arabidopsis* ADF1, actophorin, and human ADF are reported to bind to ADP-G-actin with about 30 to 80 fold higher affinity than they bind to ATP-G-actin at physiological ionic strength (Carlier *et al.*, 1997; Blanchoin & Pollard, 1998; Ressad *et al.*, 1998). With the exceptions of ADF/cofilin from yeast and *Dictyostelium*, the activity of ADF/cofilin is inhibited by phosphorylation at ser3 (Morgan *et al.*, 1993; Agnew *et al.*, 1995) or its equivalent ser6 in plants (Smertenko *et al.*, 1998) or ser2 in amoeba actophorin (Blanchoin *et al.*, 2000b). The crystal structures of actophorin (Leonard *et al.*, 1997), yeast cofilin (Fedorov *et al.*, 1997), *Arabidopsis* ADF1 (Bowman *et al.*, 2000), and the NMR structure of human ADF (Hatanaka *et al.*, 1996) have been reported. All contain six β-strands and four α-helices, with the exception of human ADF, which has an additional helix 1, and *Arabidopsis* ADF1, which is missing helix-4.

Two or more ADF/cofilins are often found in the same cell in a multicellular organisms, but only one member is expressed in single cell organisms and it can function well to fulfill its need. Multiplicity of ADF/cofilins in multicellular organisms is

important for their tissue specific and differential regulation of expression. In maize, three ADF/cofilin homologues, ZmADF1-3 have been found (Lopez *et al.*, 1996). ZmADF1 and ZmADF2 are expressed in pollen, whereas ZmADF3 is expressed in tissues other than pollen. ZmADF3 is involved in the reorganization of actin filaments during root sprouting (Jiang *et al.*, 1997). The expression of ADF and cofilin also show a temporal and tissue specific regulation in animals. In chick muscle cells, ADF and non-muscle cofilin decrease as muscle develops whereas muscle cofilin levels increase (Shinagawa *et al.*, 1993). In chick brain, ADF levels are much higher than cofilin levels throughout development (Devineni *et al.*, 1999). The tissue- and temporal-specific expression of each ADF/cofilin homologue indicates there is differential gene regulation. Over-expression of β-sm actin, a mutant human actin that does not polymerize normally, in mouse myoblasts (C2 cells) selectively down-regulates expression of mouse ADF and actin at both the protein and mRNA level, but not the expression of cofilin or several other actin binding proteins (Minamide *et al.*, 1997). These results demonstrate ADF but not cofilin expression responds to the actin monomer pool. In degenerating denervated and dystrophic muscles, cofilin, but not ADF, is significantly increased in amount (Shinagawa *et al.*, 1993). Thus, one reason for the multiple isoforms of these proteins is to allow different mechanisms for regulating their expression.

The pH dependence on activity differs from one ADF/cofilin homologue to another. Human ADF showed a greater pH-dependent F-actin depolymerizing activity than *Arabidopsis* ADF1 (Ressad *et al.*, 1998), whereas actophorin showed no pH sensitivity (Maciver *et al.*, 1998). In cells, ADF co-localizes more with G-actin at higher pH (7.4) and with F-actin at lower pH (6.8), whereas cofilin/actin co-localization is less

pH-dependent in the same cells (Bernstein *et al.*, 2000).

Some quantitative differences have been reported among ADF/cofilin homologues for their binding to actin. Human ADF has higher binding affinity to ATP-G-actin than does actophorin (Maciver *et al.*, 1998) suggesting human ADF, but not actophorin, might be one of the sequestering protein to increase the predominantly MgATP-actin monomer pool *in vivo*. The extent of enhanced barbed end assembly by different ADF/cofilins varies. *Arabidopsis* ADF1 and yeast cofilin, but not actophorin, have been reported to promote filament assembly at barbed ends by increasing assembly rate and/or nucleation (Carlier *et al.*, 1997), although an increased number of ends through severing could also explain these results.

We previously reported that there are quantitative differences between chick ADF and chick cofilin in their interactions with G- and F-actin that could be functionally important. Chick ADF has only a 2-4 fold difference in affinity for binding MgATP-actin vs. MgADP-actin, whereas cofilin shows a 20-40 fold difference, suggesting that ADF, but not cofilin, could play a role as a sequestering protein for the primarily ATP-G-actin monomer pool. Chick ADF is also more effective in filament severing and depolymerization than chick cofilin and shows a greater pH-dependence in these activities. There is so far no obviously defined structure-function correlation that predicts the differences in biochemical properties of ADF/cofilin proteins. This study attempts to classify ADF/cofilin proteins based on their biochemical activities.

Materials and Methods

Proteins

CaATP-G-actin was extracted from chick muscle acetone powder and purified according to Pardee & Spudich (1982). G-actin was gel filtered over Sephadex G-150 in G-buffer (2 mM Tris, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.01% NaN_3, pH 8.0), and the final ATP was adjusted to 0.5 mM prior to freezing 100 µl aliquots in liquid nitrogen. MgATP-G-actin was prepared from CaATP-G-actin by incubation with 0.2 mM $MgCl_2$ and 0.1 mM EGTA. The MgATP-G-actin was converted to MgADP-actin by the addition of 1 mM glucose and 20 units/ml hexokinase and incubation for 1 hour (Gershman *et al.*, 1989). Recombinant *Xenopus* XAC and human ADF were prepared as described (Abe *et al.*, 1996; Hawkins *et al.*, 1993) with a final binding and elution from Green A-agarose as described for chick ADF (Giuliano *et al.*, 1988). Recombinant *Drosophila* twinstar was expressed in *E. coli* strain BL21pLysS, and purified by chromatography on DEAE-cellulose and Green A resin, similar to the purification scheme for recombinant chick ADF (Adams *et al.*, 1990). Recombinant *Arabidopsis* ADF1 was expressed in *E. coli* strain BL21DE3 as a GST-ADF1 fusion protein, purified on a glutathione column, and cleaved by thrombin. *Acanthamoeba* actophorin, *Saccharomyces* cofilin, starfish depactin, *Dictyostelium* cofilin, and *Caenorhabditis elegans* UNC-60A and UNC-60B were kindly provided by Drs. Blanchoin, Drubin, Mabuchi, Condeelis, and Ono, respectively. ADF/cofilins were dialyzed against 2 mM Tris, pH 8.4 with 0.5 mM DTT before incubation with actin for non-denaturing polyacrylamide gel electrophoresis (PAGE). The concentration of actin was determined spectrophotometrically using the extinction coefficient of 0.63 l/g at 290nm (Houk & Ue,

1974). The extinction coefficients at 280 nm are 19540 $M^{-1}cm^{-1}$ for *Xenopus* XAC1, 16530 $M^{-1}cm^{-1}$ for *Drosophila* twinstar, 12690 $M^{-1}cm^{-1}$ for *Arabidopsis* ADF1, 12530 $M^{-1}cm^{-1}$ for *Acanthamoeba* actophorin, and 8040 $M^{-1}cm^{-1}$ for *Caenorhabditis* UNC-60A and UNC-60B, respectively, calculated by the method of Gill & von Hippel (1989). The concentrations of ADF/cofilins were first calculated by spectrophotometric measurement in G-buffer, then checked by quantification on Coomassie Blue R-250 stained SDS-polyacrylamide gels using actin for generating a standard curve. AC concentrations by this method were always within 20% and usually within 01% of the spectrophotometrically determined value.

Gelsolin-actin 1:1 complex was prepared by mixing equal molar ratio of gelsolin and G-actin in the presence of 10 mM $CaCl_2$, and incubated for 10 minutes. Then 30 mM EGTA was added. The complex was gel filtered over Sephadex G-150 in 10 mM HEPES, pH 7, 50 μM $MgCl_2$, 0.1 mM EGTA, 1 mM NaN_3, and 0.2 mM DTT. Human Vitamin D-binding protein (DBP), also called Gc-globulin, was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA). Spectrin:actin:protein 4.1 complex was kindly provided by Drs. James Casella and Susan Craig.

Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was used to study the effect of actin bound nucleotide and divalent cation on the interaction between ADF/cofilin and G-actin. Details are described in Chapter Two. In brief, ADF/cofilins at concentrations ranging from 0 to 10 μM were mixed with 5 μM G-actin in 2 mM Tris, pH 8.4 and 0.5 mM DTT containing either 0.2 mM ATP or ADP. The mixtures were incubated for 15

minutes, the free actin and the complex of actin-ADF/cofilin were then separated by electrophoresis on 7.5% non-denaturing polyacrylamide gels at 4°C. The running buffer contained 50 mM bicine, 40 mM triethanolamine, pH 8.4, 0.2 mM ATP or ADP at a calculated ionic strength of 34 μ. If assaying for the interaction with Mg-actin, 5 mM EGTA was included. Gels were stained with Coomassie Blue R-250. An internal actin standard of 2.5 μM was run on each gel to normalize the staining on each gel in order to quantify the concentration of free actin and ADF/cofilin-actin complex. Digitized images were obtained with a Photometrics chilled CCD camera, band densities were quantified using 1D Phoretix software (Non Linear Dynamics Ltd, England), and analyzed by Kaleidagraph analysis/graphics software (Synergy Software, Reading, PA). An estimate of the apparent dissociation constant (K_{Dapp}), which is defined by the equation (K_D = $[Actin]_f [ADF/cofilin]_f / [Actin\text{-}ADF/cofilin]$), was obtained by curve fitting to the transformed equation: ([AC-actin] = ([AC]$_t$+K_D+A)/2– ([AC]$_t$*[AC]$_t$+2*(K_D-A)*[AC]+(K_D+A)*(K_D+A)))^0.5/2. Since this is not a true equilibrium system, measurements are reported only as $K_D$app.

Job Plot

The method of continuous variation, also known as the Job plot, is an efficient approach to determine the binding stoichiometry of two components (Huang, 1982). Because ADF/cofilin proteins bind to G-actin at a 1:1 molar ratio (Giuliano *et al.*, 1988; Nishida *et al.*, 1984), the Job plot was used to determine if the different ADF/cofilin preparations and actin were equally active. The total molar concentration of ADF/cofilin and actin was held constant at 5 μM, but the mole fraction of each was varied. CaATP-

actin was used in the interaction with XAC1 and CaADP-actin was used in the interaction with twinstar, actophorin, *Arabidopsis* ADF1, UNC 60A, UNC 60B, and human ADF. The molar ratio of ADF/cofilin to actin at maximum binding would show a substantial deviation from its position of 1:1, if preparations contain more than 20% of inactive protein, but no deviation from a 1:1 ratio was found for any of the ADF/cofilins.

Timed sedimentation assay

The steady-state filament length distribution in the presence of ADF/cofilins was studied by a timed sedimentation assay. Actin at 20 μM was polymerized alone or in the presence of 20 μM ADF/cofilins in F-buffer at pH 8.1 (15 mM Tris / 15 mM PIPES, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM EGTA, 0.2 mM ATP) containing an ATP regenerating system (10 u/ml creatine phosphokinase, and 10 mM creatine phosphate). The samples were incubated at 4 °C overnight and then centrifuged at 4°C from 5 minutes to 60 minutes at 436,000g in a Beckman TLA100 rotor. The total supernatant was removed and mixed to insure uniform sampling, and the pellet was resuspended in SDS-containing buffer (0.25 M Tris, pH6.8, 10% glycerol, 10% β-mercaptoethanol, and 1% SDS). Fractions from supernatant and pellet were analyzed for the amount of actin by SDS-PAGE.

SDS-PAGE

Samples with unknown protein concentration and 5 actin standards (0.1 μg to1.6 μg) were loaded on an 18-well SDS-polyacrylamide mini gel (15% T, 2.67% C) (Laemmli, 1970). After electrophoresis, the gel was stained with Coomassie Blue R-250.

The gel image was digitized using a CCD camera and analyzed using the 1D Phoretix program (Synergy Software, Reading, PA). The amount of protein in each sample was obtained from the actin standard curve, which was determined to be in the linear range for analysis.

Measurement of filament severing

Filament severing by different ADF/cofilins was measured by relative depolymerization from pointed ends. In this assay, spectrin:actin:protein 4.1 complex at 60 nM was mixed with 3.3 μM actin containing 5% pyrene label in F-buffer (100 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, and 10 mM PIPES at pH 6.8 or 10 mM Tris at pH 7.8) to nucleate filament growth from the barbed ends. In preliminary experiments following assembly for 60 minutes, it was determined that steady state was reached in 6 minutes. When steady state was reached, 200 nM gelsolin-actin 1:1 complex and 0.6 μM ADF/cofilin were added and the sample incubated for 10 minutes. Gelsolin-actin 1:1 complex will cap free barbed ends including initial barbed ends and those generated by severing. DBP, a potent actin monomer sequestering protein, was then added to sequester monomers and thus induce depolymerization from the pointed end of the filaments by lowering the actin concentration to below the critical concentration. Changes in fluorescence intensity were monitored at 18 °C using AVIV ATF 105 spectrofluorometer. The wavelength was 365 nm for excitation and 404 nm for emission. The bandwidth was 2 nm for excitation and 4 nm for emission.

Results

Binding of ADF/cofilin proteins to G-actin

The purity of actin and ADF/cofilin proteins used here was obtained from densitometric scanning of Coomassie Blue R-250 stained SDS-polyacrylamide gels (Fig. 3.1). The purity was estimated to be 87% for human ADF, 97% for XAC1, 94% for XAC2, 95% for chick ADF(S3E), and greater than 98% for the other ADF/cofilin proteins and actin.

Job plots of ADF/cofilin binding to G-actin were used before each experiment to verify that each protein was active and homogeneous with respect to actin binding. The maximum formation of the ADF/cofilin-actin complex was reached at 1:1 molar ratio of ADF/cofilin to actin for *Xenopus* XAC1 (Fig. 3.2a-b), *Drosophila* twinstar (Fig. 3.2c-d), *Acanthamoeba* actophorin (Fig. 3.2e-f), *Arabidopsis* ADF1 (Fig. 3.2g-h), *Caenorhabditis* UNC-60A (Fig. 3.2i-j), UNC-60B (Fig. 3.2k-l), and human ADF (Fig. 3.2m-n). Chick ADF and chick cofilin were shown previously (chapter 2) to bind actin homogeneously and with 1:1 stoichiometry. Thus, our preparations of ADF/cofilin and actin do not contain a significant portion of inactive protein. We were unable to characterize yeast cofilin using this assay since there was not a significant mobility shift between the complex and G-actin.

The effect of actin bound nucleotide on the interaction between G-actin and ADF/cofilin.

Non-denaturing PAGE was used to determine the apparent dissociation constants for different ADF/cofilins from G-actin in a titration binding analysis. Under low ionic

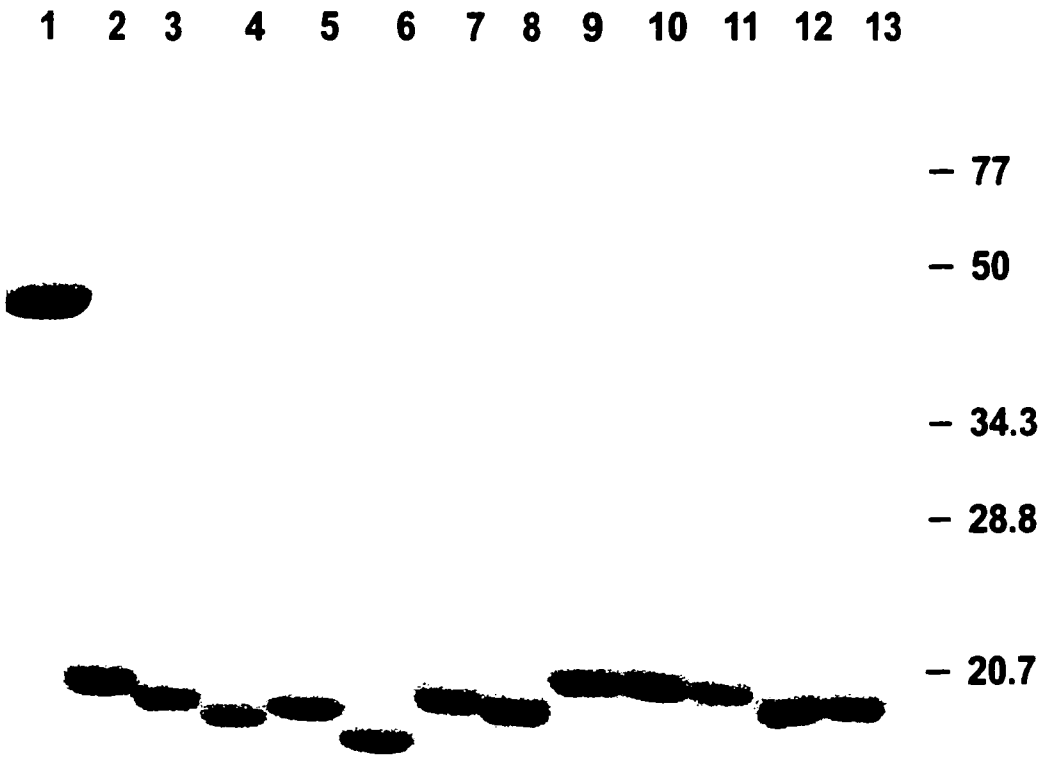
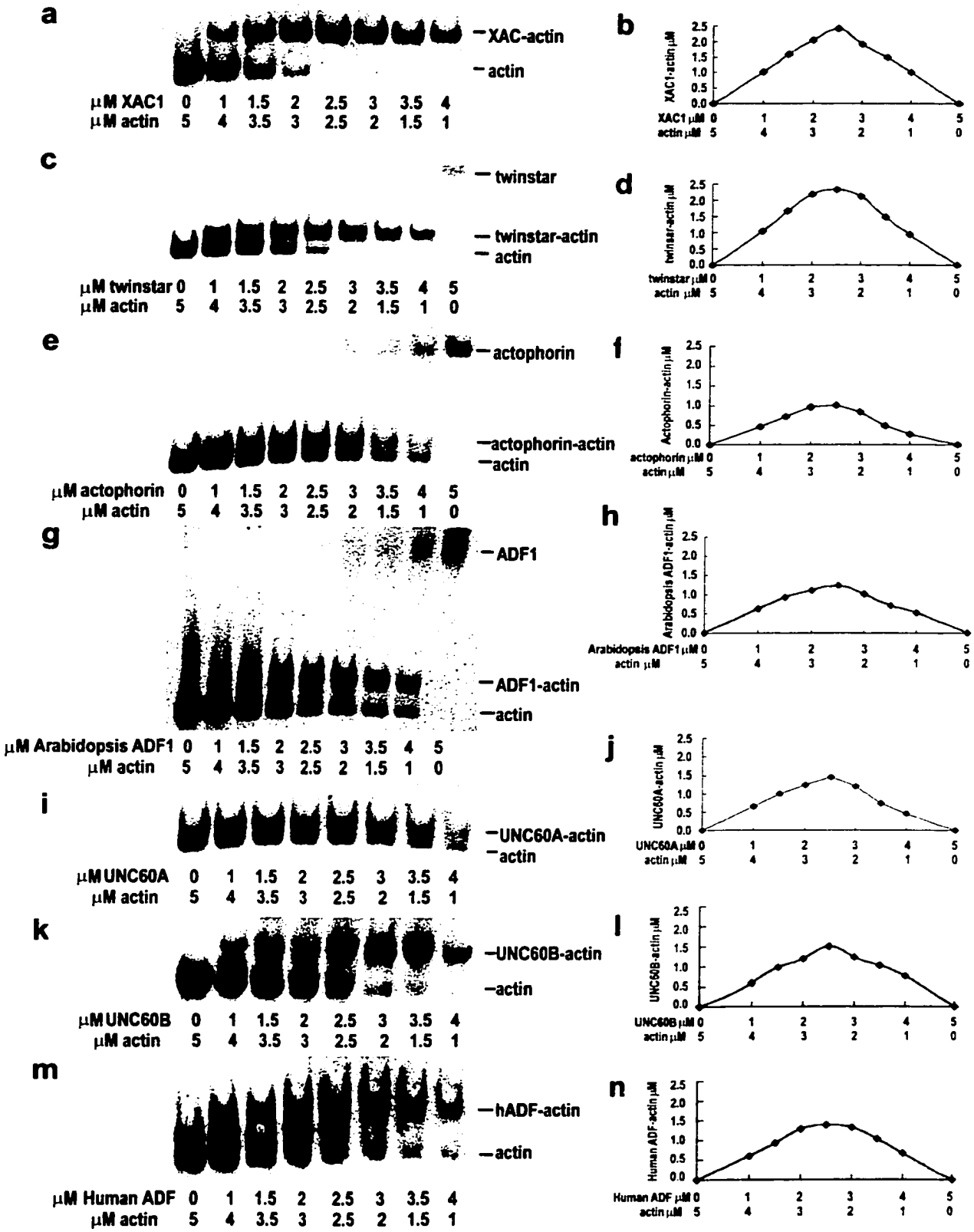


Figure 3.1
SDS-PAGE of actin and ADF/cofilin proteins. The protein and its amount in each lane are: 1.6 μg of actin (lane 1), 1.2 μg of endogenous chick brain ADF (lane 2), 0.8 μg of recombinant yeast cofilin (lane 3), 0.7 μg of recombinant *Dictyostelium* cofilin (lane 4), 0.9 μg of recombinant *Acanthamoeba* actophorin (lane 5), 0.9 μg of recombinant *Toxoplasma gondii* ADF (lane 6), 1.0 μg of human ADF (lane 7), 1.1 μg of endogenous starfish depactin (lane 8), 1.0 μg of recombinant chick cofilin (lane 9), 0.9 μg of recombinant *Xenopus* XAC1 (lane 10), 0.6 μg of recombinant *Xenopus* XAC2 (lane 11), 1.0 μg of recombinant chick ADF (lane 12), and 0.9 μg of recombinant chick ADF(S3E) mutant, respectively. The 15% gel was stained with Coomassie Blue R-250. Positions of molecular weight standards (kD) are shown.

Figure 3.2

Xenopus XAC1, *Drosophila* twinstar, *Acanthomoeba* actophorin, *Arabidopsis* ADF1, *Caenorhabditis* UNC 60A, UNC 60B, and human ADF are equally active. Job plots were used to determine the binding stoichiometry of G-actin to the ADF/cofilin protein. A decreasing amount of actin was incubated with an increasing amount of XAC1 (a), twinstar (c), actophorin (e), *Arabidopsis* ADF1 (g), UNC-60A (i), UNC-60B (k), or human ADF (m). The complexes of actin with each ADF/cofilin were separated from free actin by non-denaturing PAGE and stained with Coomassie blue R-250. The amount of complex, determined by quantitative densitometry of the bands from (a, c, e, g, i, k and m), was plotted against the protein concentration (b, d, f, h, j, l and n). Maximum complex formation occurred at a 1:1 ratio of actin to each ADF/cofilin.



strength (calculated 34 μ) conditions, as increasing amounts of *Xenopus* XAC1 were incubated with 5 μM actin, the free actin band decreased and the complex of actin and XAC1 increased (Fig. 3.3). The apparent K_D, calculated by fitting the curve to the binding equation, ranges from 0.4 μM to 0.8 μM for XAC1 complexes with MgADP-actin, MgATP-actin, CaADP-actin, and CaATP-actin (Fig. 3.3). The nature of the actin bound nucleotide and cation did not significantly affect the binding of XAC1 to G-actin. Thus, XAC1 behaved like chick ADF (see Fig.2.3, chapter 2).

The interactions between actin and *Drosophila* twinstar (Fig. 3.4), *Acanthomoba* actophorin (Fig. 3.5), *Arabidopsis* ADF1 (Fig. 3.6), or *Caenorhabditis* UNC-60B (Fig. 3.7) were affected by actin bound nucleotide and cation. These ADF/cofilins in general had similar high affinity binding (K_{Dapps} ~ 0.5 to 3.8 μM) to MgADP-actin, CaADP-actin, and CaADP-actin (Table 3.1). However, they differed from chick ADF and *Xenopus* XAC1 in that their K_{Dapp} for MgATP-actin was > 16 μM, much more similar to chick cofilin (see Fig 2.4, chapter 2; Table 3.1). When increasing amounts of *Arabidopsis* ADF1, *Drosophila* twinstar, actophorin, or UNC-60B were incubated with 5 μM MgATP-actin, free actin decreased or disappeared without a corresponding increase in the amount of complex suggesting that these ADF/cofilins might nucleate assembly similarly to chick cofilin (see Fig. 2.4 to 2.6, chapter 2).

ADF enhances filament numbers compared to cofilin

A timed sedimentation assay at steady state, physiological ionic strength, and pH 8.1 with 20 μM actin was used to estimate the distribution of filament lengths in mixtures of actin incubated with each member of the ADF/cofilin family in the presence of an

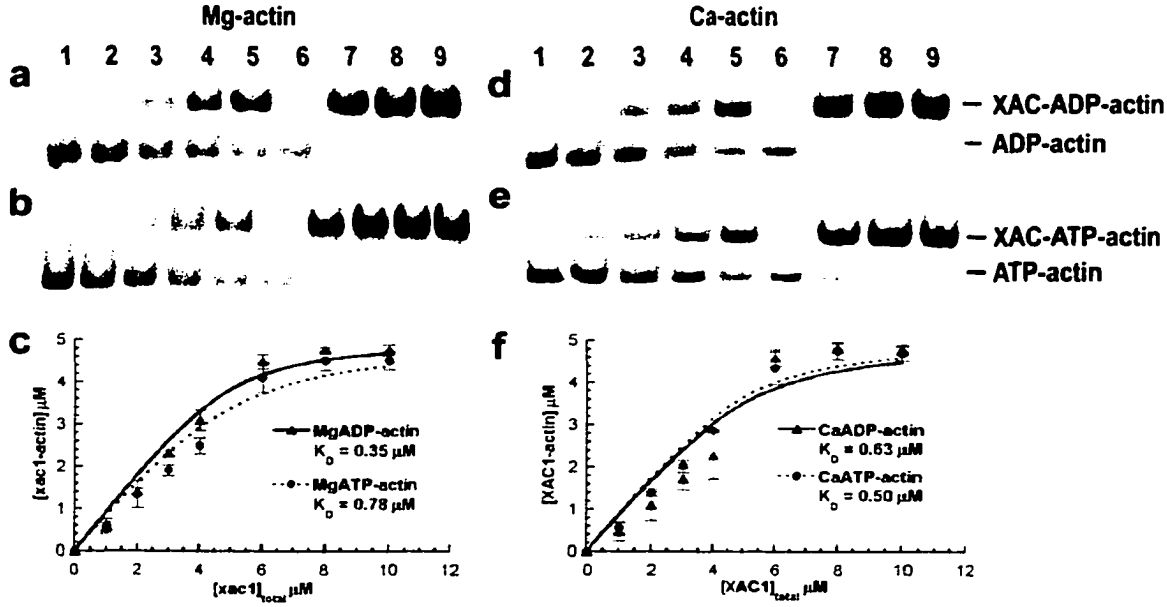


Figure 3.3

The interaction of *Xenopus* XAC1 with G-actin is not significantly affected by actin bound nucleotide and divalent cation. XAC1 at 0, 1, 2, 3, 4, 6, 8, 10 μM (lane 1, 2, 3, 4, 5, 7, 8, 9) was incubated with: (a) 5μM MgADP-actin; (b) 5μM MgATP-actin; (d) 5μM CaADP-actin; (e) 5μM CaATP -actin. The complex was separated from free actin by non-denaturing PAGE and stained with Coomassie Blue R. The binding curves were obtained by quantitative densitometry of gels. The amount of complex was plotted against total XAC1 as shown in (c) for the Mg-actin and in (f) for the Ca-actin. The lines shown are the best fit to the binding equation.

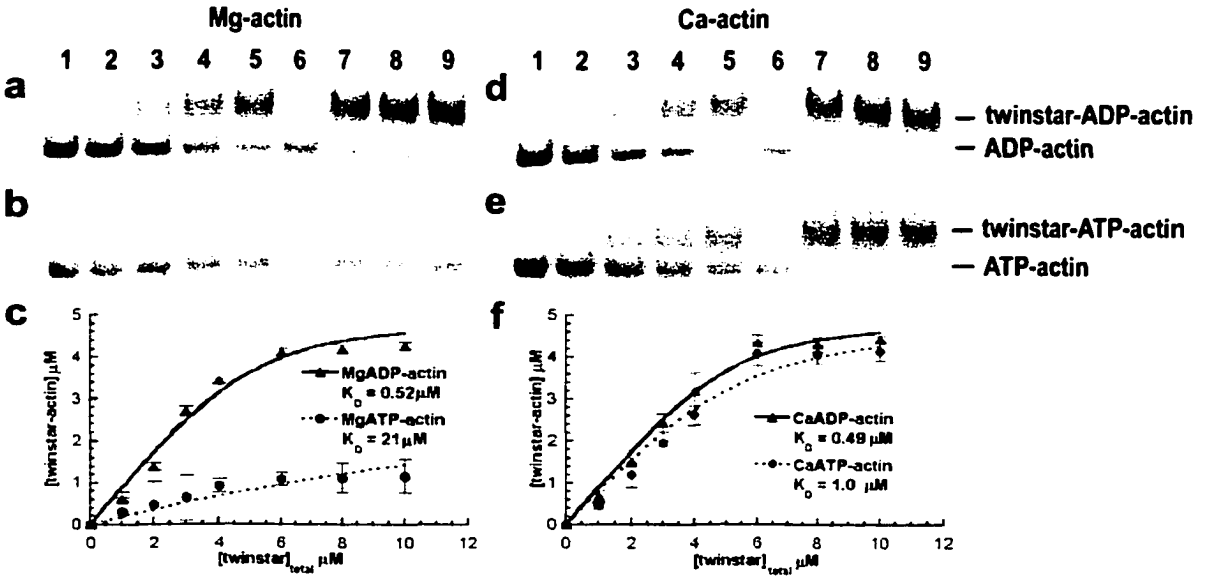


Figure 3.4

The interaction of *Drosophila* twinstar with G-actin is affected by actin bound nucleotide and divalent cation. *Drosophila* twinstar at 0, 1, 2, 3, 4, 6, 8, 10 μM (lane 1, 2, 3, 4, 5, 7, 8, 9) was incubated with: (a) 5μM MgADP-actin; (b) 5μM MgATP-actin; (d) 5μM CaADP-actin; (e) 5μM CaATP -actin. The complex was separated from free actin by non-denaturing PAGE and stained with Coomassie blue R-250. Binding curves obtained by quantitative densitometry of gels are shown in (c) for Mg-actin and in (f) for Ca-actin. The lines shown are the computer fit to the binding equation.

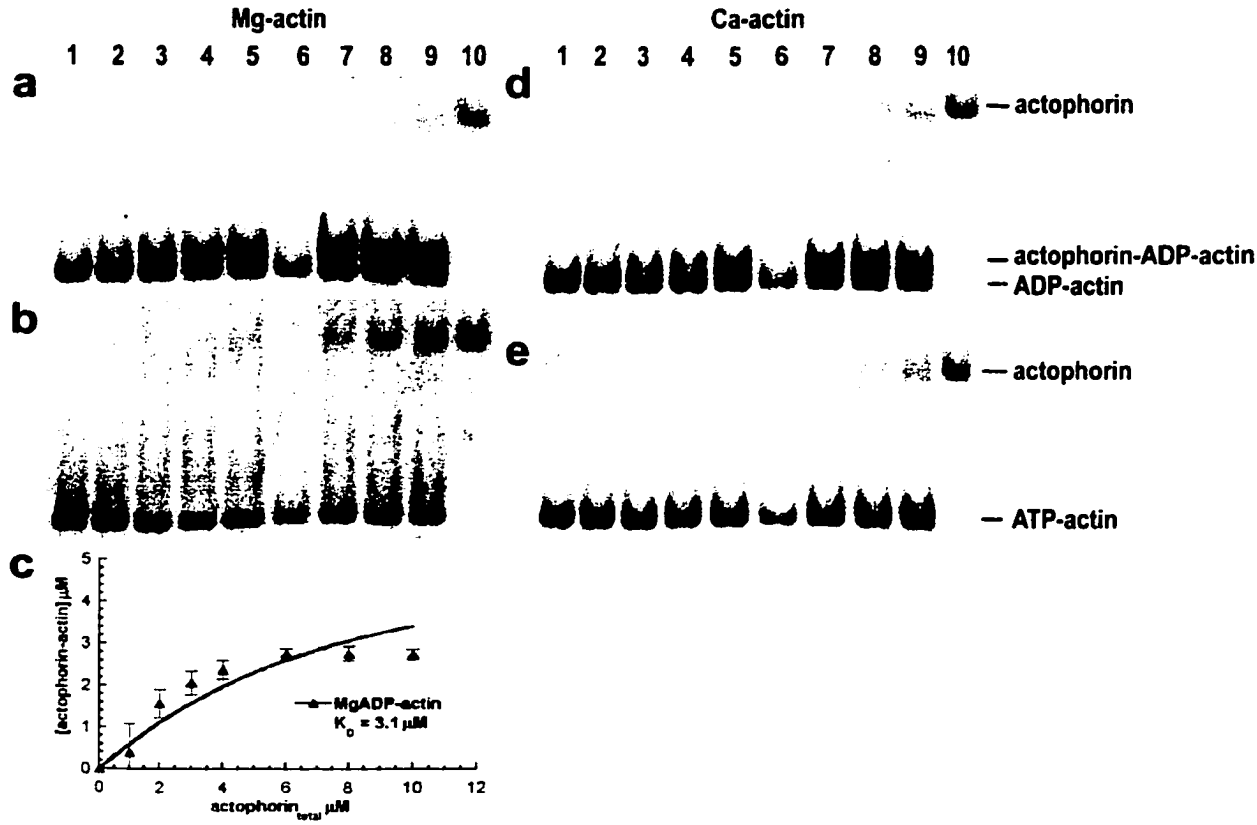


Figure 3.5

The interaction of *Acanthamoeba* actophorin with G-actin is affected by actin bound nucleotide and divalent cation. *Acanthamoeba* actophorin at 0, 1, 2, 3, 4, 6, 8, 10 μM (lane 1, 2, 3, 4, 5, 7, 8, 9) was incubated with: (a) 5μM MgADP-actin; (b) 5μM MgATP-actin; (d) 5μM CaADP-actin; (e) 5μM CaATP -actin. An internal standard of 2.5 μM actin was loaded in lane 6. Actophorin at 10 μM was loaded in lane 10. The complex was separated from free actin by non-denaturing PAGE and stained with Coomassie blue R-250. Binding curves obtained by quantitative densitometry of gels are shown in (c) for actophorin binding to MgATP-actin. A complex of actophorin and Ca-actin was formed (d, e) since free actophorin in actophorin-Ca-actin mixtures (lane 9) was much less than actophorin alone (lane 10). But the complex was not separated from free actin under the electrophoresis condition. The line shown is the computer fit to the binding equation.

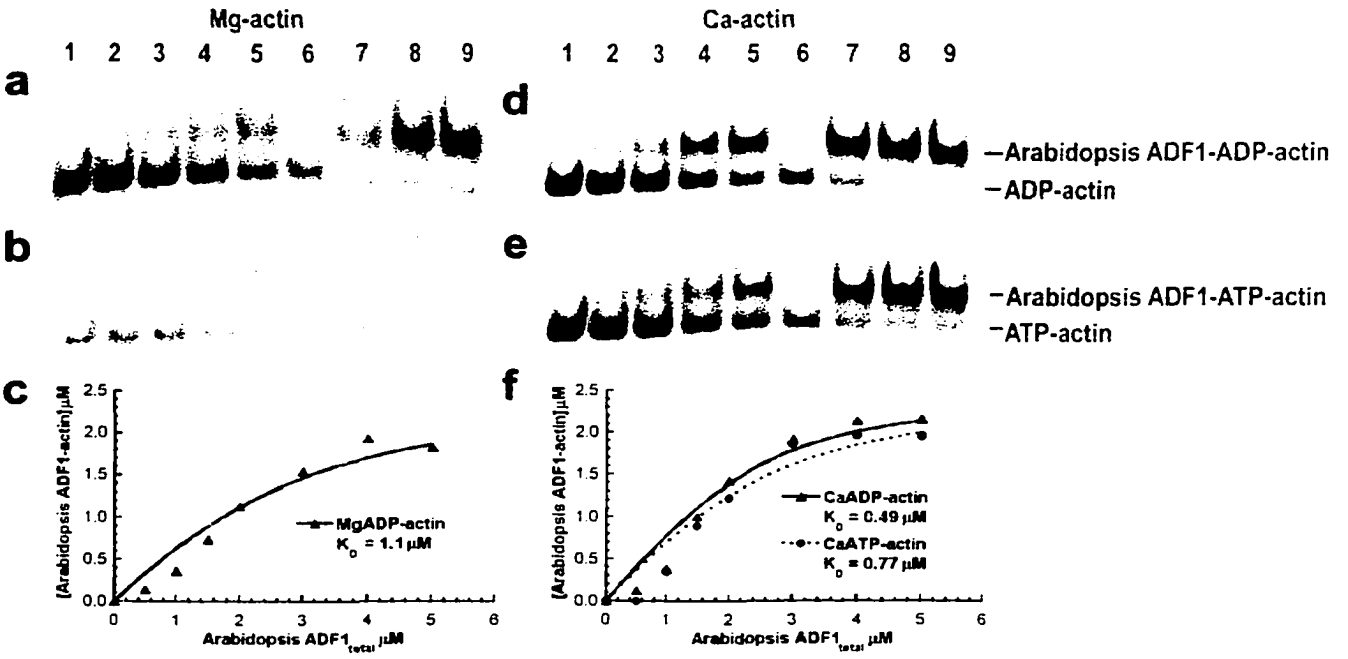


Figure 3.6

The interaction of *Arabidopsis* ADF1 with G-actin is affected by actin bound nucleotide and divalent cation. *Arabidopsis* ADF1 at 0, 0.5, 1, 1.5, 2, 3, 4, 5 μM (lane 1, 2, 3, 4, 5, 7, 8, 9) was incubated with: (a) 2.5 μM MgADP-actin; (b) 2.5 μM MgATP-actin; (d) 2.5μM CaADP-actin; (e) 2.5μM CaATP-actin. An internal standard of 1.25 μM actin was loaded in lane 6. The complex was separated from free actin by non-denaturing PAGE and stained with Coomassie blue R-250. Binding curves obtained by quantitative densitometry of gels are shown in (c) for MgADP-actin and in (f) for Ca-actin. No detectable complex band was formed when ADF1 was mixed with MgATP-actin. The lines shown are the computer fit to the binding equation.

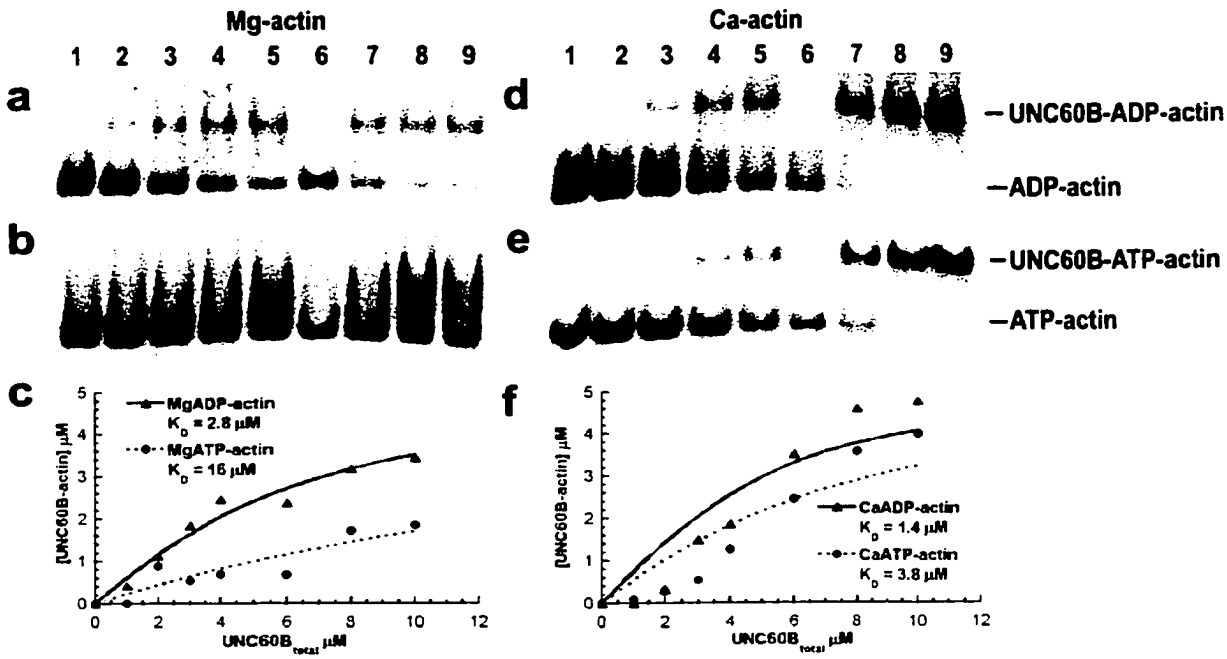


Figure 3.7

The interaction of *C. elegans* UNC-60B with G-actin is affected by actin bound nucleotide and divalent cation. *C. elegans* UNC-60B at 0, 1, 2, 3, 4, 6, 8, 10 μM (lane 1, 2, 3, 4, 5, 7, 8, 9) was incubated with: (a) 5μM MgADP-actin; (b) 5μM MgATP-actin; (d) 5μM CaADP-actin; (e) 5μM CaATP -actin. An internal standard of 2.5 μM actin was loaded in lane 6. The complex was separated from free actin by non-denaturing PAGE and stained with Coomassie blue R-250. Binding curves obtained by quantitative densitometry of gels are shown in (c) for Mg-actin and in (f) for Ca-actin. A smear and not a sharp band, was seen when UNC-60B was mixed with MgATP-actin, suggesting the complex had low affinity and dissociated during migration. The lines shown are the computer fit to the binding equation.

Table 3.1: The apparent dissociation constants of ADF/cofilin proteins for G-actin at ionic strength $\mu = 34$:

	MgATP-actin	MgADP-actin	CaATP-actin	CaADP-actin
chick ADF	1.0 μM (4)	0.5 μM (4)	0.5 μM (3)	0.4 μM (3)
chick cofilin	18 μM (3)	0.6 μM (3)	0.9 μM (3)	0.7 μM (3)
Xenopus XAC1	0.8 μM (4)	0.4 μM (3)	0.5 μM (3)	0.6 μM (3)
Twinstar	21 μM (4)	0.5 μM (4)	1.0 μM (3)	0.5 μM (3)
plant ADF	* (1)	1.1 μM (1)	0.73 μM (1)	0.49 μM (1)
UNC 60B	>16 μM (1)	2.8 μM (1)	3.8 μM (1)	1.4 μM (1)
Actophorin	* (3)	3.1 μM (3)	* (3)	* (3)

* indicates no measurable AC-actin complex. Number in ( ) indicates number of experiments.

ATP regenerating system. When 20 μM actin was mixed with 20 μM human ADF, *Xenopus* XAC1, starfish depactin, or *Caenorhabditis* UNC-60A, less than 50% of the total actin was pelleted after a 5 min centrifugation at 436,000g. The supernatant actin decreased as centrifugation time increase until the steady state supernatant actin pool (critical concentration) was reach at about 35 min (Fig. 3.8a, 3.8c). The incremental sedimentation of filaments over this time period suggested the filaments in these mixtures were heterogeneous in size. These proteins behave like chick ADF (Fig. 3.8c). When 20 μM actin was mixed with 20 μM *Acanthamoeba* actophorin, *Dictyostelium* cofilin, *Caenorhabditis* UNC-60B, yeast cofilin, *Arabidopsis* ADF1, or *Drosophila* twinstar, more than 70% of the actin was pelleted within 5 min, suggesting long filaments predominate in these mixtures (Fig. 3.8a, 3.8b). These proteins behave like chick cofilin (Fig. 3.8b). The short filaments could arise from the greater severing activity of proteins in the ADF subgroup, but they could also arise from depolymerization and/or nucleation of new filaments as predicted by Ressad *et al.*, (1999).

ADFs show greater pH-dependence and have more severing activity than cofilin

A severing assay was performed with 3.3 μM F-Actin containing 5% pyrene-labeled actin. The filaments were nucleated by spectrin:actin:protein 4.1 complex which capped all pointed ends and the barbed end was capped by excess gelsolin:actin 1:1 complex added after assembly reached steady state. The depolymerization rate upon addition of DBP following severing by different ADF/cofilins was measured at pH 7.8 and pH 6.8 relative to that for actin alone (Table 3.2). There is a quantitative difference

Figure 3.8

Timed sedimentation analysis of steady state mixtures of F-actin with different ADF/cofilins. (a) Actin-ADF/cofilin mixtures (20 μM each) were incubated overnight in an ATP-regenerating actin assembly buffer and then centrifuged in a Beckman TLA100 rotor at 436,000g for times from 0 to 60 minutes. Samples of the supernatant and pellet fractions were subjected to SDS-PAGE and stained with Coomassie Blue R-250. (b, c) The amount of actin remaining in the supernatant was plotted against the time of centrifugation. For the ADF/cofilins shown in (b), more than 70% of the actin was pelleted within 5 min. For the ADF/cofilins shown in (c), less than 50% of the actin was pelleted within 5 min. The steady state unpolymerized actin pool (critical concentration) was not reached until 35 min of centrifugation at 436,000g. The error bars show the standard deviation from triplicate experiments.

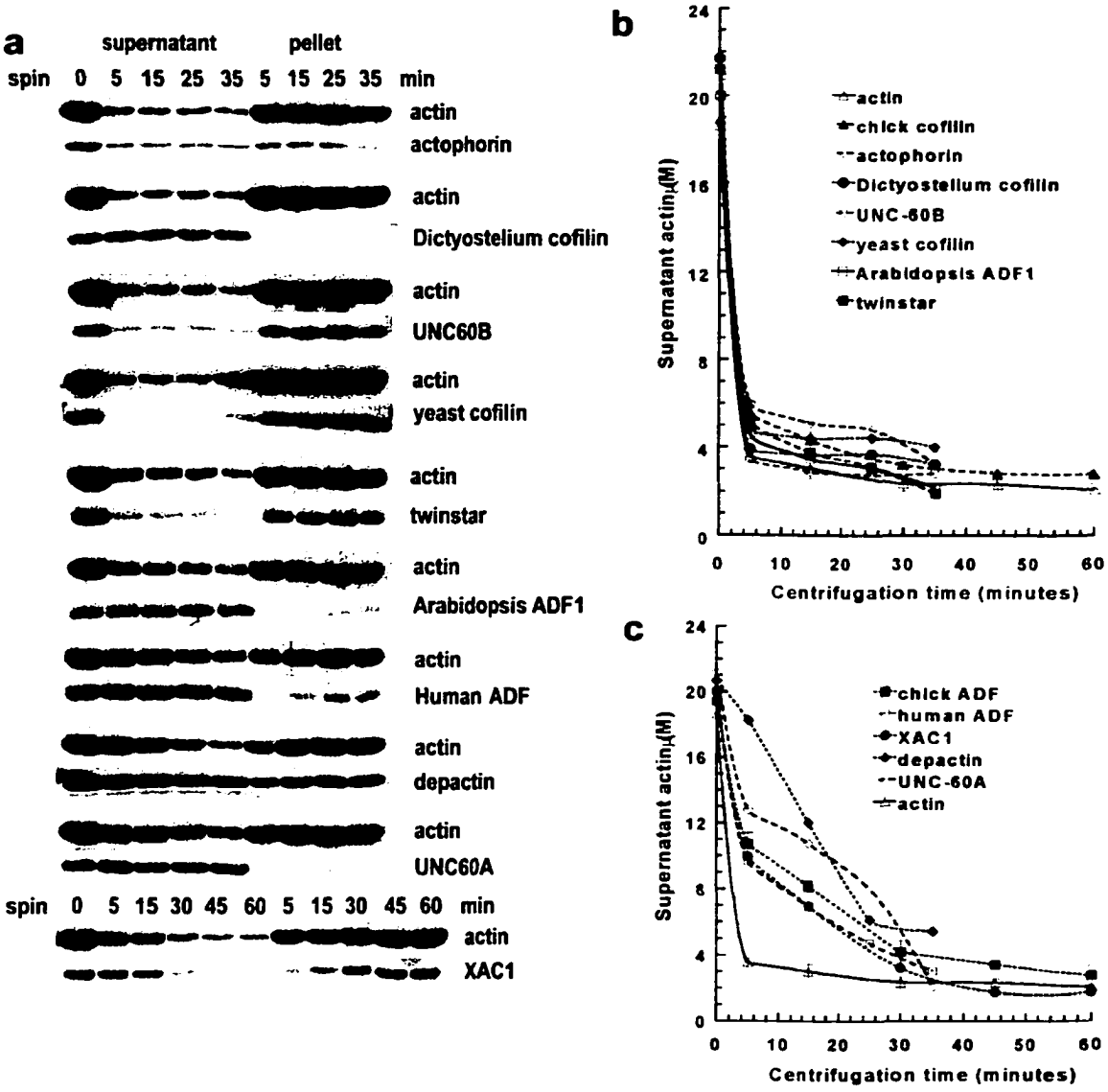


Table 3.2. The relative initial rate of depolymerization compared to actin alone following severing by different ADF/cofilins.

	pH7.8	pH6.8	Ratio of rates 7.8/6.8
actin	1	1	1
chick ADF	22	11	2.0
Xenopus XAC1	29	9.6	3.0
Xenopus XAC2	25	6.8	3.7
depactin	15	6.0	2.5
chick cofilin	2.8	3.8	0.7
Acanthomoeba actophorin	0.2	3.4	0.06
Caenorhabditis UNC-60B	6.5	3.6	1.8
Dictyostelium cofilin	4.5	3.2	1.4
Arabidopsis ADF1	2.6	2.1	1.2
Drosophila twinstar	3.8	4.3	0.9
yeast cofilin	55	10	5.5
human ADF	3.9	4.4	0.9
Caenorhabditis UNC-60A	3.2	3.3	1.0
Caenorhabditis UNC-60B(Δ152)	4.9	4.4	1.1
Caenorhabditis UNC-60B(s1307)	1.7	1.5	1.1

among ADF/cofilins from different species that fall into two groups. The initial rates of depolymerization in the presence of chick ADF, XAC1, XAC2 and depactin relative actin alone were above 15 at pH 7.8 and above 6 at pH 6.8. The initial depolymerization was 2 to 4-fold faster at pH 7.8 than at pH 6.8 suggesting a pH dependent severing. The initial rates of depolymerization in the presence of chick cofilin, actophorin, UNC-60B, twinstar, *Arabidopsis* ADF1, and *Dictyostelium* cofilin relative actin alone were below 7 at pH 7.8 and below 4 at pH 6.8. Thus, these proteins had weaker severing and were less pH dependent than the ADF subgroup.

There are three ADF/cofilins not falling into the ADF and cofilin subgroups. Yeast cofilin has greater pH-dependent severing/depolymerization (Table 3.2), behaving like chick ADF by the severing assay but behaving like chick cofilin in the timed-sedimentation assay (Fig. 3.8). Human ADF and *Caenorhabditis* UNC-60A had less filament severing/depolymerization (Table 3.2) behaving like chick cofilin by the severing assay but behaving like chick ADF in the timed-sedimentation assay (Fig. 3.8).

Caenorhabditis UNC-60B(Δ152) is a C-terminal truncation mutant of UNC-60B that has been reported to be non-severing compared to the UNC-60B wild type (Ono *et al.*, 2001). However, we find no difference in the severing activity of UNC-60B(wt) and the Δ152 mutant (Table 3.2), although both are only weak severing proteins. *Caenorhabditis* UNC-60B(s1307) is a point mutant S112F in putative actin-binding helix that shows strong severing activity in a nucleation assay (Ono *et al.*, 1999). However, our severing assay showed UNC-60B(s1307) to be less active than UNC-60B(wt) (Table 3.2).

Discussion

Actin is a highly conserved protein

Actins form a family of eukaryotic proteins whose sequences are highly conserved. All actins share more than 82% amino acid sequence identity (Sheterline & Sparrow, 1994). Except for a series of variable acidic residues at the N-terminus, the amino acid sequences of muscle and cytoplasmic actins of animals and protozoa differ by less than 5 % (reviewed in Pollard & Cooper, 1986). Plants have multiple actins that differ from each other and from animal actins by 6-22 % (reviewed in Pollard & Cooper, 1986).

Because actin is such a highly conserved protein and because muscle actin is relative easy to obtain with high purify and in large quantity, skeletal muscle actin is commonly used to study the properties of actin binding proteins from various species. Actin has been purified with relative low yield from several other species, including amoeba (Pollard, 1984) and *Caenorhabditis* (Ono, 1999). Amoeba actophorin binds slowly to and dissociates from amoeba actin filaments in a simple bimolecular reaction, but binding to muscle actin is cooperative (Blanchoin & Pollard, 1999). The assembly and critical concentration of actins from muscle and from *Caenorhabditis* are similar, but *Caenorhabditis* UNC-60B shows greater depolymerization of *Caenorhabditis* actin than rabbit muscle actin (Ono, 1999). It is ideal to study the interaction of actin and ADF/cofilin from the same species, but it is very difficult to get a sufficient quantity of actin in high purity from each species that corresponds to each ADF/cofilin. Furthermore, the differences reported above for the interaction of the ADF/cofilin with its homologous

actin could arise from either some contaminants in the actin preparations or from the additional steps required in its purification. The proper folding of actin by eukaryotic chaperonin containing T-complex polypeptide 1 is essential for actin to function (Gao *et al.*, 1992), and thus no one has been able to produce active recombinant actin from bacteria. Therefore, we use chick muscle actin to screen and compare ADF/cofilin from various organisms in their interaction with G-actin and F-actin.

Quantitative differences between ADF/cofilin proteins

Saccharomyces cerevisiae expresses a single ADF/cofilin gene product (yeast cofilin) that is essential for survival. However both porcine ADF and porcine cofilin can rescue the yeast cofilin knock out mutant (Iida *et al.*, 1993) demonstrating that both porcine ADF and cofilin can functionally substitute for cofilin in the activities requiring actin dynamics in yeast. Thus, both ADF and cofilin have qualitatively similar activities.

Here we show there are quantitative differences between the ADF and the cofilin subgroups. Proteins in the ADF subgroup have greater severing and depolymerizing activities than those in the cofilin subgroup and they bind to MgATP-G-actin with higher affinity. Proteins in the cofilin subgroup enhance nucleation. Members of both subgroups have been shown to enhance the filament turnover *in vivo* that is important for cell growth, motility, cytokinesis, endocytosis and exocytosis, but the extent of turnover may vary depending on the species, cell type, and developmental stage.

The relatively weak severing activity of members of the cofilin subgroup determined *in vitro* might be significantly enhanced *in vivo*. Cofilin is essential for the generation of free barbed ends at the leading edge of extending lamellipods in stimulated

metastatic rat mammary adenocarcinoma cells (Zebda *et al.*, 2000). The mechanism for barbed end formation arises from filament severing by cofilin (Zebda *et al.*, 2000) and pointed end capture by the Arp2/3 complex (Bailly *et al.*, 1999). Cofilin has weaker severing activity than ADF *in vitro*, so its severing *in vivo* might be aided by the actin binding protein, actin interacting protein 1 (Aip1). In yeast, filament depolymerization by cofilin is enhanced by Aip1 to achieve the rapid filament turnover observed *in vivo* (Rodal *et al.*, 1999). In *Dictyostelium*, Aip1 also plays an important role in controlling actin depolymerization (Konzok *et al.*, 1999). Aip1-null cells are impaired in growth and their rates of fluid-phase uptake, phagocytosis, and movement are reduced in comparison to wild-type. *Dictyostelium* overexpressing Aip1 by ~ 20-fold has normal motility, an identical rate of pinocytosis, and a 50% increase in phagcytosis compared to wild-type (Konzok *et al.*, 1999). These data suggest that cofilin alone is unable to enhance filament turnover rapidly enough for normal cellular function and that Aip1 plays an important regulatory role in the rapid remodeling of actin filaments. A mammalian homologue of Aip1, called WDR-1, has been identified (Adler *et al.*, 1999). Its expression and regulation could be used to modulate cofilin's activity in cellular processes.

In cells expressing ADF as the predominant member of the AC family, the filament severing and depolymerizing activities of ADF alone are likely to produce enough new ends to efficiently recycle G–actin. Aip1, if it occurs in these cells, might be required to further enhance filament severing needed only during certain developmental stages. Thus, Aip1 might bring the activity of cofilin closer to that of ADF. Micro-injection of Aip1 into *Xenopus* blastomeres arrests their development and abolishes the strong cortical staining of both actin and XAC (Okada *et al.*, 1999), suggesting Aip1

might greatly enhance the activity of XAC (an ADF) to almost completely fragment the cortical actin filament network. Recent unpublished results from our lab suggest Aip1 caps the barbed ends of ADF or cofilin severed filaments, limiting their ability to reanneal and thus enhancing the efficiency of AC severing (Okada et al., in preparation). However the Aip1 capped filaments are also limited in their ability to nucleate filament growth suggesting additional regulatory steps are still to be discovered.

The quantitative difference between ADF and cofilin in filament severing/depolymerization can make a big difference in filament organization in cells where these proteins are overexpressed. Over expressing the active and non-phosphorylatable XAC(A3)-GFP causes cells to gradually round up and lose their ability to adhere to the substratum as expression increases, suggesting the assembled actin pool is decreased (Bamburg, 1999). In our assays, XAC belongs to the ADF subgroup, which has a significant affinity for binding MgATP-G-actin and is effective in filament severing/depolymerization. In contrast, over-expression of *Dictyostelium* cofilin, a member of the cofilin subgroup by our assays, caused an increase in F-actin and little change in G-actin (Aizawa *et al.*, 1996). The over-expression of cofilin also stimulated cell motility and membrane ruffling suggesting that enhanced filament dynamics can accompany an increase in F-actin.

In cells that co-express ADF and cofilin, these proteins might work cooperatively to enhance filament turnover. ADF is best at severing filaments, both ADF and cofilin enhance off-rates from pointed ends to recycle G-actin, and cofilin promotes nucleation.

Possible structural basis for the pH dependent filament severing

Many proteins show a pH sensitivity in their activity. For some proteins that show this pH sensitivity over the physiological pH range of about 6.5 to 7.5, alterations in the ionization of histidine side chains are often responsible. The imidazole side group of histidine has a pKa of about 6.1 in an aqueous environment, but this can be shifted up or down depending on the local environment provided by the protein. Plasminogen activator inhibitor 1 has 13 histidine residues and shows pH-dependent inhibition of plasminogen activator. Converting the his364 to thr abolishes this pH-dependence, but changing any of the other 12 his residues does not affect the pH dependence (Mangs *et al.*, 2000). Similarly, the introduction of a histidine to replace tyrosine 31 in the cellulose binding domain of the enzyme cellobiohydrolase (Cel7A) introduces a pH sensitivity into this enzyme that was previously absent (Linder *et al.*, 1999). Thus, alterations in the ionization state of histidines may be responsible for the pH sensitivity of many proteins.

Removal of C-terminus (I152) of *Caenorhabditis* UNC-60B eliminates F-actin binding activity indicating the C-terminus of ADF/cofilin is important for interacting with F-actin (Ono *et al.*, 2001). By closer inspection of the sequence alignment of ADF/cofilin family and the crystal structures of four known ADF/cofilin proteins, Bowman *et al* (2000) speculated the aromatic:aromatic interactions resulting from two phenylalamine residues (human ADF 161 and 68) stabilize the conformation of the C-terminus. We inspected the crystal structure of human ADF (PDB 1AK7) using the RasMol program (http://www.umass.edu/microbio/rasmol) and compared the location of histidine residues in a large number of different ADF/cofilins based upon their structural homologies. All of the ADF/cofilins with strong pH-dependent severing/depolymerizing

activity have a histidine residue in the vicinity of the C-terminal-stabilizing interaction between residues 161 and 68. These include his70 of human ADF and chick ADF on the surface of helix 2, his22 of XAC1 and XAC2 on the surface of helix 1', his 85 of depactin in a loop between the β-strands 4 and 5, and a C-terminal his in yeast cofilin. In our assays, these proteins were the ones to show the greatest pH-dependency. No histidine residues were present in regions that could interact with the C-terminal stabilizing domains of the other ADF/cofilins that did not show a strong pH-dependent activity.

Chapter Four

Effects of actin binding drugs on the interaction between actin binding proteins and actin

Summary

Latrunculin and jasplakinolide, two membrane permeable natural products from different species of sponge, have been extensively used to study actin-dependent processes *in vivo*. Latrunculin A is an inhibitor of actin polymerization that works by sequestering G-actin in a 1:1 molar complex. Jasplakinolide binds to F-actin and stabilizes filaments. The effects of these actin-binding drugs on the interaction between actin and other actin-binding proteins are not well characterized. Non-denaturing polyacrylamide gel electrophoresis was used to study the competition between latrunculin A and two actin binding proteins, chick ADF and bovine thymosin β4. Latrunculin A competes with ADF and thymosin β4 for binding to G-actin. Jasplakinolide protects filaments from depolymerization by chick ADF and latrunculin A as measured by light scattering, pyrene-actin fluorescence, and sedimentation. The molar ratio of bound ADF to actin was measured in the presence of a 3–fold molar excess of ADF to actin following centrifugation at 436,000g for 40 minutes by quantitative SDS-PAGE of proteins in the pellet. The molar ratio of bound ADF to F-actin is reduced from 1.8 in the absence of jasplakinolide to 0.9in presence of sub-saturating level of jasplakinolide (1:5 molar ratio)

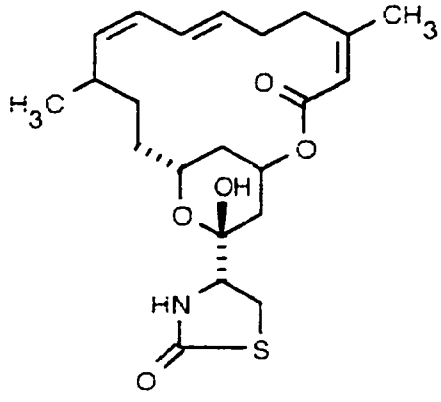
to 0.3 in presence of a saturating level (4:1 molar ratio) of jasplakinolide. In conclusion, latrunculin A competes with ADF and thymosin β4for binding G-actin, and jasplakinolide blocks filament depolymerization induced by ADF and latrunculin A and can promote filament assembly in the presence of ADF.

Introduction

Latrunculins are marine toxins, first isolated from the Red Sea sponge, *Latrunculia magnifica* (Kashman *et al.*, 1980). Both latrunculin A (Lat A; Fig. 4.1a) and latrunculin B, at submicromolar concentrations, disrupted actin filament organization and altered the morphology of mouse neuroblastoma and fibroblast cells (Spector *et al.*, 1983). The effect was reversible upon removal of the toxin. Lat A affects actin polymerization *in vitro* by forming a non-polymerizable 1:1 molar complex with G-actin that has a dissociation constant of 0.2 μM (Coue *et al.*, 1987). Lat A, which is membrane permeable, has been extensively used as an actin monomer-sequestering drug to study the role of filament turnover *in vivo*. The rapid filament turnover plays an important role in maintaining cell shape and cell polarity, endocytosis, and cell growth (Ayscough *et al.*, 1997). Lat A inhibits nucleotide exchange on G-actin in a dose-dependent manner (Ayscough *et al.*, 1997), similar to the effects of ADF and cofilin (Hayden *et al.*, 1993; Nishida, 1985). When this study was undertaken, the effect of Lat A on the interaction of actin with actin-binding proteins had not been reported.

Jasplakinolide (JAS; Fig. 4.1b) is a naturally occurring cyclodepsipeptide from the marine sponge *Jaspsi species*. It was first identified as an antifungal agent (Scott *et al.*, 1988). JAS induces actin polymerization and stabilization *in vitro*, and it binds to

a



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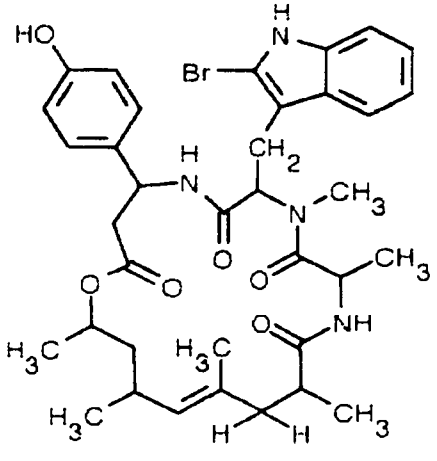


Figure 4.1
The structure of latrunculin A (a) and jasplakinolide (b) from Molecular Probes (www.probes.com).

F-actin competitively with phalloidin with a dissociation constant of approximately 15 nM (Bubb *et al.*, 1994). JAS, at concentrations higher than 3 μM, inhibits the growth of green alga *Micrasterias* and induces abnormal accumulation of actin filaments (Holzinger & Meindl, 1997). The effect of JAS on the binding of actin to actin-binding proteins has not been reported.

The ADF/cofilins are a group of actin filament binding, depolymerizing and severing proteins that is important for enhancing actin filament turnover *in vivo*. The molar ratio of ADF/cofilin binding to F-actin subunits as determined by Beckman Airfuge sedimentation (178,000g for 30 min) has been reported to be 1.3 for human ADF (Maciver *et al.*, 1998) and 1 for actophorin (Maciver *et al.*, 1998) and chick ADF (Hayden *et al.*, 1993), suggesting there is one ADF binding site on each actin subunit. Helical reconstruction analysis of AC proteins bound to F-actin also demonstrated a 1:1 binding (McGough *et al.*, 1997). A recent study on the structure of ADF saturated filaments that utilizes a new method for helical filament reconstruction suggests that there are two ADF binding sites on each actin subunit (Galkin *et al.*, 2001), but no biochemical measurement has been reported to support this model.

In this study, an *in vitro* binding assay was used to determine the competitive binding between Lat A and either chick ADF or thymosin β4 for actin. Light scattering, fluorescence, and sedimentation assays were used to study the protection by JAS of filament depolymerization in the presence of chick ADF or Lat A. The results help to explain the cellular effects of these drugs treatment. In addition, sedimentation studies of ADF binding to F-actin suggest an additional site per actin subunit. How this finding

might enhance our understanding of the JAS competition and the mechanism of severing is discussed.

Materials and Methods

Materials

Latrunculin A and jasplakinolide were obtained from Molecular Probes (Eugene, OR). Recombinant chick ADF was prepared as described by Adams et al. (1990). Recombinant chick cofilin was prepared as described by Abe et al. (1990) with a final binding and elution from Green A-agarose as described in chapter 2. Thymosin β4 was kindly provided by Dr. H. L. Yin.

Non-denaturing polyacrylamide gel electrophoresis

The amount of actin displaced by Lat A from ADF-MgATP-actin 1:1 complex or thymosin β4-MgATP-actin 1:1 complex was measured using non-denaturing gel electrophoresis. To form these complexes, 5 μM MgATP-actin and 10 μM ADF or 10 μM thymosin β4 were incubated in 2 mM Tris, pH 8.4, 0.5 mM DTT, 0.2 mM ATP, 0.21 mM $MgCl_2$, and 0.2 mM EGTA for 10 min on ice. A 2:1 molar ratio of ADF or thymosin β4 to actin was used to drive most of the actin into complex. Increasing concentrations of Lat A were incubated with the ADF-actin complex or thymosin β 4-actin complex for 20 minutes at room temperature. The proteins were then separated on a 7.5% non-denaturing polyacrylamide gel at 4°C using 50 mM bicine, 40 mM triethanolamine, 0.2mM ATP, and 0.2 mM EGTA, pH 8.4 as the running buffer. The gel was stained with Coomassie Blue R250. Digitized gel images were obtained with a Photometrics chilled CCD camera

(Tucson, AZ) and band densities were analyzed using 1D Phoretix gel analysis software (Non Linear Dynamics Ltd, England).

Light scattering and fluorescence assays

CaATP-Actin at 5 μM in the presence of 60 nM gelsolin was polymerized in F-buffer (10 mM PIPES, pH 6.8, 0.1 M KCl, 0.21 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM ATP, and 0.5mM DTT) at room temperature for 4 hours. After F-actin was incubated with 10 μM JAS for 10 minutes at room temperature, 10 μM ADF was added. The light scattering signal of the mixture was monitored at 18° C with an AVIV ATF 105 spectrofluorometer at 400 nm. The excitation bandwidth was 2 nm and the emission bandwidth was 4 nm.

CaATP-actin (5 μM) containing 38 % pyrene label was polymerized in F-buffer at room temperature either in the absence or presence of 5 μM JAS. The polymerization in the absence of JAS reached steady state after 2 hours and the polymerization in the presence of JAS reached steady state within 5 minutes. At steady state, the fluorescence signals of F-actin alone and JAS bound F-actin are at same level. Lat A at 40 μM was added at steady state to sequester actin monomer. The fluorescence of the mixture was monitored at 18° C with an AVIV ATF 105 spectrofluorometer, set for excitation 365 nm and emission at 404 nm with a 40 sec sampling interval. Bandwidths were 2 nm for excitation and 4 nm for emission.

Sedimentation assay

The protection of filaments by JAS from depolymerization by chick ADF and Lat A was studied using a sedimentation assay. Actin at 5 μM was polymerized alone or in the presence of 1, 5, or 20 μM JAS in F-buffer at pH 7.8 or pH 6.8. At steady state, 15 μM ADF or 40 μM Lat A was added until another steady state was reached at room temperature. In one experiment 5 μM actin was polymerized at pH 7.8 in the presence of 15 μM ADF prior to addition of 5 μM JAS. The samples were centrifuged for 40 minutes at 436,000g in the Beckman TLA100 rotor. The total supernatant was removed and mixed for sampling, and the pellet was resuspended in SDS-containing buffer (0.25 M Tris, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, and 1% SDS). Fractions from both supernatant and pellet were subjected to SDS-PAGE, and stained by Coomassie R-250. Digitized gel images were obtained and analyzed as described for non-denaturing gels.

Results

Latrunculin A and ADF compete for binding to G-actin

Lat A competes with ADF for binding to monomeric actin (Fig. 4.2). Free actin and actin bound to Lat A run identically under these non-denaturing gel electrophoresis conditions (Fig. 4.3c). Adding increasing amounts of Lat A to actin complexed with ADF results in the appearance of a band at the position of actin or Lat A-actin complex, suggesting Lat A competes with ADF for binding to G-actin. Lat A at over 30 μM does not show a dose-dependent competition. Lat A inhibits nucleotide exchange on G-actin in a dose-dependent manner (Ayscough *et al.*, 1997). ADF also inhibits nucleotide

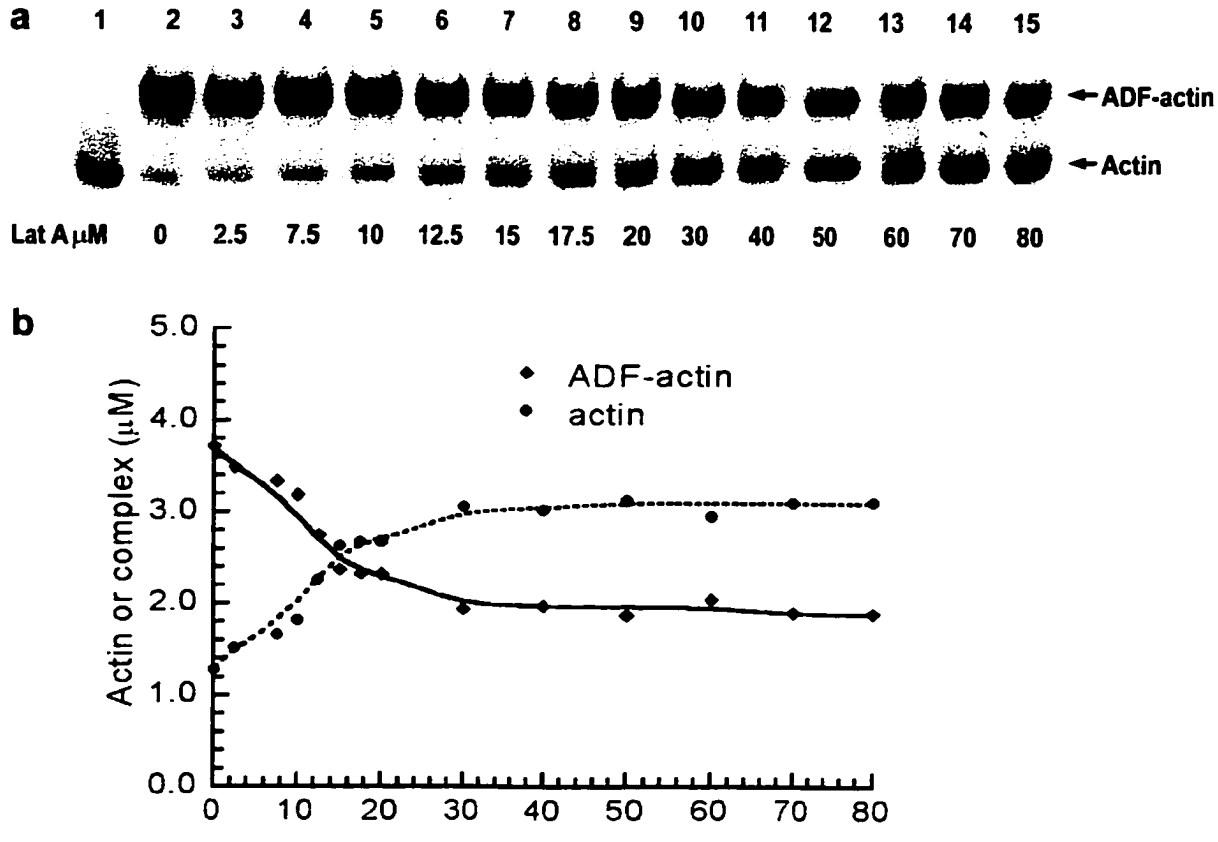


Figure 4.2. Lat A displaces actin from ADF-actin complex. (a) Lanes 2 to 15: increasing concentrations of Lat A were incubated with 5 μM MgATP-actin and 10 μM ADF. Lane 1: 5 μM MgATP-actin alone. The ADF-actin complex was separated from free or Lat A-bound actin by non-denaturing PAGE and stained with Coomassie blue R. (b) The amount of ADF-actin complex in each lane was determined by quantitative densitometry and plotted against the concentration of Lat A.

exchange on actin monomer (Hayden *et al.*, 1993). Thus, one possible mechanism to explain these results is that the binding of Lat A to G-actin might require nucleotide exchange and its binding to G-actin in the presence of ADF might take hours and not just the 10 minutes as was used in these experiments.

Latrunculin A and thymosin β4 compete for binding to G-actin

Lat A and thymosin β4 compete for binding to G-actin (Fig. 4.3). At the low ionic strength used for electrophoresis, the ratio of apparent dissociation constants for thymosin β4-actin and Lat A-actin is 0.86 meaning that thymosin β4 binds more tightly to actin than Lat A at the 34μ ionic strength used for gel analysis.

Jasplakinolide protects filaments from depolymerization by Lat A

Upon addition of 40 μM Lat A to 5 μM pyrene F-actin, the fluorescence signal decreased rapidly suggesting that Lat A either quenches the pyrene fluorescence or sequesters monomer and induces filament depolymerization or both (Fig. 4.4a). Preincubation of the filaments with JAS blocked the rapid decrease in fluorescence following Lat A addition suggesting that quenching of the pyrene fluorescence is not the cause of the fluorescence decline with Lat A alone. Sedimentation analysis was performed to verify the above observation. In the absence of JAS, Lat A induced the depolymerization and 71% of total actin after overnight incubation was G-actin. An equal molar amount of JAS to actin prevented most of the Lat A-induced depolymerization, with only 11% of total actin as G-actin (Fig. 4.4b). Thus, an equal molar amount of JAS to actin subunits, prevents filament depolymerization by an 8-fold molar excess of Lat A.

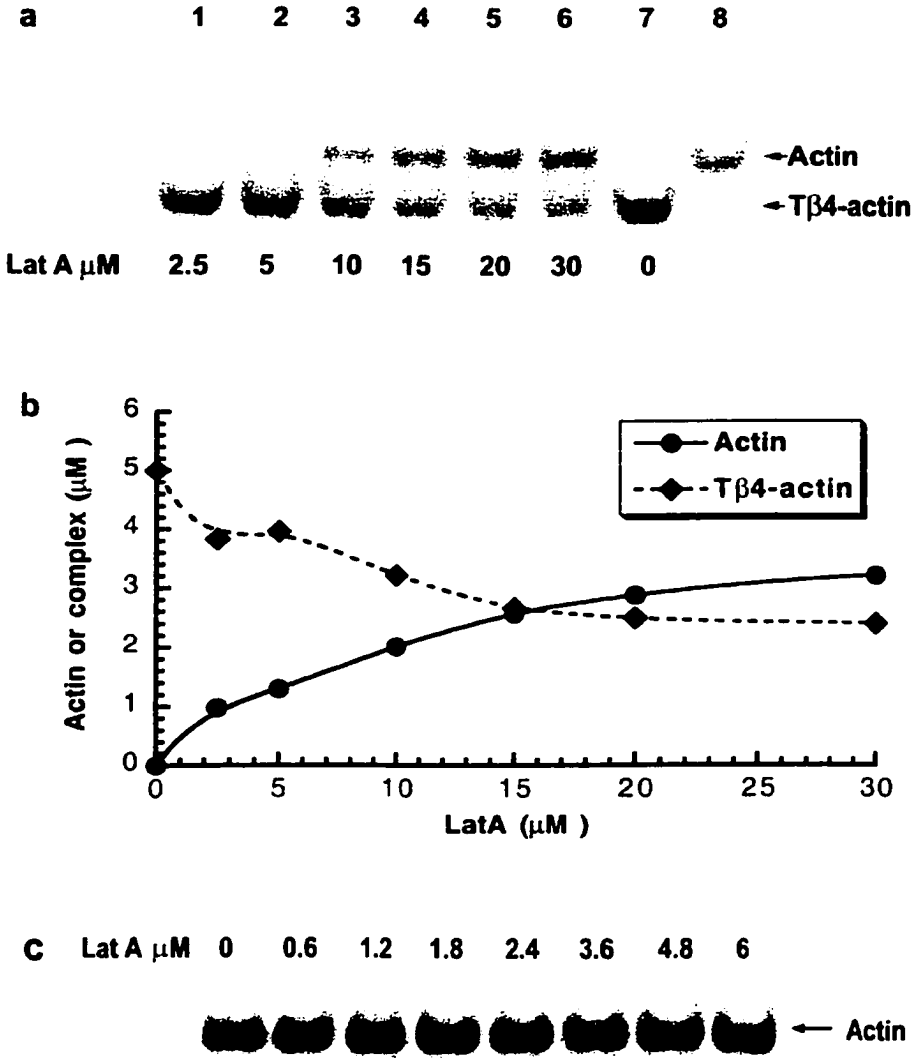


Figure 4.3. Lat A displaces actin from thymosin β4-actin complex. (a) Lanes 1 to 6: increasing concentrations of Lat A were incubated with 5 μM MgATP-actin and 10 μM thymosin β4. Lane 7: 5 μM MgATP-actin and 10 μM thymosin β4. Lane 8, 5 μM MgATP-actin alone. The thymosin β4-actin complex was separated from free or Lat A-bound actin by non-denaturing PAGE and stained with Coomassie blue R. (b) The amount of thymosin β4-actin complex in each lane was determined by quantitative densitometry and plotted against the concentration of Lat A. (c) Increasing concentrations of Lat A (0 to 6 μM) were incubated with 5 μM actin, and subjected to non-denaturing polyacrylamide gel electrophoresis, then stained with Coomassie blue R. Only one species was observed on the non-denaturing gels suggesting that free actin and Lat A-bound actin ran identically.

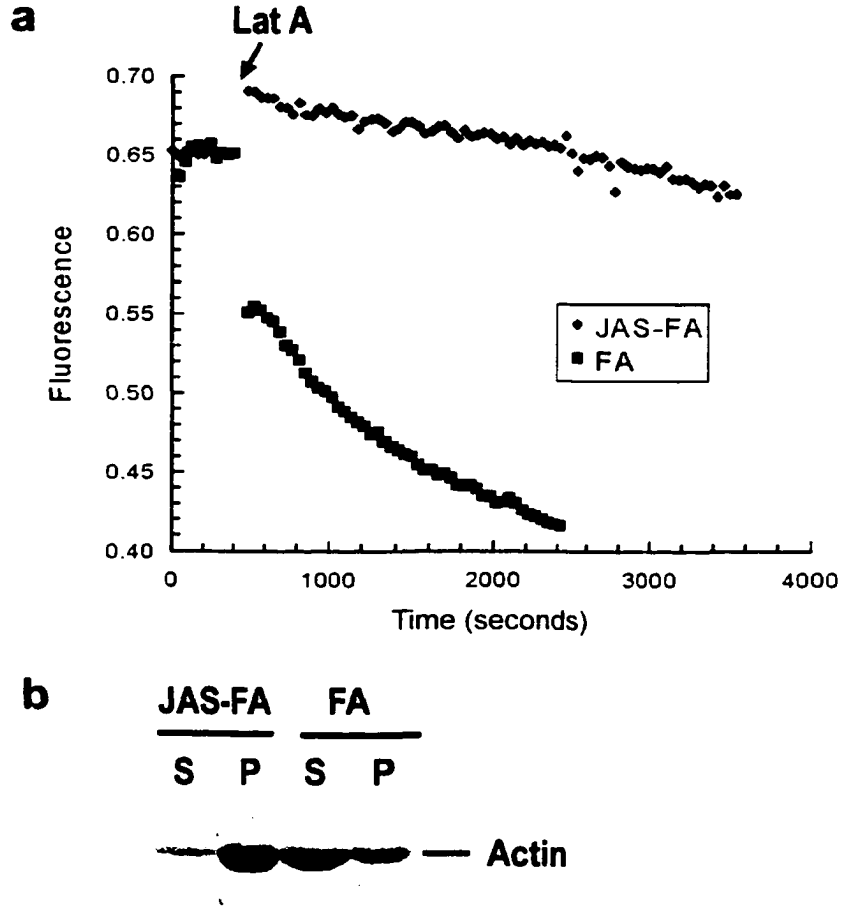


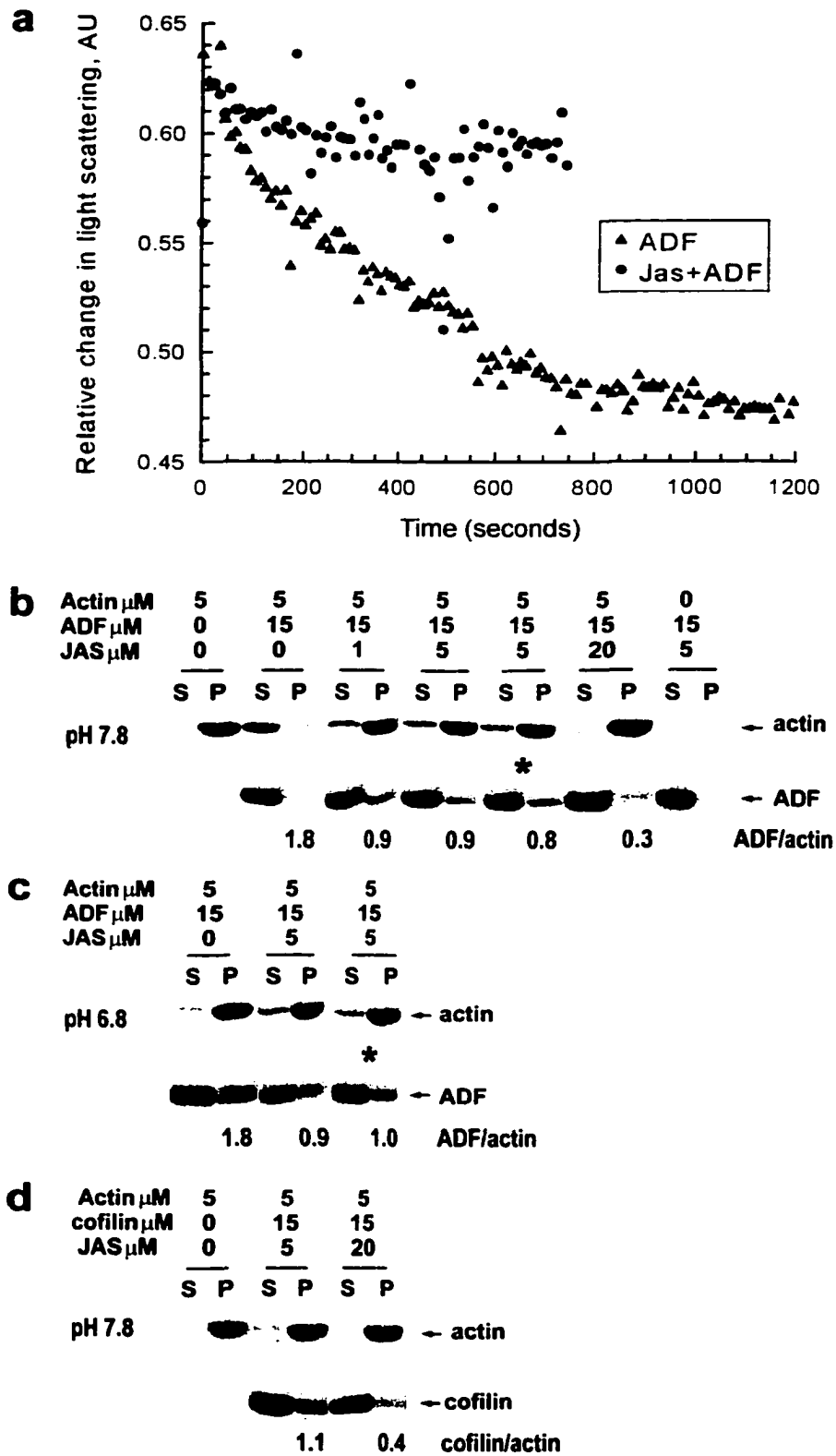
Figure 4.4. JAS protects filaments from depolymerization by Lat A. (a) F-actin at 5 μM containing 38% pyrene label was polymerized in F-buffer at pH 6.8 either with or without 5 μM JAS. At steady state, 40 μM Lat A was added to sequester G-actin to induce filament depolymerization. The decrease in fluorescence signal was followed for 30 min. (b) Samples from (a) were incubated overnight and then were centrifuged in Beckman TLA100 rotor at 436,000g for 40 minutes. The supernatant and resuspended pellet corresponding to equivalent fractions of the two pools were run on SDS-PAGE and stained with Coomassie Blue R-250. Lat A sequestered G-actin and enhanced the supernatant actin pool. JAS pretreatment counteracted the effect of LatA on the filaments.

ADF and cofilin bind to F-actin at close to 2:1 stoichiometry

Sedimentation assays were performed to look at the maximum binding ratio of ADF/cofilin to F-actin. The samples were centrifuged in Beckman TLA 100 at 436,000g for 40 min to sediment short actin filaments and actin oligomers. About 97% of the actin sedimented at pH 7.8 when 5 μM total actin was used. In the presence of 3-fold molar excess of ADF, 94% of the 5 μM actin sedimented at pH 6.8 but only 10% sedimented at pH 7.8 (Fig. 4.5). This demonstrates ADF induced depolymerization through severing and/or enhancement of off-rate from the minus end of filaments in a pH-dependent manner and confirms the critical concentration for ADF-actin of close to 5 μM as previous measured by light scattering (chapter 2, Fig. 2.6). The molar ratios of ADF to actin in the pellet from 2 assays were 1.6 and1.8 at pH 6.8, and 1.8 and 2.3 at pH 7.8 (Fig. 4.5). This stoichiometry was surprising given that all previous reports on ADF/cofilin binding to F-actin show values closer to 1:1 (Maciver *et al.*, 1998; Hayden *et al.*, 1993). We confirmed this value in additional experiments with similar running condition. Higher actin concentration and higher molar ratios of ADF to actin were used to rule out any error from the weak staining of actin and ADF in the pellet at pH 7.8 and to achieve the maximum binding of ADF to actin. At pH 7.8, the molar ratio of ADF to actin in the pellet was 1.94 ± 0.33 (n=4) using 16 μM of actin and a 5-fold molar excess of ADF. The molar ratio of cofilin to actin in the pellet was 1.54 ± 0.02 (n = 4) using 14 μM or 16 μM of actin and a 2.5-fold molar excess of cofilin. These data indicate there are two ADF/cofilin binding sites on each actin subunit of the filaments as predicted by Galkin et al (2001). The second binding site is occupied to a greater extent at pH 7.8 than at pH 6.8, and ADF binds more effectively to this second site than cofilin at pH 7.8.

Figure 4.5. JAS protects filaments from depolymerization by ADF. (a) Gelsolin (60 nM) capped F-actin (5 μM) was incubated either with or without 10 μM JAS at pH 6.8. Then, 10 μM ADF was added to induce the depolymerization of F-actin. The decrease in light scattering was monitored at 400 nm for 30 min. (b-d) Actin at 5 μM was polymerized in F-buffer at pH 7.8 (b,d) or pH 6.8 (c) either in the absence or presence of JAS. At steady state (40 minutes later), 15 μM ADF (b,c) or 15 μM cofilin (d) was added until another steady state (40 minutes later) was reached. In samples with asterisk (*), actin and ADF were incubated first and JAS was added second. The samples were centrifuged in Beckman TLA 100 rotor at 436,000g for 40 minutes. The supernatant and resuspended pellet were run on SDS-PAGE and stained with Coomassie Blue R-250. ADF and cofilin co-sediment with filaments even when filaments are stabilized with substoichiometric amounts of JAS, but JAS at high concentration competes with ADF and cofilin for binding to filaments.

Figure 4.4



Jasplakinolide protects filaments from depolymerization by ADF

Light scattering was used to follow the depolymerization of 60 nM gelsolin-capped 5 μM F-actin treated with a 2 fold molar excess of ADF (Fig. 4.5a). ADF induced depolymerization of the F-actin. Addition of a 2 fold molar excess of ADF to filaments pre-incubated with a 2 fold molar excess of JAS blocked the decline in light scattering (Fig. 4.5a) suggesting that JAS inhibited the depolymerization induced by ADF.

Sedimentation assays were performed to verify whether ADF can bind to JAS treated filaments (Fig. 4.5b-c). At pH 7.8 in the absence of JAS, 97% of total actin sedimented but only 10% sedimented in the presence of 3 fold molar excess of ADF (Fig. 4.5b), indicating ADF induced filament depolymerization. The ADF/actin molar ratio in the pellet is 1.8. Upon addition of 1 μM or 5 μM JAS to ADF-actin mixtures, actin in the pellet increased to about 80% of total actin and the molar ratio of ADF to actin in the pellet decreased to 0.8 to 0.9. These results demonstrate that even substoichiometric amounts of JAS (1:5 molar ratio to actin) can have a stabilizing effect on actin filaments and inhibit the action of ADF. The same equilibrium mixture was obtained when actin was incubated first with ADF and then with JAS, demonstrating that JAS can induce assembly of actin in the presence of ADF. In the presence of 20 μM JAS, more than 98% of total actin was sedimented and the molar ratio of ADF to actin in the pellet decreased to 0.3. At pH 6.8, the molar ratio of ADF to actin in the pellet was lowered from 1.8 in the absence of JAS to 0.8 in the presence of 5 μM JAS (Fig. 4.5c). Similar experimental conditions were used to examine the competition between cofilin and JAS for F-actin binding (Fig. 4.5d). The molar ratio of cofilin to actin in the pellet was lowered from

greater than 1.5 in the absence of JAS to 1.1 in the presence of 5 μM JAS to 0.4 in the presence of 20 μM JAS at pH 7.8. These data demonstrate JAS competes with both ADF and cofilin for binding to F-actin. The substoichiometric amounts of JAS stabilizes filaments and prevents ADF from depolymerizing them even though ADF can still bind to filaments. However, the binding of ADF and cofilin to filaments decreases in a JAS-dependent fashion.

Discussion

Although actin binding drugs, Lat A and JAS, have been widely used to explore actin-dependent processes *in vivo*, their effects on the interaction of actin and actin binding proteins are not well characterized. The effect of Lat A on three actin monomer binding proteins has recently been reported (Yarmola *et al.*, 2000). Lat A inhibited actin binding to thymosin β4, but not binding to profilin or DNase I. Our results conform that Lat A inhibited actin binding to thymosin β4. In addition, we found that Lat A competes with chick ADF for binding to G-actin. JAS competes with ADF for binding to F-actin and JAS protects filament from depolymerization by chick ADF, chick cofilin, and Lat A. These data have helped in interpreting the effects of the drugs on cortical contraction in *Xenopus* oocytes following fertilization.

The effects of Lat A and JAS during *Xenopus* fertilization

Lat A, a membrane permeable drug, was used to characterize the role of actin polymerization during fertilization in *Xenopus laevis* (Boyle et al., 2001). In *Xenopus*, 80% of the actin in the unfertilized eggs is unpolymerized (Merriam & Clark, 1978).

bound to ATP, and sequestered primarily by a thymosin β4-like protein (Rosenblatt *et al.*, 1995). The thymosin β4-bound actin can readily undergo polymerization in the presence of profilin (Pantaloni & Carlier, 1993), but Lat A prevents filament assembly. Some behavioral differences were observed between *Xenopus* embryos treated with 1 μM Lat A and 5 μM Lat A (Boyle et al., 2001). *In vivo*, 5 μM Lat A disrupts actin filament organization, resulting in changes in cell morphology and inhibition of actin filament mediated events including cortical contraction, cortical granule exocytosis, and fertilization body formation. At 1 μM Lat A, cortical contraction occurred at rates comparable to controls, but the plasma membrane was often visibly distorted and abnormal accumulation of pigment granules was observed. These findings suggest that actin filament polymerization plays an important role in cell processes accompanying *Xenopus* fertilization including cortical granule exocytosis, cortical contraction and cell shape maintenance. The differences the drug effects at the two concentrations might be explained by the difference in amount of unpolymerizable Lat A-actin complex formed through displacing actin from thymosin β4-actin complex. We calculated the percentage of actin remaining uncomplexed with Lat A that can undergo polymerization during fertilization, using the values of 0.2 μM for the K_D of the Lat A-actin complex (Coue *et al.*, 1987), and 2 μM for the K_D of the thymosin β4-actin complex (Carlier *et al.*, 1993), and a concentration of actin and thymosin of 32 μM and 40 μM respectively (Pollard *et al.*, 2000) in yolk-free ooplasm. At 5 μM Lat A, only 38% of the actin is uncomplexed with Lat A, whereas at 1 μM Lat A 69% of the actin is uncomplexed with Lat A. This 30% difference in the pool of dynamic actin could explain why the lower concentration of Lat A failed to inhibit rates of cortical contraction.

JAS, a filament stabilizing drug, was used prior to Lat A treatment to study the role of filament dynamics during fertilization in *Xenopus laevis* (Boyle et al., 2001). JAS alone did not prevent sperm incorporation, cortical granule exocytosis or cortical contraction, suggesting these events can occur without depolymerization of existing, stabilized filaments. Preincubation of eggs in 3 μM JAS before 1 μM Lat A mitigated the effects of Lat A (Boyle *et al.*, 2001). Here we show that JAS protected filaments from depolymerization induced by Lat A *in vitro* even when Lat A is at 40 μM. JAS thus prevents Lat A from depolymerizing the actin filaments in vivo.

Competition between Lat A and ADF

In yeast, Lat A was used as an actin monomer sequestering drug to study the effect of ADF/cofilin on F-actin turnover (Lappalainen & Drubin, 1997). Upon addition of 400 μM Lat A, most of the actin patches disappeared within 2 minutes in wild-type cells, but most actin patches persisted after 10 minutes in cells expressing a mutant (inactive) yeast cofilin. The concentrations of F-actin and G-actin are 2 μM and 0.01 μM respectively in S. cerevisae (Pollard et al., 2000). Yeast cofilin is the only member of the ADF/cofilin family found in yeast. Lat A at 400 μM could immediately bind free unpolymerized actin and displace yeast cofilin from actin. Thus all yeast cofilin could readily bind to filaments to both sever and enhance minus end dissociation. The competitive binding between Lat A and ADF/cofilin for G-actin would enhance the effect of cofilin on filament depolymerization since it will be displaced from monomeric actin allowing it to recycle to F-actin.

Competitive F-actin binding among ADF/cofilin, JAS and phalloidin

JAS induced actin polymerization and competitively inhibited the binding of phalloidin, an F-actin stabilizing peptide, to filaments (Bubb *et al.*, 1994). The F-actin binding and depolymerizing activities of ADF are inhibited by phalloidin (Hayden *et al.*, 1993), and rhodamine-phalloidin does not stain ADF saturated filaments (Minamide *et al.*, 2000). The competitive binding might due to the stabilization of a twisted filament conformation upon ADF/cofilin binding because the binding sites of ADF/cofilin and phalloidin on actin are distinct (McGough *et al.*, 1997). A recent study on the structure of ADF saturated filaments by a novel helical reconstruction method (Egelman, 2000) suggested there are two ADF binding sites on each actin subunit (Galkin *et al.*, 2001). The second binding site had fewer surface contact interactions with ADF compared to the previously characterized binding site but both sites show cooperative binding. The percent of the F-actin that had the second ADF bound varied from 30% to 70% depending on pH. The binding of ADF to the second binding site tilted the actin subunits ~12° and decreased interactions along the filament axis. Here, the molar ratio of AC to F-actin was measured as 1.8 for chick ADF, much higher than the previously reported 1.3 for human ADF and 1 for actophorin (Maciver *et al.*, 1998) and chick ADF (Hayden *et al.*, 1993). This is the first time a biochemical measurement has shown a significant deviation of the 1:1 binding for AC proteins on F-actin and lends support to the model of Galkin *et al.* (2001). Why may others have missed this? Probably the most highly saturated fragments are those that have severed and become very short such that airfuge sedimentation for 20-30 minutes is insufficient to pellet these small pieces.

JAS competitively inhibited the binding of ADF to filaments. The molar ratio of ADF to F-actin is reduced from 1.8 (in the absence of JAS) to 0.8 (in presence of 1 μM, sub saturation level of JAS) and further to 0.3 (in the presence of 20 μM JAS). The overall depolymerization by ADF was blocked by JAS at concentrations as low as 1 μM (1:5 molar ratio with actin subunits). The inhibition of ADF depolymerization by low JAS might due to one or a combination of the following explanations. JAS might preferentially bind to filament ends and prevent enhanced off-rate by ADF. JAS binding may interfere with getting long runs of ADF/cofilin bound filaments, which may be necessary for severing. JAS might change the conformation of the actin filament and thus block one of the two binding sites for ADF. Binding of ADF to the second site might be crucial for ADF to sever and/or depolymerize filaments. The conformation of JAS saturated filaments probably disrupts both binding sites for ADF.

Chapter Five

General Discussion

Summary

The first objective of this work was to identify functional differences between ADF and cofilin, homologues of the same family that are crucial for enhancing filament dynamics *in vivo*. I compared chick ADF and chick cofilin in their interactions with chick muscle actin. ADF and cofilin have qualitatively similar effects on actin but they differ quantitatively in their interaction with actin. The binding of ADF to MgATP-G-actin is 10-fold higher than cofilin, suggesting ADF could be one of the monomer sequestering proteins that maintains the Mg-ATP-G-actin pool. Both ADF and cofilin sever filaments and enhance the off-rate from pointed ends in a pH dependent manner, but ADF is more effective than cofilin in these activities. The filament severing is favored at alkaline pH, and enhancement of off-rate is favored at acidic pH.

A second objective of this work was to compare ADF/cofilin proteins from different organisms to see if they can be classified as ADF-like or cofilin-like proteins. Based on their G-actin binding, filament severing and filament length distribution, most of ADF/cofilins from different organisms fall into these two categories. ADF-like proteins include chick ADF, Xenopus XAC1 and XAC2, and starfish depactin. They bind to MgATP-G-actin with higher affinity and sever filaments to a greater extent in a

pH dependent manner. Cofilin-like proteins include chick cofilin, *Caenorhabditis elegans* UNC 60B, *Arabidopsis* ADF1, *Drosophila* twinstar, *Dictyostelium* cofilin, and *Acanthamoeba* actophorin. They bind to MgATP-G-actin with lower affinity and/or sever filaments to a more limited extent.

A third objective of this work was to investigate the effect of Lat A and JAS on the interaction of actin with actin binding proteins, ADF and thymosin β4. Lat A competes for G-actin binding with the ADF and thymosin β4. JAS stabilizes filament and inhibits filament depolymerization by Lat A. JAS at subsaturating levels inhibits filament depolymerization by ADF even though ADF may still bind to filaments. However, the binding of ADF to filaments is displaced in a JAS-dependent fashion.

Future Experiments

Complete the classification of ADF/cofilin proteins

In chapter three, the G-actin binding and the F-actin depolymerizing activities of many ADF/cofilins were compared using non-denaturing gel electrophoresis, timed sedimentation, and fluorescence severing assays. The fluorescence severing and depolymerizing assay modified from Moriyama and Yahara (1999) need to be performed to compare the severing and enhancement of off-rate from pointed ends at both pH 6.8 and pH 7.8. Further work is needed to sort out a few ADF/cofilins that cannot fit into the current two categories. Human ADF and *Caenorhabditis* UNC-60A show heterogeneous filament distribution by timed sedimentation suggesting these are ADF-like, but they depolymerize filaments slowly in the fluorescence severing assay, behaving more like cofilin. Yeast cofilin has long filaments that predominate at steady state in cofilin-actin

mixtures but it depolymerizes F-actin rapidly in the fluorescence severing assay. Additional experiments need to be performed to determine if these discrepancies are just due to the particular batch of protein being analyzed or if they represent proteins with some intermediate types of activity. *Toxoplasma gondii* ADF is the smallest protein in the AC family containing only 118 amino acids (Allen *et al.*, 1997). *Toxoplasma gondii* ADF binds to G-actin as determined by gel filtration and depolymerizes filaments as determined using a sedimentation assay. Further work will focus on determining its binding stoichiometry to G-actin and F-actin, its filament severing, and its ability to enhance the off-rate from pointed ends.

Lat A competes with ADF for binding to G-actin

In chapter four, Lat A was shown to displace ADF from ADF-actin complex, but addition of more than 30 μM Lat A to the mixture of 5 μM actin and 10 μM ADF did not show a decrease in ADF-actin complex accompanying the increasing amount of Lat A. Lat A inhibits nucleotide exchange on G-actin in a dose-dependent manner (Ayscough *et al.*, 1997). ADF also inhibits nucleotide exchange on actin monomer (Hayden *et al.*, 1993). Thus, one possible mechanism to explain these results is that the binding of Lat A to G-actin might require nucleotide exchange and its binding to G-actin in the presence of ADF might take hours and not just the 10 minutes as was used in these experiments. Further experiments will be performed with longer incubation times (more than 2 hours), with different actin bound cation (Ca-actin vs. Mg-actin), and by starting first with Lat A-actin and titrating with ADF.

Aip1 interaction with cofilin and ADF

Actin interacting protein 1 (Aip1) was first identified in a two-hybrid screen against yeast actin (Amberg *et al.*, 1995). It is a 65 kD protein and binds with a maximum stoichiometry of 1:2:2 to AC and actin (Okada *et al.*, 1999). Aip1 is localized to regions of cell cortex including lamellipodia in *Dictyostelium* where actin dynamics are high (Konzok *et al.*, 1999). Aip1 has no significant F-actin severing or depolymerization activity on its own, but it greatly enhances filament severing promoted by yeast cofilin or *Xenopus* XAC (Rodal *et al.*, 1999; Okada *et al.*, 1999). In chapter two, cofilin is shown to have weaker severing activity than ADF. In the leading edge of ruffling membrane, filament severing is necessary for the generation of free barbed ends and the protrusion of membrane. Inhibiting cofilin activity blocks lamellipodial extension (Zebda *et al.*, 2000). Future work should examine if Aip1 enhances severing activity of ADF or cofilin to similar extents. Is Aip1 required to enhance the severing activity of cofilin and, if so, is this enhancement required for rapid lamellipodial extension?

"Knock in" either ADF or cofilin genes to substitute for the other in mouse development

In yeast, the cofilin null mutant can be rescued by porcine ADF or porcine cofilin (Iida *et al.*, 1993). However, yeast may not require the subtle difference in regulating actin dynamics that might be required in metazoans. In multicellular organism, it is not known if one homologue of ADF/cofilin can functionally replace the others. Future work could focus on "knock in" experiments in which either the ADF or cofilin gene is substituted for the other but without altering the promoter. By following mouse development it will then be possible to determine if the nature of the AC protein

expressed during different stages of development is important or is it the spatial/temporal control of the expression that led to development of multiple AC genes in metazoans.

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