A BIOCHEMICAL INVESTIGATION OF ACTIN DISASSEMBLY MECHANISMS

BY

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DISSERTATION

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ABSTRACT

The dynamic nature of the actin cytoskeleton enables the rapid shape changes that are necessary for processes such as wound healing, motility and division of cells. Disassembly of actin filaments is extremely critical for the reorganization of cell shape. The cell possesses several factors that depolymerize actin filaments in an environment that has a high concentration of polymerizable monomer. However current microscopic techniques preclude the direct observation of the dynamics of individual actin filaments that usually exist as part of highly crosslinked networks inside cells. Therefore, the mechanism(s) by which actin filaments disassemble inside cells remains unclear.

In this work we use a combination of single filament imaging of fluorescently labeled actin filaments as well as pyrene and FRET-based spectroscopy in order to reconstitute cellular disassembly in vitro in the presence of three factors: cofilin, coronin and Aip1. These three factors have been shown to be principally responsible for the disassembly activity of thymus extract. We describe here our discoveries regarding catastrophic whole filament destabilization of actin in the presence of the three factors. We also reinvestigated the role of Aip1 alone in cofilin-mediated depolymerization of actin filaments. We showed that Aip1 is not an actin capping protein as was previously thought, however it can destabilize cofilin-saturated stable filaments and potentiate cofilin's severing and depolymerization activity. During the course of our work we also uncovered some insights on the biophysics of filament severing in the presence of cofilin.

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CHAPTER 1

GENERAL INTRODUCTION

Division, directional movement, polarized growth and secretion: these are seemingly mundane processes in a cell's life. However, in order to carry out these functions, spatial organization of cellular material is critical. For example, in order to be able to divide quickly, faithfully and symmetrically, cells must segregate their chromosomes to two distinct poles. In order to be able to move or grow directionally, cells must designate a top, bottom, front and back.

Cytoskeletal polymers are the executors of spatial organization in eukaryotic (and even bacterial) cells. These are filamentous polymers made up of smaller subunits that can readily be assembled into various structures, and disassembled easily to be reconfigured into different structures. These are as vital to cells (and consequently, tissues and organisms) as systems that mediate processes like energy production or vesicular transport.

Eukaryotic cells have three main polymer systems that endow cells with the properties of organization, structural stability and rapid reorganization of cell shape. These are: actin, microtubules and intermediate filaments. Actin and microtubules possess an intrinsic structural and biochemical polarity, whereas intermediate filaments are non-polar.

Actin is the most abundant protein in eukaryotic cells and can polymerize into dynamic filamentous polymers. These filaments can form a variety of arrays (Figure 1.1) (Blanchoin et al., 2014). Bundled filaments are found in the basal stress-fibers and in the finger-like sensory filopodia that the cell extends in order

to sense its environment. Actin filaments are also found in crosslinked arrays at the leading edge of the cell and it is the polymerization and the pushing of these filaments against the membrane that provides the force for cellular motility. Actin is also coupled to several transmembrane molecules that are utilized by cells in order to adhere to one another and to their substrates. Molecular motors can "walk" on this system of tethered filaments and generate contractile forces. Therefore, actin (along with adhesion molecules and motors) is a major component of the force generation/transmission system of cells. Consequently, the actin cytoskeleton is a versatile system that has a major role in processes including cellular motility and division, cell polarity and directional transport, cell adhesion and endocytosis. Dysfunction of these processes can be lethal for cells.

Biochemical characteristics of actin filaments

Actin filaments are formed by the polymerization of monomeric subunits (G-actin) that can bind ATP. Actin displays a structural asymmetry as the nucleotide binding cleft is found on one face of the molecule and this lends an intrinsic polarity to the filament (Figure 1.2). Polymerization of G-actin activates the nucleotide hydrolysis activity of the protein 40,000-fold (*for review see* Reisler and Egelman, 2007). Incorporation of ATP-G-actin into a filament is followed by ATP-hydrolysis and phosphate release. This is likely associated with changes in the structure of the filament although the precise nature of these is still debated. The energy of ATP hydrolysis is used to destabilize actin filaments

and therefore an ADP-actin filament is less stable than an ATP-filament and will shrink faster. Exchange of monomers occurs at the ends of the filament and actin filaments have two ends with distinct properties, a more dynamic end (also called the barbed end, or the plus end) which adds and loses monomer faster than the less dynamic end (also known as the pointed end or the minus end) (Pollard, 1986). The rate at which monomers are exchanged at the two ends also depends upon the concentration of the monomers in solution. At a monomer concentration above 0.12 µM (the barbed end *critical concentration)* the barbed end grows faster than it shrinks and at a concentration above 0.6 µM, the barbed as well as the pointed end grow. However, when the concentration of monomer is in the range of 0.12- 0.6, the filament preferentially grows at the barbed end and loses monomer at the pointed end. The filament therefore undergoes cycling or “treadmilling” until all the ATP in the solution is exhausted (Wegner, 1976).

In cells, although there is a high concentration of polymerizable monomer (Pollard, 2004) and exposed barbed ends would have the propensity to grow, unregulated polymerization of actin is unproductive. Some steps that ensure that filaments nucleate only in specific regions of the cell are as follows.

Firstly, the actin filament possesses an intrinsic kinetic barrier to polymerization. The “nucleation” step, or formation of an actin trimer (nucleus/ seed) that can elongate in the presence of monomer, is thermodynamically unfavorable (Figure 1.3)(Alberts et al., 2008). Most of the G-actin in the cell exists bound to monomer sequestering factors that limit the unregulated

polymerization of actin. For example, one such factor, profilin, does not allow spontaneous nucleation of actin, but only allows polymerization at existing free barbed ends (Pantaloni and Carlier, 1993). Secondly, polymerization is usually activated at membranes and in response to extracellular signals. Lastly, and most pertinently, the actin in cells constantly undergoes turnover. Photo-activation of a fluorescently caged derivative of actin showed that the F-actin in cells has a half-life of ~30 seconds (Theriot and Mitchison, 1991). An attractive possibility is therefore that actin assembly is locally regulated in cells however the rest of the cytoplasm has depolymerizing characteristics. By utilizing mechanisms that make them less susceptible to disassembly, actin filaments and arrays may be able to persist in the cytoplasm.

Actin disassembly inside cells

In pure solution at steady state, filaments are thought to undergo a phenomenon known as treadmilling (Wegner, 1976). It would be reasonable to assume that actin filaments also treadmill inside cells, however, the *in vitro* rate constants are 2 orders of magnitude too slow to account for the fast rates of disassembly seen inside cells (Theriot and Mitchison, 1992). This discrepancy can be explained by the fact that cells express factors that can accelerate actin disassembly.

Although the view of the mechanism of disassembly inside cells continues to evolve, most models until recently have relied on the assumption that monomer loss occurs mainly at the pointed ends of actin, much like treadmilling

in vitro. Functional studies of actin disassembly proteins, thus far, have been slightly biased by this view.

The most essential of filament depolymerization factors, ADF/cofilin, was purified from brain extract in a search for proteins that could maintain actin in the nonfilamentous form (Bamburg et al., 1980). Since then cofilin and cofilin-homologs have been found to be necessary for viability in several organisms (*for review see* Bamburg and Bernstein, 2010). Cofilin increases filament turnover or "dynamizes" filaments (Carlier et al., 1997) by severing them into smaller fragments (Maciver et al., 1991), the pointed ends of which could lose monomer. The increase in the number of pointed ends due to severing was initially interpreted as an increase in the pointed end off-rate (Carlier et al., 1997). However, this has since been disproved. Cofilin does not significantly increase the off-rate at filament ends (Andrianantoandro and Pollard, 2006) and thus is unlikely to affect the rate of individual filament treadmilling.

Cofilin binding to actin changes the twist of actin filaments (McGough et al., 1997). The interfaces between cofilin-bound twisted sections of actin and cofilin-free sections of actin are thought to be unstable and this leads to severing (De La Cruz, 2009).

Cofilin also binds ADP-actin with greater affinity than ATP-actin (Carlier et al., 1997), thus leading to the view that assembly and disassembly at the leading edge of the cell are spatially separated (Pollard and Borisy, 2003) (Figure 1.4). In this view, newly polymerized filaments move rearward by retrograde actin flow

and become competent to bind cofilin after hydrolyzing ATP. They are then depolymerized by severing and pointed end monomer loss.

There is a high concentration of polymerizable monomer inside cells. Therefore, depolymerization would only be effective if the dynamics of the exposed barbed ends were restricted. High affinity barbed end capping factors such as Capping protein (CP) (Wear et al., 2003) and Aip1(Okada et al., 2002) are thought to be responsible for occluding the barbed ends of newly severed filaments in order to bias filament dynamics toward pointed-end disassembly. This is a rather convenient view of filament depolymerization inside cells that is consistent with the behavior of some factors *in vitro*. However there are a number of experimental observations that are not readily explained by the textbook model.

Firstly, not all barbed ends in the leading edge of the cell are capped. In 2002, Watanabe and Mitchison expressed a low level of gfp-labelled actin in cells and observed clusters of the fluorescent molecules or “speckles” to infer the dynamics of single actin filaments. Speckles persisted within the field of view if they were incorporated into an actin filament. Appearance of speckles could be used to track a polymerization event whereas disappearance of a persistent speckle could report on disassembly. They found that new fluorescent speckles appeared not just at the membrane, but all throughout the leading edge of the cell (Watanabe, 2002; Brieher, 2013). This indicated that there were uncapped filament ends all throughout the leading edge of the cell.

Forcibly capping these ends by the use of a barbed-end capping drug such as cytochalasin D reduced depolymerization kinetics rather than accelerated them, contrary to what would be predicted if filaments were disassembling solely through their pointed ends (Kueh et al., 2008). This indicated that barbed ends of filaments contributed to filament turnover.

Observation of the branched organization of actin filaments in the lamellipodium of keratocytes and fibroblasts and in the actin comet tail of the bacterium *Listeria monocytogenes* by electron microscopy showed that pointed ends were anchored in Y-junctions and colocalized with the actin filament branching protein Arp2/3 (Svitkina and Borisy, 1999; Cameron et al., 2001). Although free barbed ends were visible, free pointed ends could not be visualized readily. This called into question the assumption that loss of monomer occurs mainly at the pointed ends of actin filaments inside cells.

Cofilin-mediated severing alone does not adequately explain disassembly behavior of cellular actin

Cofilin-mediated severing alone does not appear to be an efficient way to depolymerize actin filaments. When actin filaments are bound by high concentrations of cofilin, they are stabilized in an alternative twisted configuration (McGough et al., 1997). Cofilin-mediated severing does not obey the law of mass action and filament severing occurs most efficiently at low ratios of cofilin to actin (Andrianantoandro and Pollard, 2006; Suarez et al., 2011).

A number of observations indicate that cofilin-mediated severing is more critical for polymerization rather than depolymerization. Locally photo-activating a caged, inactive cofilin leads to a burst of polymerization and local protrusion (Ghosh et al., 2004). Expressing a severing-deficient mutant of cofilin in yeast prevents the assembly of actin patches (Chen and Pollard, 2013). Thus, severing appears to contribute to the generation of new mother filaments for elongation.

The amount of cofilin in cells can be high, ranging from 3->20 μM in cells (Rosenblatt et al., 1997; Chen and Pollard, 2013; Brieher et al., 2006). However, adding recombinant cofilin to *Xenopus* egg extract to 4 times the amount of endogenous cofilin present in the extract does not impede disassembly (Rosenblatt et al., 1997). This indicates that it is not the amount but the ratio of cofilin to actin and perhaps other actin-binding/depolymerization factors that leads to a depolymerization event.

It is difficult to temporally and spatially resolve disassembly in a cell at the single filament level due to the highly crosslinked nature of filaments inside cells. However, fluorescence decay profiles of bulk actin present in *Listeria* actin comet tails or in the lamellipodium of the cell can be used to infer the mechanism of disassembly inside cells (Figure 1.5). The kinetics of cellular actin disassembly can be fit by a single exponential curve (Theriot and Mitchison, 1991; Kueh et al., 2010). This means that disassembly is a stochastic process like other processes that show single exponential decay such as radioactive decay. It implies that a newly polymerized filament is as likely to disassemble

within the population as an old filament and filaments do not undergo an ageing process that increases the probability of their destruction. Therefore, the fact that cofilin can only bind aged ADP-rich actin filaments *in vitro* does not seem to have much bearing on disassembly inside cells. This is also consistent with experimental observations that polymerization and depolymerization are not spatially separated in the cell (Ponti et al., 2003).

Mathematical modelling of various disassembly mechanisms showed that severing followed by pointed-end disassembly did not produce exponential decay of actin filaments (Kueh et al., 2010). The two mechanisms that complied with the exponential decay model included treadmilling and catastrophic filament disassembly along the length of the filament.

Moreover, if cells relied on severing alone, this might compromise the mechanical integrity of actin networks as fragmentation of filaments would lead to extremely short filaments that may not be able to perform load-bearing functions (Kueh et al., 2010)

Disassembly activity of cellular extract

Cell extract is more efficient than pure cofilin at disassembing actin filaments and arrays. Additionally there is substantial evidence that current models of disassembly that involve severing/pointed end disassembly cannot fully explain the disassembly activity of cells. This motivated the search for factors that could potentiate cofilin-mediated disassembly (Brieher et al., 2006). By following the disassembly activity of thymus extract by biochemical

fractionation, two factors that potentiate cofilin-mediated disassembly *in vitro* were isolated namely, coronin and Aip1.

Coronin 1A was first identified in *Dictyostelium* as an actin binding protein that participates in the reorganization of the cytoskeleton (De Hostos et al., 1993; Gerisch et al., 1995). Mutants of coronin have defects in cytokinesis and cell-migration. Coronin has also been found to be important for debranching of actin at the leading edge of fibroblasts (for review see Gandhi and Goode, 2008). Genetic interactions have been detected for coronin with cofilin in yeast (Goode et al., 1999). A possible molecular mechanism was put forth in the fractionation study; coronin could increase cofilin-binding on *Listeria* actin comet tails (Brieher et al., 2006).

Aip1 is an actin and cofilin-interacting protein (Rodal et al., 1999) that was found to increase cofilin-mediated disassembly *in vitro* (Okada et al., 1999; Rodal et al., 1999). Mutations in Aip1 or perturbation of its function in cells leads to ectopic accumulation of F-actin (Ren et al., 2007; Kato et al., 2008), defects in actin turnover dynamics (Ono, 2003), and depletion of the actin monomer pool that fuels actin assembly (Okreglak and Drubin, 2010). The dominant model of Aip1's activity is that it can cap newly severed cofilin-actin filaments (Okada et al., 2002; Balcer et al., 2003), however there is some work that calls this into question (Ono et al., 2004).

When the fractionated factors (coronin, cofilin and Aip1) were combined together *in vitro*, they showed an eccentric disassembly mechanism that was termed 'bursting' (Figure 1.6) (Kueh et al., 2008). Bursting filaments lost large

chunks of filaments in an end-biased manner without the detection of any short filament intermediates. The authors proposed that the filaments were depolymerizing directly to monomer, and bursting behavior unlike severing could produce exponential decay of actin (Kueh et al., 2010). Moreover, bursting was resistant to polymerizable monomer, indicating that it could be relevant in the cellular environment.

However, there remains a controversy whether bursting is a distinct mechanism from filament severing. Moreover the contribution of each factor in the reaction is not known, nor is it known how these factors offer resistance to polymerizable monomer. As novel roles for factors come to light, it is important to reinvestigate old views and mechanisms of disassembly. With the advent of single filament imaging by TIRF and wide-field fluorescent microscopy we are able to view the effects of defined combinations of factors on actin filaments. With the inclusion of each additional factor to the mix, we increase the number of distinct molecular species and kinds of depolymerization reactions that occur. The ability to view these reactions thus gives us many insights into processes that could only be studied indirectly in the past.

We describe here our discoveries regarding catastrophic whole filament destabilization of actin. We developed FRET-based spectroscopic assays to give us insight into the bulk behavior of filament disassembly in the presence of cofilin, coronin and Aip1. We also investigated the role of Aip1 in cofilin-mediated depolymerization of actin filaments. During the course of our work we also

uncovered some insights on the biophysics of filament severing in the presence of cofilin by single-filament imaging and these are described in the appendix.

Figures

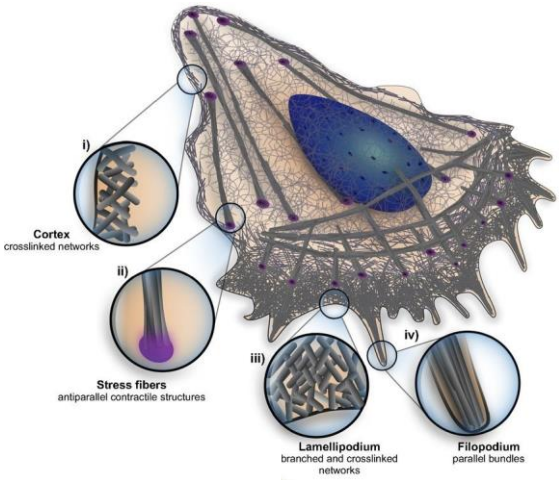

Figure 1.1 Actin filaments in cells are found in a variety of arrays. Bundled filaments are found in the stress fibers and filopodia – sensory projections at the front of the cell; whereas crosslinked networks of filaments are found in the lamellipodium or leading edge of the cell and the cortex (Blanchoin et al., 2014).

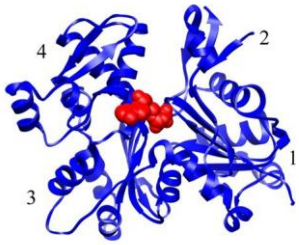

Figure 1.2 The nucleotide binding cleft in actin is found on one face of the molecule and this lends to the protein an intrinsic structural asymmetry (Reisler and Egelman, 2007).

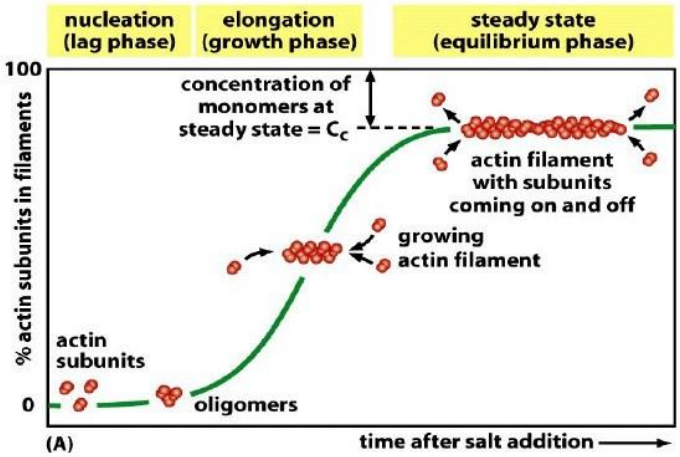

Figure 1.3 The formation of the actin trimer (oligomer) is the rate limiting step of actin assembly and involves a kinetic lag (Alberts et al., 2008) .

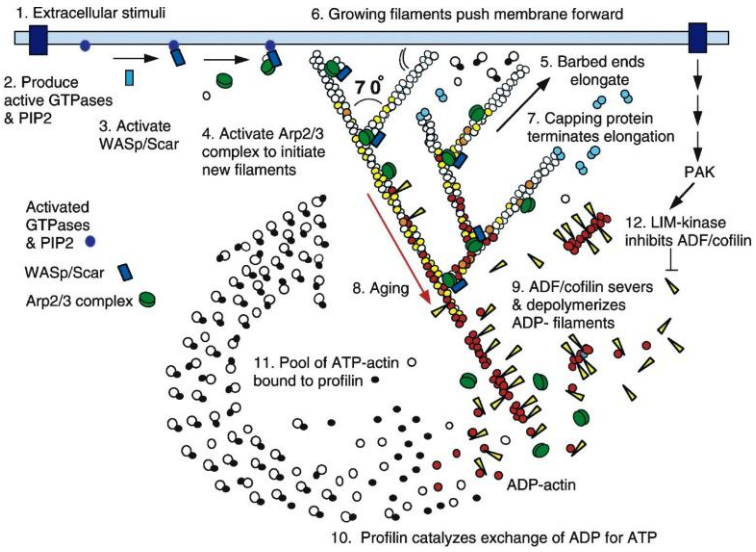

Figure 1.4 A popular model of actin disassembly involves the spatial separation of polymerization and depolymerization and relies on cofilin-mediated severing and capping of free barbed ends (Pollard and Borisy, 2003).

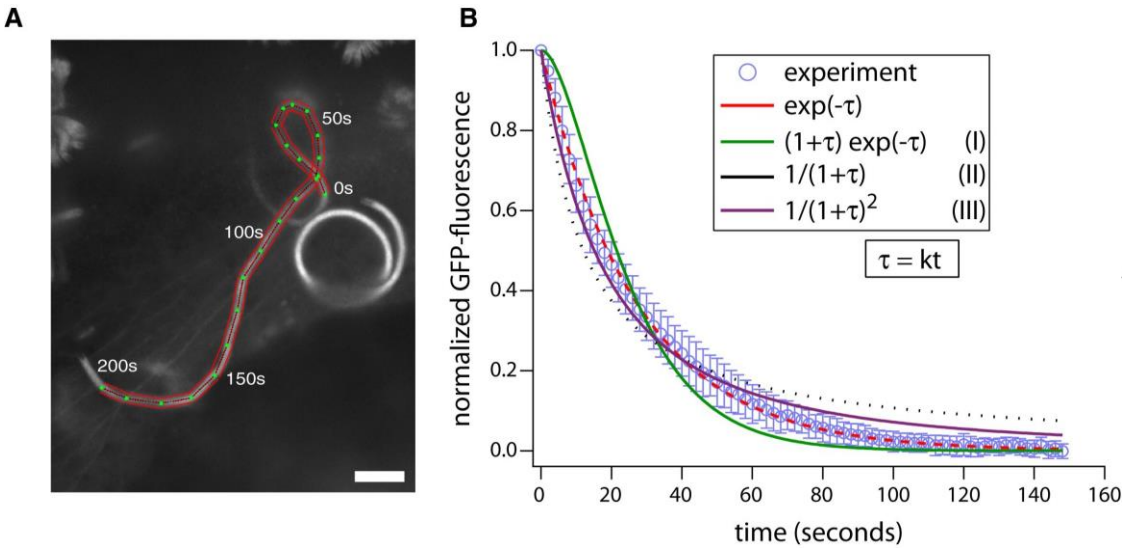

Figure 1.5 Polymer mass in a *Listeria* actin comet tail is fit by a simple exponential curve. (A) Maximum intensity projection of GFP-actin of a comet tail from a time lapse movie of *Listeria* in a BSC- 1 cell (B) Experimental data (blue circles) is best fit by a simple exponential (red dotted line) (Kueh et al. 2010).

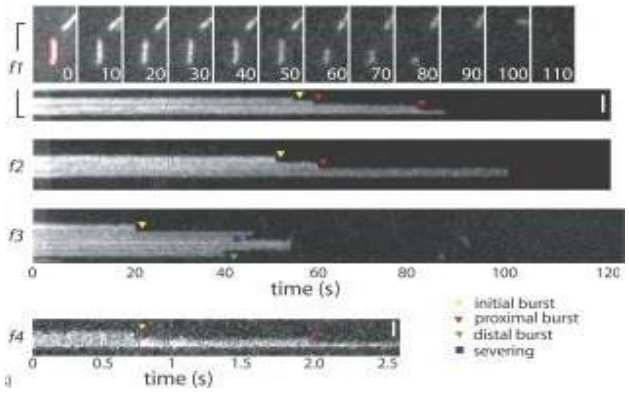

Figure 1.6 End-biased depolymerization of 'bursting' filaments. Frames from a movie of a single actin filament depolymerizing in the presence of the bursting factors is shown with a corresponding kymograph of filament length over time. The kymograph shows abrupt losses of chunks of filaments (arrowheads) (Kueh et al., 2008) .

CHAPTER 2

SPECTROSCOPIC ASSAYS TO INVESTIGATE ACTIN DISASSEMBLY IN THE PRESENCE OF COFILIN, CORONIN AND AIP1

Introduction

I*n vitro* spectroscopic studies of actin labeled with a fluorescent probe *N*-(1-pyrene) iodoacetamide (referred to as pyrene-actin) have provided important information about the properties of the polymer (Kouyama and Mihashi, 1981). Emitted fluorescence of pyrene-actin increases with an increase in polymer mass. Kinetic measurements of pyrene-actin fluorescence can provide us with association and dissociation rates of actin monomer, as well as information about critical concentration(Cooper et al., 1983). Pyrene-based bulk assays are also important because they offer insights on the behavior of F-actin in combination with other factors at steady state. Bulk assays can often complement microscopic filament imaging assays especially about processes that occur on very fast time scales.

However, as pyrene is an environmentally sensitive fluorophore, factors used in combination with actin can sometimes alter its behavior in undesirable ways. For example, fluorescence of pyrene-actin is quenched by the binding of cofilin – an essential depolymerizing protein in eukaryotes (Carlier et al., 1997). Pyrene can report usefully on cofilin-binding to actin, as degree of quenching is proportional to amount of cofilin bound. However, it cannot reliably answer questions about polymer mass or depolymerization rates.

Single filament assays have also been used extensively to study the effects of cofilin on actin (Andrianantoandro and Pollard, 2006). The information

derived from these often contradicts that obtained by bulk pyrene assays. For example, spectroscopic assays indicate a change in critical concentration of actin by cofilin whereas single filament assays do not (Carlier et al., 1997; Andrianantoandro and Pollard, 2006). It is necessary to develop better assays that can report on bulk actin disassembly and address these differences. Moreover, bulk assays can often over come the spatial and temporal limitations of light microscopy as depolymerization reactions can occur rapidly and with the generation of intermediates that may be unresolvable by light microscopy.

In order to reliably assess the behavior of actin in the presence of cofilin and other depolymerizers, and to circumvent the problems of quenching of fluorescence, we used a FRET-based spectroscopic method that reports on actin dynamics. FRET has been used in the past to report on actin assembly (Wang and Taylor, 1981; Taylor et al., 1981) and on filament assembly and disassembly of the bacterial actin homolog, ParM (Garner et al., 2004). We discovered that the FRET signal is unperturbed by the binding of cofilin and therefore can be used to report on rate and extent of disassembly in the presence of cofilin and other depolymerizers.

In order to supplement the FRET assay we also developed a monomer generation assay which was based on the principle of quenching of Oregon green actin fluoresence. Fluorescein is an environmentally sensitive fluorophore whose fluorescence is quenched or undergoes a red-shift when bound by anti-fluorescein antibodies or when fluorescein-tagged proteins non-specifically bind other proteins, certain amino acids and molecules like iodide in solution (Watt

and Voss, 1977, 1979; Ferenčík, 1993). We observed that the fluorescence of Oregon green 488 actin monomer was quenched by the binding of Vitamin D-binding protein (DBP), a high affinity actin monomer sequestering protein (Van Baelen et al., 1980; Lees et al., 1984). Vitamin D-Binding Protein has been known to increase the fluorescence of monomeric pyrene-actin and 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole (NBD)-actin (Lees et al., 1984; Detmers et al., 1981). In these studies, as well in our work, the actin was labelled either on cysteine 373 or lysine 372 which indicates that these residues might be close to the interaction surface of DBP.

We describe the development of the FRET and the monomer-generation assay. We used these assays to investigate disassembly in the presence of the bursting factors and to study differences between catastrophic filament disassembly or “bursting” and other mechanisms of actin disassembly.

Materials and Methods

Protein purification: Actin was purified as previously described (Pardee and Spudich, 1982) and gel filtered on a Sephacryl S-300 (GE-Healthcare) column. Recombinant human cofilin-1 was purified as previously described with modifications (Brieher et al., 2006). Briefly, cofilin was expressed in *E.coli* BL21 cells by IPTG induction. The supernatant of the lysed bacterial cells was passed over a DE-52 column (Whatman) equilibrated in 20 mM TRIS pH 8.0, 50 mM NaCl, 1 mM EDTA and 1 mM PMSF (Buffer A). Cofilin was contained in the flowthrough and relevant fractions were passed over a Q-column (GE Healthcare) equilibrated in Buffer A. Cofilin, contained in the flow-through, was purified by ultracentrifugal concentration and gel filtration on a Sephacryl S-300 column. Aip1 was purified using the same series of ion exchange, hydrophobic interaction and gel filtration columns as described previously (Brieher et al., 2006). ActA (Skoble et al., 2000) and CapZ (Soeno et al., 1998) were purified as previously described. Coronin 1a was purified commercially from baculovirus (Blue Sky Bioservices). Arp2/3 was purified as described (Brieher et al., 2004) with some modifications. Briefly bovine thymus was homogenized 1:2 (w/v) in buffer (20 mM TRIS pH 7.4, 20 mM KCl, 5 mM MgCl2, 2 mM EGTA, 5 mM BME, 1 mM PMSF) in a standard Waring Blender and passed over a DE-52 column (Whatman) equilibriated in the same buffer. The flowthrough was dialyzed into buffer B (20 mM HEPES pH 7.0, 20 mM NaCl, 5 mM BME) and passed over a 70mL S-column (GE Healthcare). Activity was followed by *Listeria* tail formation

assays. Arp2/3 positive fractions were pooled and dialyzed to pH 8.5 and applied to a Source Q column (GE). Positive fractions were then gel filtered.

Fluorescent labeling of actin: Pyrene-actin and Oregon Green 488 actin were prepared as described (Bryan and Coluccio, 1985). Briefly, G-actin was labeled on cysteines with a stoichiometric amount of N-pyrene or Oregon Green 488 (Molecular Probes, Invitrogen). Actin was immediately polymerized by addition of 100 mM KCl, 2 mM MgCl2 and 0.5 mM ATP. The reaction was allowed to proceed overnight at room temperature (N-Pyrene maleimide) or at 4ºC (Oregon Green 488 maleimide). Filaments were collected by centrifugation at 140,500 X g for 2 hours, resuspended in G-buffer, and dialyzed exhaustively against G-Buffer (containing DTT). G-Actin was subsequently gel filtered on a Sephacryl S- 300 column (GE Healthcare). Pyrene actin was 80% labeled and Oregon Green actin was 60-80% labeled. 80% TMR-actin was prepared as described (Tang and Brieher, 2012).

FRET assay: Aliquots of labeled actin were diluted to 20 µM in G buffer (pH 7.4) and spun the next day at 227,900 X g for 20 minutes. 35-40% Tetramethylrhodamine labelled actin and 12-15% Oregon green 488 labelled actin were premixed at 20 µM.

For the initial controls for polymerization assays (200 µl in a 96-well plate), polymerization was initiated by diluting G- actin 3 µM in 1x F-buffer(10 mM HEPES 7.8, 50 mM KCl, 0.5 mM EGTA, 1 mM MgCl2, 1 mM ATP). To monitor assembly, 0.5 µM 80% labeled spun pyrene-actin was added to the reaction as a tracer. Total actin in the reaction was 3.5 µM. Excitation wavelengths for Pyrene

and OG488 were 365 nm and 490 nm respectively. Fluorescence intensity was detected at 410 nm and 530 nm on a Spectramax M2 fluorimeter (Molecular Devices).

For depolymerization reactions in the presence of the bursting factors actin was prepolymerized at 10 μM by the addition of 1x F-buffer(10 mM HEPES 7.8, 50 mM KCl, 0.5 mM EGTA, 1 mM MgCl2, 1 mM ATP). The final concentration of actin in the reaction was 1 μM. Spectroscopic monitoring of fluorescence quenching of OG488 (λEx = 490 nm, λEm = 530 nm) over time was used to report on assembly/disassembly on a Spectramax M2 fluorimeter (Molecular Devices). Final concentrations of disassembly proteins were 1.25 μM cofilin, 0.75 μM coronin and 0.1 μM Aip1, unless indicated otherwise in graphs. Data was normalized using values of 1 as actin polymer and 0 as actin monomer.

Monomer generation assay: Actin for this assay was prepared identically as the FRET assay except for the exclusion of TMR-actin from the reaction. TMR -actin was replaced by unlabelled G-actin. 1 μM Vitamin D-binding protein (Goldbio) was added to monomeric or polymeric actin to sequester actin monomer and the fluorescence was monitored (λEx = 490 nm, λEm = 530 nm) over time on a Spectramax M2 fluorimeter (Molecular Devices). Concentrations of proteins were as follows: 1 μM actin 1.25 μM cofilin, 0.75 μM coronin 0.1 μM Aip1 and 0.1 μM CapZ when applicable.

Seeding assay: Actin filaments treated with various combinations of depolymerizers as described in the FRET assay. The reaction was allowed to

proceed to completion and roughly 1/10 of the reaction (125 nM) was used to seed new pyrene G-actin assembly (25% labelled, 1 μM, preincubated in G-buffer). Fluorescence was monitored (λEx = 365 nm, λEm = 410 nm) over time.

Measurement of Critical Concentration using FRET: F-actin was prepolymerized at 10 μM and diluted to the desired concentrations in 1x F-buffer, in the presence of the indicated concentrations of the depolymerizers. Reactions were allowed to incubate overnight (12 hr) before reading. This was compared to equimolar actin concentrations where there was no FRET. Data were converted to E values as described previously by using the equation E*[actin]=Emax[actin]-Emax*Cc (Bugyi et al., 2006).

Results

FRET assay reports on cofilin-mediated disassembly not cofilin-binding

Excitation energy of fluorophores can be transfered non-radiatively to acceptor fluorophores that have an excitation wavelength overlapping with the emission spectrum of the donor fluorophore in a process known as Förster Resonance Energy Transfer (FRET) (Stryer and Haugland, 1967). We used FRET to report on the kinetics of actin polymerization by covalently labeling actin with two different fluorescent probes- Oregon Green 488 (OG488) and tetramethylrhodamine (TMR). This is manifested as a 'quenching' or decreased emission of donor fluorescence that can be measured over time.

We mixed green and red fluorescently labeled actin at a high ratio of acceptor to donor. In order to show that the covalent labeling of the actin in no way altered the kinetics of assembly, we added a small amount of pyrene actin to the mix as a tracer (Figure 2.1A). The labeled actin shows the canonical lag prior to polymerization. The addition of actin nucleators, *Listeria monocytogenes* surface protein ActA and Arp2/3 significantly reduces the lag phase (Figure 2.1B).

After we established that the assay was reporting on the kinetics of actin polymerization, we asked if it could report on bulk actin disassembly. We first examined the effect of cofilin alone on actin, using FRET. The addition of cofilin to 2 µM prepolymerized F-actin at pH 7.8 caused an initial decrease in FRET, representing a decrease in polymer mass, in a dose-dependent manner over 25 min (Figure 2.2A). This is consistent with the fact that cofilin binds to the newly

dissociated ADP actin monomers with an affinity of 150 nM and suppresses ADP to ATP exchange (Blanchoin and Pollard, 1998; Ressad et al., 1998).

Cofilin changes the conformation of actin filaments upon binding and also causes severing and depolymerization of actin (McGough et al., 1997; Maciver et al., 1991). We considered the possibility that the decrease in FRET could simply be a result of conformational changes that occur upon the binding of actin by cofilin. In order to test if the decrease in FRET indicated disassembly of F-actin, we carried out the reaction with cofilin at pH 6.7. At this pH, cofilin is able to bind to actin and change the conformation of the filament but is unable to sever actin (McGough et al., 1997). In agreement with previously published data, we observed that the cofilin binds to the polymer at pH 6.7 and quenches pyrene fluorescence. However, it does not significantly alter the fluorescence of the Oregon green labeled actin, suggesting that the filaments bind cofilin but are not disassembled by it (Figure 2.2B). As an additional control to verify if the FRET assay was indeed reporting on the disassembly of actin, we measured change in FRET on addition of a monomer sequestering protein, DNase I (Hitchcock, 1980) (Figure 2.2C). No FRET was observable 45 minutes after the addition of 15 µM DNase I as all the F-actin had been depolymerized to monomer.

Only the triple mix of bursting factors alters both the rate and extent of disassembly of actin

After establishing that the FRET assay was indeed reporting on polymer mass, we used the assay to investigate if different combinations of factors could

alter the rate and extent of actin disassembly. 1.25 µM cofilin alone in combination with 1 µM actin caused a 30-40% decrease in polymer mass consistent with cofilin's ability to sever actin and bind ADP-G-actin monomer (green curve). Addition of 0.1 µM Aip1 to this reaction increased the initial rate of depolymerization roughly by five times (yellow curve), consistent with Aip1's ability to potentiate cofilin-mediated depolymerization. Conversely when 1 µM coronin was added to the cofilin-actin it led to an apparent increase in the polymer mass and a decrease in the depolymerization rate (blue curve). This is consistent with coronin's ability to stabilize filaments against depolymerization (Galkin et al., 2008) by binding the filaments and also by increasing cofilin loading on actin and producing a cofilin saturated hyper-twisted filament (Brieher et al., 2006, unpublished data). While the behavior of any two factors approximated the disassembly characteristics of cofilin alone, when all three bursting factors were combined the FRET signal declined rapidly, leading to a near complete conversion of actin polymer to monomer with $t_{1/2} \approx 100$ seconds (pink curve). Once disassembled, the actin did not reassemble over a period of 25 minutes, demonstrating that the triple mix dramatically raises the critical concentration. No other combination of factors was able to produce this behavior (Figure 2.3A).

In order to get a more accurate measure of the rate at which actin monomer was being produced, we used a monomer generation assay based on the principle that the fluorescence of Oregon green actin would be quenched on binding vitamin D-binding protein, a high affinity monomer sequestering factor. In

the FRET assay, the rate of depolymerization is a convolution of both the rate of dissociation of monomer as well as re-association of actin monomer in the presence of the depolymerization factors. However, as DBP acts as a passive 'sink' for actin monomer, it isolates and reports on solely the depolymerization rate.

When green actin monomer alone was combined with an equimolar concentration of DBP the fluorescence was completely quenched, however OG488 still fluoresced if it was incorporated in actin polymer (light yellow and purple curves respectively) (Figure 2.3B). If DBP was added to polymeric actin, the fluorescence gradually decayed reporting on the depolymerization rate. The addition of cofilin to this reaction generated more ends and rate of monomer generation was 3x that of pure actin (green curve). Capping the more dynamic barbed end of actin reduced the depolymerization rate of cofilin-actin, thereby validating the assay (blue curve). When the 3 bursting factors were combined with actin, 1 μM actin was depolymerized extremely rapidly with a $t_{1/2} \cong 50$ s (pink curve).

Products of the bursting reaction do not seed new actin assembly

The fact that actin failed to reassemble in the presence of the bursting factors over a period of 25 minutes led us to ask if the products of the reaction would be able to seed new actin assembly. We used the end products of the depolymerization reaction to nucleate a pyrene-actin polymerization reaction (Figure 2.3C). If the end products of the depolymerization were short filaments,

the lag phase of the actin polymerization reaction would be diminished. However, actin monomer or fragments with occluded ends would not serve as seeds. Unseeded pyrene actin showed a canonical lag phase prior to polymerization whereas cofilin, cofilin-Aip1 and cofilin-coronin treated, severed filaments yielded a more effective seeding mixture as there were more ends for elongation, consistent with the results of the FRET reaction. However, the 3x mix displayed the same lag phase to polymerization as unseeded actin thus indicating that the end products of the polymerization reaction were incompetent to seed new actin assembly.

The triple mix of factors raises the critical concentration of actin

By rapidly depolymerizing actin and generating products that were unable to seed new actin assembly it appeared that the bursting factors were able to raise the critical concentration of actin. We used FRET to directly measure the critical concentration of actin in combination with the bursting factors. We compared the fluorescence of increasing concentrations of G- and F-actin. The concentration at which we first start to observe polymerization signifies the critical concentration (Cc) of actin. The Cc of pure actin was 0.2 µM. To validate the assay, we measured the Cc in the presence of actin and a high affinity barbed end capping factor CapZ, which shifted the Cc close to the Cc of the pointed end, consistent with previous measurements (Howard, 2001). While other combinations of factors along with actin caused a modest increase in the

Cc, (described in Chapter 3), the bursting factors raised the critical concentration to 1.85 µM, approximately five times greater than pure actin.

In order to gain further insight into the mechanism of depolymerization, we varied the concentration of each factor in the presence of fixed concentrations of the remaining two factors. When actin was disassembled with increasing concentrations of cofilin alone, maximum rates of disassembly were achieved at a ratio of two actin molecules to every one or two cofilin molecules (refer to Figure 2.2A). In the presence of all three factors, however, increasing concentrations of any factor lead to ever-faster rates of disassembly and actin monomer formation (Figure 2.4A). Consistent with coronin's ability to suppress the depolymerization of actin, increasing amounts of coronin led to decreased rates of disassembly. Coronin functioned at an optimum ratio of 5 to 12.5:1 coronin to cofilin. This is also consistent with other experimental observations that coronin works at lower ratios of coronin to cofilin (Tang & Brieher, unpublished data). When we varied the concentration of Aip1 while keeping the amounts of cofilin and coronin constant, we found that Aip1 could exert its function at vanishingly low ratios to the other factors, almost 100 times less than the other factors, saturating at a ratio of 1:10 Aip1 to cofilin. This behavior is consistent with observations that Aip1 is acting as a "trigger" for the bursting reaction (Tang and Brieher, unpublished). It also indicated to us that that Aip1 was not acting in a manner similar to a high affinity barbed end capping factor. A capping factor that binds the more dynamic barbed end might have the effect of suppressing and not elevating the disassembly rate.

Discussion

We utilized FRET to report on bulk actin polymerization and cofilin-mediated depolymerization and whole filament destabilization or 'bursting'. The FRET assay offered several insights to supplement our understanding of bursting. Firstly as demonstrated previously, we showed that the triple mix generates actin monomer at a rapid rate (roughly 1 μM with a $t_{1/2}$ of 50 s), consistent with previous observations (Kueh et al., 2008). Although cofilin and Aip1 can cause rapid initial depolymerization of actin, only in the presence of all three factors is both the rate and extent of disassembly high. The bursting factors also are able maintain actin in the depolymerized state and the final products of this reaction are incompentent to seed filament elongation. This has two possible implications: either the reaction produces solely actin monomer, or the final molecular products of bursting are short filaments with unconventional/ occluded ends. We favor a combination of the two models. We posit (on the basis of unpublished supporting electron microscopic data) that filament disassembly in the presence of the three factors occurs along the length of the filament in a catastrophic manner. This causes dramatic disruption of the structure of the filament that would produce actin monomer and short oligomers.

Previous work also showed that bursting causes the formation of unconventional ends that do not readily grow (Kueh et al., 2008). However, their results argued against a high affinity macromolecular complex that remains on the ends of the actin filaments after dilution.

The fact that the bursting end products cannot seed new actin assembly contradicts previous results. It is conceivable that the bursting factors participate in a macromolecular complex that can also serve to occlude filament ends. Recently published single molecule TIRF studies (Jansen et al., 2015) also suggest that severed ends of filaments in the presence of the 3 factors do not elongate as often as those formed by severing in the presence of only 2 out of the 3 factors. However, our results require further investigation and a possible molecular characterization of the final product/s of the bursting reaction.

Previous work showed that the three factors were able to depolymerize actin in the presence of excess polymerizable actin (Brieher et al., 2006; Kueh et al., 2008). Our results validate this and show that the bursting factors raise the critical concentration to 1.8 μM. As polymerization only occurs at concentrations of monomer above the Cc, this rise in the critical concentration by the bursting factors could potentially be important in the cellular context, where actin assembly is tightly regulated and there exists a high concentration of polymerizable actin.

The disassembly reaction was robust within some range of concentrations of the individual proteins. In the presence of cofilin alone, lower ratios of cofilin to actin were more effective at severing. However in the presence of coronin and Aip1, even higher ratios of cofilin (2.5x that of actin) were able to disassemble actin at rapid rates. In the presence of coronin and Aip1, saturating concentrations of cofilin were able to disassemble filaments. This indicates that bursting is a distinct mechanism of actin disassembly than severing, which is

thought to occur at boundaries of bare and cofilin-decorated stretches of actin (Suarez et al., 2011).

Coronin was able to exert its functions at extremely low ratios of coronin to cofilin (as low as 1:12). Previous work showed that coronin was able to increase the amount of cofilin-loading on *Listeria* actin comet tails (Brieher et al., 2006) Coronin could have a filament “priming” function, allowing actin to bind cofilin more readily. Recent work has shown that coronin can diminish the time lag of cofilin binding to actin filaments (Jansen et al., 2015). However, increasing concentrations of coronin decrease the rate of disassembly. This is consistent with the observation that coronin can bind filaments and protect them against dilution-induced depolymerization (Galkin et al., 2008) and also with observations that coronin and cofilin compete for the same binding site (Cai et al., 2007, Tang and Brieher, unpublished observations).

Coronin’s role in increasing cofilin binding to actin also has the effect of suppressing filament severing (Jansen et al., 2015). As more cofilin binds actin, the number of heterotypic junctions between bare and decorated segments of filaments decreases. Filaments are stabilized in an alternative hyper-twisted configuration. This raises the question of how these stable filaments make the transition to become unstable.

The answer seems to lie in the action of Aip1. Aip1 was thought to be a cofilin dependent capping factor however its role in catalyzing bursting cannot be explained by the phenomenon of capping. A capping protein would passively occlude the dynamic end of a cofilin-coronin decorated filament, suppressing

and not elevating disassembly. The fact that Aip1 can exert its actions at concentrations 10-100 times less than the amount of cofilin or coronin in the reaction lends evidence to the idea of Aip1 as a “trigger” for the bursting reaction that could potentially act in amounts stoichiometric to filament ends rather than sides.

Coronin has only been recently implicated to directly interact with cofilin-actin. Therefore, potential biochemical links between coronin and cofilin and coronin and Aip1 must be elucidated. Our work also calls for a reinvestigation of the function of Aip1 within the context of actin disassembly.

The FRET assay provides us with interesting observations, however in order to convincingly determine that bursting is a distinct mechanism of disassembly than severing it would be necessary to directly observe of filament structure in the presence of the bursting factors. The FRET results are also consistent with a model of rapid filament severing and high affinity barbed end capping. Therefore the FRET assay is unable to distinguish whether the initial observations of filaments disassembling by losing long stretches of actin polymer are true.

Figures

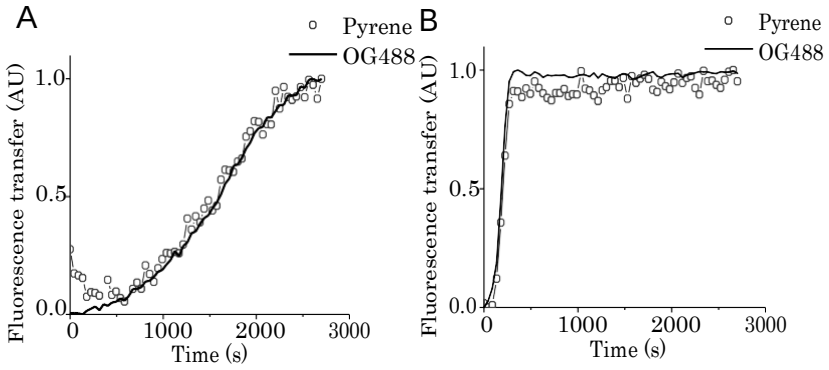

Figure 2.1 FRET assay reports on kinetics of actin assembly. (A) Polymerization of 3.5 μM actin measured by FRET (black line) and pyrene (gray circles). (B) Addition of Arp2/3 (500 nM) and ActA (200 nM) significantly reduces the lag phase of both the pyrene and FRET signals. Normalized data is plotted against time. Representative kinetic data from n=3 experiments is shown.

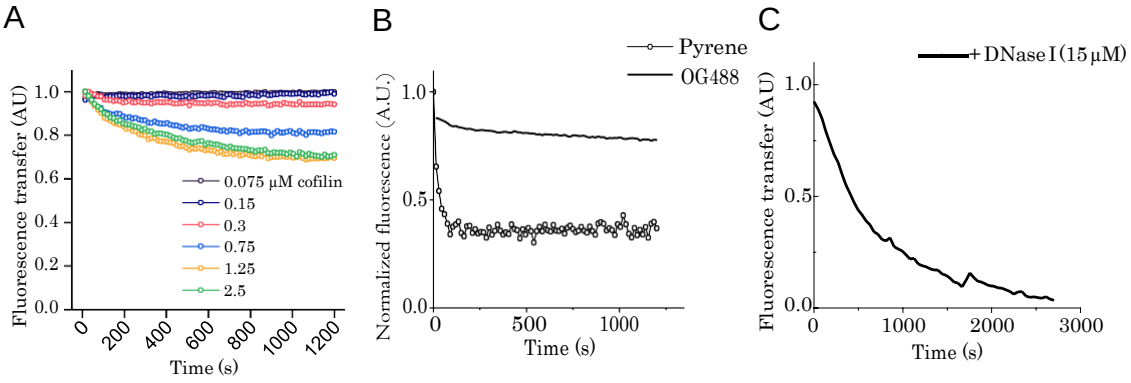

Figure 2.2 FRET assay reports on cofilin mediated depolymerization and not cofilin-binding. (A) Increasing concentrations of cofilin cause an initial disassembly of actin in a dose-dependent manner. Pre-polymerized F-actin (2 μM) was mixed with varying amounts of cofilin, and the fluorescence of Oregon green actin was measured over time. The decrease in FRET represents loss in polymer mass.

Figure 2.2 (cont.) (B) At pH 6.7, cofilin binds to F-actin and quenches pyrene fluorescence (gray open circles) whereas the FRET signal is not significantly changed indicating there is little depolymerization (black line). (C) The assay reports on polymer loss. DNase I (15 μM) depolymerizes all F-actin to monomer, as reported by decrease in FRET.

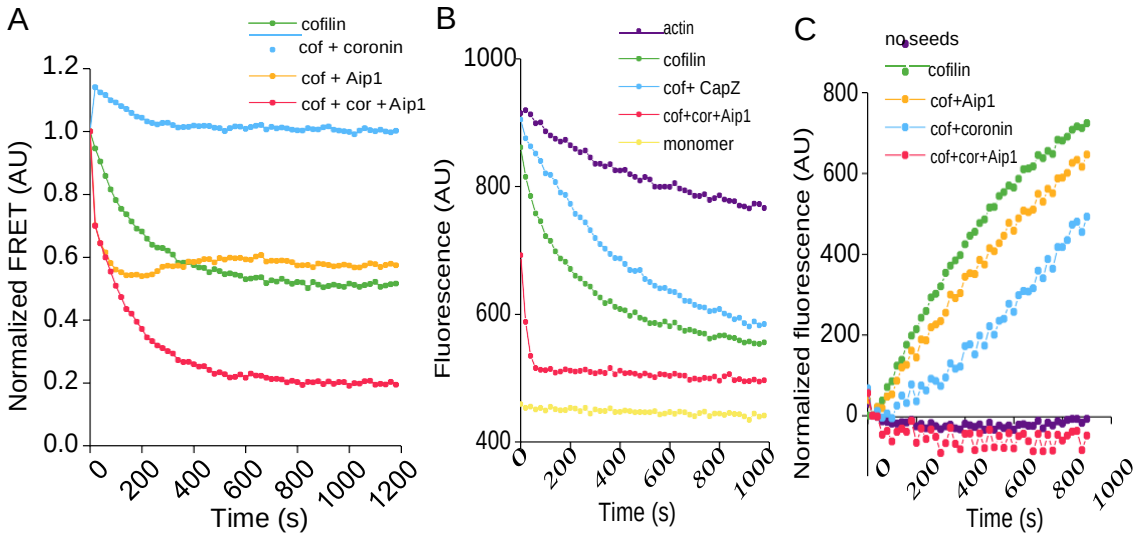

Figure 2.3 The bursting reaction proceeds with rapid kinetics to generate monomer and/or products incompetent to seed actin assembly. (A) Disassembly kinetics of 1 μM actin was monitored by loss of FRET signal in the presence of various combinations of factors. In the presence of cofilin alone, cofilin + coronin and cofilin + Aip1 (green, blue and yellow curves) the polymer mass was decreased by 30-40%. The triple mix of bursting factors decreased the polymer mass by 80% in 250 seconds and the actin did not reassemble over 25 mins. (B) Rate of monomer production was monitored by quenching of Oregon green -488 actin by Vitamin D-binding protein over time. This validates the results of

Figure 2.3 (cont.) the FRET showing that the triple mix causes complete de-polymerization of 1 µM actin over 100 s. (C) Pyrene based seeding assay shows that products of severing by cofilin (green), cofilin+ coronin (blue) and cofilin + Aip1 (yellow) can seed elongation of filaments however the products of bursting (pink) cannot.

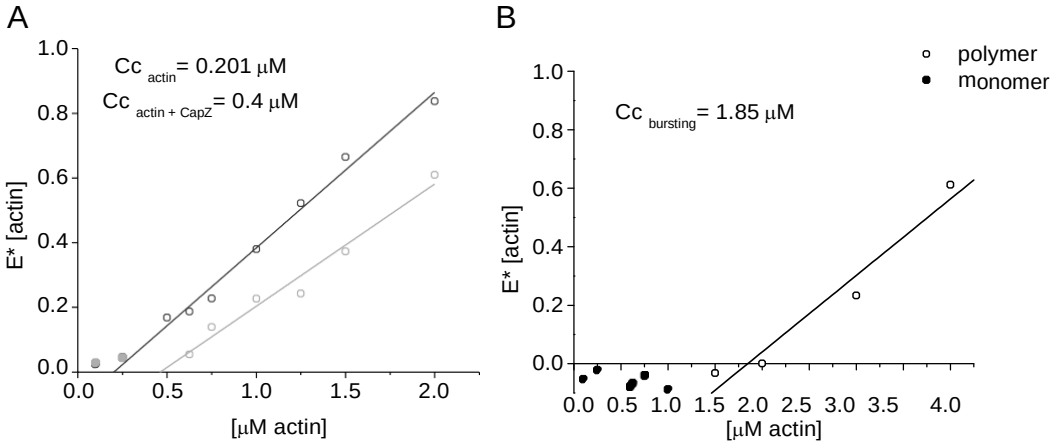

Figure 2.4. The bursting factors alter the critical concentration of actin. (A) FRET assay was used to determine the critical concentration (Cc) of actin as a function of cofilin, coronin and Aip1, or actin alone. Fluorescence intensity in the presence of polymerizing and non-polymerizing conditions was measured at various actin concentrations and the equation [E] = Emax* [(actin 2 Cc)/actin] was used to fit the data. The x-intercept represents the critical concentration. In (A), the black line shows the condition actin alone dark-gray line shows the condition actin in the presence of 0.1 µM CapZ which represents the pointed end Cc. (B) In the presence of 1 µM cofilin 0.75 µM coronin and 0.1 µM Aip1, the critical concentration was raised over 5-fold to 1.84 µM. Thus, the bursting factors alter the critical concentration.

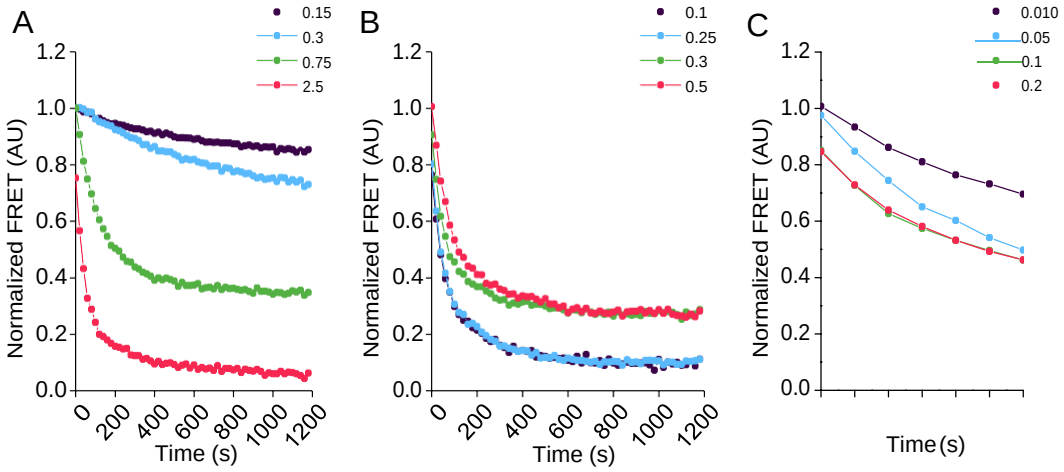

Figure 2.5 Dose-response curves of the bursting factors in the presence of constant amounts of the remaining factors. (A) Cofilin dose response shows increasing disassembly with increasing amounts of cofilin in the presence of 0.75 µM coronin and 0.1 µM Aip1.(B) Lower ratios of coronin: cofilin show better depolymerization kinetics (purple and blue curves) in the presence of 1.25 µM cofilin and 0.1 µM Aip1 whereas higher ratios (green and pink) may stabilize actin filaments and inhibit depolymerization (C) Initial rates (120 s) show a concentration dependence of the reaction for Aip1 concentration (indicated).

CHAPTER 3

ATTRIBUTING NOVEL FUNCTIONS TO AIP1 IN ACTIN DISASSEMBLY[1]

Introduction

Actin filament severing activity detected in vitro with pure cofilin alone cannot account for the behavior of Listeria actin comet tails, which disassemble faster with increasing concentrations of cofilin (Rosenblatt et al., 1997; Carlier et al., 1997). Furthermore, the intrinsic cofilin severing and actin depolymerization rates do not account for the rapid actin turnover rates of yeast actin patches (Sirotkin et al., 2010; Berro et al., 2010). Thus, cofilin-dependent auxiliary factors present in cytoplasm may be responsible for the destabilization of actin filaments even in the presence of saturating cofilin concentrations.

The fractionation of thymus extract has yielded factors that function to potentiate cofilin's ability to disassemble actin, namely coronin and Aip1. Among these, coronin appeared to increase the loading of cofilin on *Listeria* actin comet tails and stabilize them (Brieher et al. 2006). Aip1 was able to rapidly disassemble these filaments and previous work has indicated that Aip1 can exert its activity in the presence of higher cofilin to actin ratios (Mohri et al., 2004). This indicated to us that Aip1 was a likely candidate to explain the contradictory behaviors of pure cofilin and actin disassembly in complex cell extracts.

Aip1 facilitates cofilin-mediated actin disassembly in vitro (Ono et al., 2004; Rodal et al., 1999; Okada et al., 1999), and mutations in Aip1 or perturbation of its function in cells lead to ectopic accumulation of F-actin (Ren et al., 2007; Kato et al., 2008), defects in actin turnover dynamics (Ono, 2003),

[1]This chapter was published as an article entitled 'Aip1 Destablizes Cofilin-Saturated Filaments by Increasing Severing and Monomer Dissociation from Ends' by A.V. Nadkarni and W.M. Brieher in the journal *Current Biology,* Volume 24, Issue 23 in December 2014.

suppression of filament elongation (Michelot et al., 2013), and depletion of the actin monomer pool that fuels actin assembly (Okreglak and Drubin, 2010) . Thus, the *in vitro* and *in vivo* data are consistent with Aip1 playing a role in cytoskeletal organization by enhancing cofilin-mediated filament disassembly, but the underlying molecular mechanism is not yet known.

The apparent ability of Aip1 to cap filament barbed ends led to a popular model in which Aip1 facilitates cofilin-mediated disassembly by preventing the reannealing of severed filaments (Ono et al., 2004; Okada et al., 2002, 2006). In this model, cofilin alone mediates severing, whereas Aip1 simply blocks the back reaction and does not alter the mechanism of filament destabilization. Aip1 is therefore thought to control actin filament dynamics in a manner similar to capping protein (CapZ). CapZ is a well-characterized barbed-end binding protein that caps barbed ends with nanomolar affinity thus preventing barbed end growth or shrinkage (Wear et al., 2003). This model was supported by the fact that CapZ and Aip1 exhibit strong genetic interactions in yeast where null mutations in both CapZ and Aip1 elicit a more severe disruption in actin organization than a null mutation for either gene alone (Michelot et al., 2007). However this model is at odds with Aip1's ability to disassemble stable cofilin-coronin filaments. A capping factor would passively occlude filament ends, not actively destabilize them. CapZ cannot substitute for Aip1 to disassemble Listeria actin comet tails or single filaments in the presence of cofilin and coronin (Kueh et al., 2008).

In order to test our hypothesis that Aip1 is responsible for the disassembly of filaments in the presence of stabilizing concentrations of cofilin, and to reinvestigate the existing model of Aip1 function, we used single-filament imaging of actin and fluorescence resonance energy transfer (FRET)- based bulk actin depolymerization assays.

Materials and Methods

Imaging of actin single filaments: Single actin filaments were either prepolymerized, flowed into perfusion chambers and imaged in solution (Figures 3.1, 3.2 and 3.3) or polymerized in the chamber and attached to coverslips via filamin (Figures 3.4 and 3.6). Relevant combinations depolymerizers were flowed into the perfusion chamber and filaments were imaged in a buffer containing oxygen-scavengers (1xPhotoBuffer). Severing events were enumerated as the number of visual breaks per second normalized to the amount of polymer measured in microns. Depolymerization rates were calculated from kymographs generated using Fiji software. Elongation of polymerized Oregon green actin filaments was carried out in the presence of 2 µM monomeric Alexa 647 actin, 150 nM cofilin and 200 nM of either CapZ or Aip1 for 60 seconds. This was compared to a control with actin alone.

Fluorescence equilibrium binding and competition assays: Quenching of pyrene fluorescence was used to quantify cofilin binding to F-actin in the presence or absence of phalloidin or Aip1 as described previously (De La Cruz, 2005; Elam et al., 2013). FRET was used to normalize for the amount of polymer present at various concentrations of Aip1.

Measurement of FRET has been described in Chapter 2: For disassembly reactions, actin was prepolymerized and combinations of depolymerizers were added to final concentrations of 2 µM cofilin, 0.2 µM Aip1 or 0.2 µM CapZ. Fluorescence of Oregon Green actin was monitored as mentioned.

Depolymerization leads to dequenching of OG488 fluorescence. Normalized readings were obtained by using Origin graphing software.

Seeding reaction with actin pyrene: Actin filaments treated with or without cofilin and increasing concentrations of Aip1 for 10 mins (Fig 6A) and 2 mins (Fig 6B) were added to a solution of 2 µM pyrene labeled G-actin. The final concentration of F-actin seeds was 0.25 µM (Fig 6A) and 0.5 µM (Fig 6B) and the final cofilin concentration was 100 nM (Fig 6B). Final Aip1 concentrations are provided in the main text.

Results

Opposing behavior of pure cofilin and cofilin in cellular extracts

Previous work has shown that the amount of cofilin in thymus extract is high (up to the order of 20 µM) (Brieher et al., 2006). We confirmed this result by quantitative western blotting and found the amount of cofilin in thymus extract to be 21 ± 6 µM (Figure 3.1A). In our severing assays on single-actin filaments, we found that recombinant human cofilin severed actin most efficiently at 1 µM (Figure 3.1B), whereas severing was inhibited at higher cofilin concentrations consistent with previous results (Andrianantoandro and Pollard, 2006). In order to test if cellular extract can sever single filaments, bovine thymus extract was diluted so that the final concentration of cofilin was 5 µM. At these concentrations, thymus extract is able to sever single-actin filaments whereas an equimolar amount of recombinant cofilin was unable to sever pure actin filaments efficiently (Figure 3.1B, right panel, Figure 3.1C). Actin filament severing rates were in fact ten times faster in the presence of thymus cytosol than the fastest rates detected with pure cofilin alone.

Aip1 can depolymerize actin filaments even in the presence of saturating amounts of cofilin

Previous work has shown that coronin increases cofilin loading on *Listeria* actin comet tails and stabilizes them. These filaments are destabilized by Aip1 (Brieher et al., 2006). Thus, Aip1 appeared to us as the most likely candidate for depolymerization of cofilin-saturated stabilized filaments and could possibly

explain this behavior of extract. To test this, we imaged single filaments in the presence of saturating amounts of cofilin, in the presence or absence of Aip1. Actin filaments did not sever in the presence of 25 μM cofilin. As a control, we also tested severing in the presence of 25 μM cofilin and 100 nM capping protein (CapZ) (Figure 3.2A, top panels) and detected no severing events. However, filaments did sever in the presence of 25 nM Aip1 and 25 μM cofilin (Figure 3.2A, bottom panels). Severing rates increased from 0 events per micron per second in the presence of 25 μM cofilin to 0.006 events per micron per second in the presence of 25 nM Aip1 (Figure 3.2B). This experiment provided us with the first evidence that Aip1 and capping protein do not appear to act by the same mechanism.

Aip1 does not displace cofilin to promote severing

Cofilin-mediated severing involves the destabilization of lateral interfaces between cofilin-bound and unbound sections of actin. Therefore, severing by Aip1 could operate by two mechanisms. Either Aip1 could be displacing cofilin from actin, thus creating additional unstable lateral interfaces and exploiting cofilin's intrinsic ability to sever, or alternatively Aip1 could be potentiating severing by a different mechanism, but in co-operation with the bound cofilin.

In order to test if Aip1 displaced bound cofilin from actin filaments, we validated that our recombinant cofilin binds co-operatively to actin as demonstrated previously (De La Cruz, 2005) (Figure 3.3A, inset). Next, we carried out competitive equilibrium binding assays on actin in the presence of a

high initial occupancy of cofilin (3 µM, corresponding to binding density υ_{cof} > 0.9) and increasing concentrations of Aip1 (Figure 3.3A). Aip1 did not affect pyrene fluorescence by itself (data not shown). As a control, we also carried out the experiment with a known competitive inhibitor of cofilin, phalloidin, as described previously (Figure 3.3B) (Elam et al., 2013). Concentrations of phalloidin as low as 0.25 µM displaced cofilin by ~20%. Increasing concentrations of phalloidin displaced cofilin from actin nearly completely. However, in the presence of Aip1 there appeared to be no displacement of cofilin at the concentrations at which we assayed its activity. There was little to no displacement even at 1:1 concentrations of Aip1: cofilin (Figure 3.3A). Thus, Aip1 does not displace cofilin from actin.

If Aip1 were to compete with cofilin for binding to F-actin, then we would predict that Aip1 should inhibit severing when cofilin is present at the optimal concentration where severing rates are highest. In our assay, filaments severed fastest in the presence of 1 µM cofilin corresponding to υ= 0.3. Using this concentration of cofilin, we observed that severing rates increased with increasing concentrations of Aip1 (Figure 3.3C). This is the opposite of what is expected if Aip1 functions by displacing cofilin.

Aip1's effect was seen even at extremely low ratios of Aip1: cofilin with a roughly four-fold increase at 10 nM Aip1. At equimolar concentrations of Aip1: cofilin, severing rates were increased over ten times the maximal severing rates achieved by any concentration of cofilin alone despite the fact that Aip1 does not displace cofilin at these concentrations (Figure 3.3A).

The displacement hypothesis predicts that Aip1 will inhibit severing at cofilin binding densities of 0.5 or less, and only accelerate severing at concentrations corresponding to υ>0.5 (Elam et al., 2013). Therefore, to further test whether Aip1 enhances severing by displacing cofilin, we compared severing rates across a range of cofilin concentrations in the presence or absence of Aip1. We observed that 10 nM Aip1 accelerated actin filament severing rates at all cofilin concentrations (Figure 3.3D). This result is inconsistent with Aip1 displacing cofilin from F-actin.

Aip1 accelerates disassembly from the barbed and pointed ends of filaments

We observed that filaments treated with Aip1 in the presence of saturating concentrations of cofilin appeared to depolymerize rapidly suggesting that Aip1 also accelerates subunit loss from actin filament ends. In order to measure depolymerization rates from ends, filaments were immobilized to the coverslip with the actin bundling protein, filamin, unlike previous experiments where the filaments were free-floating and imaged in the presence of methylcellulose.

In the presence of 2 µM cofilin and 0.2 µM Aip1, filaments rapidly disassembled by shrinking from both barbed and pointed ends. Quantitation of depolymerization rates from barbed ends of polarity marked actin filaments revealed an average rate of 8 – 10 subunits per second in the presence of 2 µM cofilin with or without 0.2 µM Aip1 (Figure 3.4A top panels, Figure 3.4B). Barbed ends of filaments in the presence of cofilin and CapZ, however, were stable, thus

once again contradicting previous hypotheses that Aip1 acts in a manner similar to CapZ (Figure 3.4A bottom panel).

We also measured the effects of increasing amounts of cofilin on depolymerization rates. In the presence of 0.2 µM Aip1, barbed end depolymerization rates increased from an average of 10 subunits/second in the presence of 2 µM cofilin to 48 subunits/second in the presence of 10 µM cofilin (Figure 3.4C, D). These results reveal a new function for Aip1 in accelerating cofilin-mediated barbed end depolymerization rates anywhere from 5-10 times those measured in the presence of cofilin alone. This is the opposite of what is expected of a high affinity barbed end capping factor and the opposite of what is observed in the presence of CapZ.

Aip1-disassembled cofilin-actin filaments also had a higher pointed end depolymerization rate. Pointed ends depolymerized at a rate of approximately 1 subunit per second in the presence of 2 µM cofilin with or without 0.2 µM CapZ. In contrast, pointed ends depolymerized approximately 4x faster on average in the presence of 2 µM cofilin and 0.2 µM Aip1 (Figure 3.4E,F).

Aip1 and CapZ have differing effects on disassembly rate and critical concentration

There were multiple lines of evidence indicating that Aip1 was not acting as a barbed end capping factor and had additional biochemical roles distinct from CapZ. To further compare Aip1 and CapZ in actin disassembly, we sought

to analyze changes in bulk actin polymer mass in the presence of high concentrations of cofilin by utilizing the FRET assay described in Chapter 2.

The addition of cofilin to 2 µM pre-polymerized F-actin at pH 7.8 caused an initial decrease in FRET, representing a decrease in polymer mass, in a dose dependent manner over 25 minutes (Ref to Figure 2.2 A). This is consistent with the fact that cofilin binds to the newly dissociated ADP actin monomers with an affinity of 150 nM and suppresses ADP to ATP exchange (Blanchoin and Pollard, 1998; Ressad et al., 1998).

We used FRET to compare the disassembly characteristics of cofilin-actin in the presence of capping protein versus Aip1 (Figure 3.5A). In the presence of cofilin and Aip1, actin polymer mass decayed roughly 6x faster but to the same extent as it did in the presence of cofilin alone. This indicated that Aip1 was accelerating the rate of actin depolymerization in conjunction with cofilin. However in the presence of cofilin and CapZ, actin polymer mass decayed more slowly than in the presence of Aip1, but it was converted nearly completely to monomer over a period of 25 minutes. Thus, CapZ appeared to affect the extent, but not the rate of actin depolymerization in the presence of cofilin, unlike Aip1.

Next, we tested the effects of Aip1 and CapZ on actin critical concentration (Cc) in the presence of cofilin by FRET. Addition of 1 µM cofilin increased the critical concentration to between 0.3 – 0.4 µM (Figure 3.5B). We conclude that cofilin has only a modest effect on the critical concentration which is consistent with previous results (Andrianantoandro and Pollard, 2006). We measured the Cc of actin in the presence of 1 µM cofilin and 0.1 µM Aip1 and

found it to be nearly identical to that in the presence of cofilin alone (Cc = 0.36 µM in 1 µM cofilin alone versus Cc = 0.33 µM in the presence of 1 µM cofilin and 0.1 µM Aip1) (Figure 3.5B). Similarly, treatment of actin with CapZ alone had only a modest effect on Cc (Ref Figure 2.4A). However, the combination of 1 µM cofilin and 0.1 µM CapZ raised the critical concentration more than five-fold relative to that of pure actin to 1.7 µM (Figure 3.5C) which is the pointed end critical concentration for ADP-actin (Howard, 2001). This is consistent with the barbed ends being capped by CapZ and ADP-G-actin in a high affinity complex with cofilin. Therefore CapZ affects the critical concentration to a greater extent than Aip1, and the two proteins are not functionally redundant.

Aip1 does not cap filament barbed ends

Differences in kinetics of cofilin-mediated depolymerization in the presence of Aip1 versus CapZ and cofilin as well as differences in the critical concentrations prompted us to directly reinvestigate whether or not Aip1 caps barbed ends. An established assay for barbed end capping is the inability of capped barbed ends to seed new actin polymerization. New actin monomer adds to the barbed ends of pre-existing short actin filament seeds thus shortening the lag phase of polymerization. Filaments with occluded barbed ends such as those bound by CapZ will not be able to reduce the lag phase of new actin assembly. When products of the disassembly reactions were used to seed new actin polymerization, F-actin seeds enhanced the initial rate of polymerization (Figure 3.6A yellow line), and cofilin-actin filaments seeded

polymerization even more efficiently due to a large number of free severed ends. As expected, CapZ-bound seeds were unable to enhance the rate of actin polymerization (black line). However, filaments depolymerized by cofilin and Aip1 seeded actin assembly as efficiently as the cofilin-actin seeding mixture (blue and pink lines). Thus, Aip1 does not form a high affinity cap on actin filaments. Additionally, the presence of increasing amounts of Aip1 in the presence of a fixed concentration of cofilin increased the number of pre-existing short filament seeds, consistent with Aip1's ability to increase severing rates in the presence of cofilin as seen by light microscopy (Figure 3.6B).

We used single filament imaging to further test whether Aip1 mediates cofilin dependent barbed end capping. Fluorescently labeled Oregon green 488-actin filaments were polymerized on a bed of filamin in a perfusion chamber, and filaments were allowed to elongate by addition of Alexa 647 G-actin monomer, in the presence of 0.15 μM cofilin and 0.2 μM of either Aip1 or CapZ (Figure 3.6C, 6D). Unlike CapZ, Aip1 did not inhibit elongation of preformed actin filaments at the level of single actin filaments. Therefore, Aip1 does not suppress barbed end growth even when it is continuously present at high concentrations along with cofilin.

Discussion

We found that thymus extracts rapidly sever and disassemble single actin filaments despite having cofilin concentrations that are too high to sever actin filaments in pure solution. We demonstrated that fast actin disassembly in the presence of saturating cofilin can be attributed to, at least in part, Aip1 but not to Capping Protein and our analysis further revealed that Aip1 alters the characteristics of cofilin-mediated filament disassembly while capping protein does not.

Previous results proposed that Aip1 caps barbed ends with a high affinity and would therefore be functionally redundant with Capping Protein (Ono, 2003). However, our results show by multiple modes that this is not the case. Aip1 does not prevent growth of free filament ends and filaments shrink at accelerated rates in the presence of Aip1, which is the opposite of what we would predict if the filaments were capped. Our results are more consistent with models proposing that Aip1's side binding and not its end-binding activity is more important for severing cofilin decorated filaments (Rodal et al., 1999; Shi et al., 2012) to create more filament ends that can grow or shrink. Interestingly, small quantities of Aip1 show a strong effect on severing cofilin-actin filaments as observed by microscopy, however, these severed filaments do not seed new growth robustly in bulk filament polymerization assays. Further studies would be required to characterize whether Aip1 possesses the ability to create some unconventional ends that are resistant to growth, as posited in previous work (Kueh et al., 2008).

While Aip1 and CapZ are biochemically distinct, they show strong genetic interactions in yeast, playing critical roles in the assembly and morphogenesis of Arp2/3-derived actin arrays by maintaining a pool of actin subunits available for assembly (Michelot et al., 2013). CapZ can help maintain a pool of assembly competent actin by suppressing non-productive barbed end elongation and funneling actin monomer towards Arp2/3 nucleation sites (Akin and Mullins, 2008). Given our results, Aip1 might help maintain a pool of assembly-competent actin by triggering fast depolymerization of cofilin-F-actin. Thus, we propose that Aip1 and CapZ genetically complement one another through distinct mechanisms.

Cofilin binding to F-actin disrupts lateral interactions between actin subunits (McCullough et al., 2011). However, stretches of actin polymer saturated with cofilin are stable (Andrianantoandro and Pollard, 2006; McGough et al., 1997) because cofilin has two actin binding sites allowing it to bridge two longitudinal subunits in the filament (Hayden et al., 1993; Lappalainen et al., 1997; Pope et al., 2000). Severing therefore occurs at junctions between decorated and undecorated polymer (Suarez et al., 2011) explaining why severing is maximal at intermediate levels of cofilin occupancy (Andrianantoandro and Pollard, 2006; Suarez et al., 2011; Elam et al., 2013). We found that Aip1 does not displace cofilin from F-actin and promotes actin filament disassembly at all cofilin occupancies. Our results imply that it is the sites of actin polymer occupied by cofilin themselves that are destabilized by Aip1 (Figure 3.7). This model is consistent with yeast two hybrid, binding, and

modeling data supporting that Aip1 forms a ternary complex with F-actin and cofilin (Rodal et al., 1999; Clark et al., 2006; Clark and Amberg, 2007).

Mutagenesis and modeling studies on Aip1 demonstrate that Aip1 contacts cofilin while bridging two contiguous actin subunits in the filament (Clark et al., 2006). Therefore, we can consider two alternative mechanisms through which Aip1 could promote cofilin mediated severing and subunit dissociation. In the first, Aip1 binding to cofilin occupied polymer might disrupt cofilin's stabilizing interaction with the adjacent actin subunit to cause severing. Mutations in cofilin that compromise its F-actin specific binding interaction increase severing (Ono et al., 2001) and the Aip1 and cofilin binding sites on actin would appear to overlap (Rodal et al., 1999; Clark and Amberg, 2007) making this an attractive model. However, studies with cofilin and Aip1 from *C. elegans* have shown that the ability of Aip1 to enhance actin disassembly requires cofilin's F-actin binding site (Mohri and Ono, 2003). An alternative possibility then is that Aip1 further distorts actin structure in the presence of cofilin to promote severing and increase subunit dissociation rates from filament ends. It has been hypothesized that a slow isomerization step follows cofilin binding (De La Cruz and Sept, 2010). If this proposed conformational change were coupled to severing, we could speculate that Aip1 catalyzes the transition between the two states to destabilize the filament. Recent work on cofilin from *Plasmodium falciparum* shows that it contacts a novel binding site on F-actin to sever filaments without decorating the polymer as human cofilin does (Wong et al., 2014). Thus, multiple cofilin binding modes might permit multiple modes of filament disassembly, and it is tempting to

speculate that Aip1 alters filament structure or induces a conformational change in cofilin allowing mammalian cofilin to access the novel *P. falciparum* binding site to destabilize the polymer.

Our results demonstrating that the combination of Aip1 and cofilin accelerate actin subunit dissociation rates offers one possible mechanism for attaining faster depolymerization rates *in vivo*. Aip1 therefore offers a potential control point to switch cofilin action from one that favors actin assembly (Ghosh et al., 2004; Andrianantoandro and Pollard, 2006) to one that favors fast depolymerization. Other factors in addition to Aip1 facilitate cofilin mediated actin disassembly. It will be important to re-examine each of these auxiliary factors to test if they, like Aip1, alter the mechanism of filament disassembly.

Figures

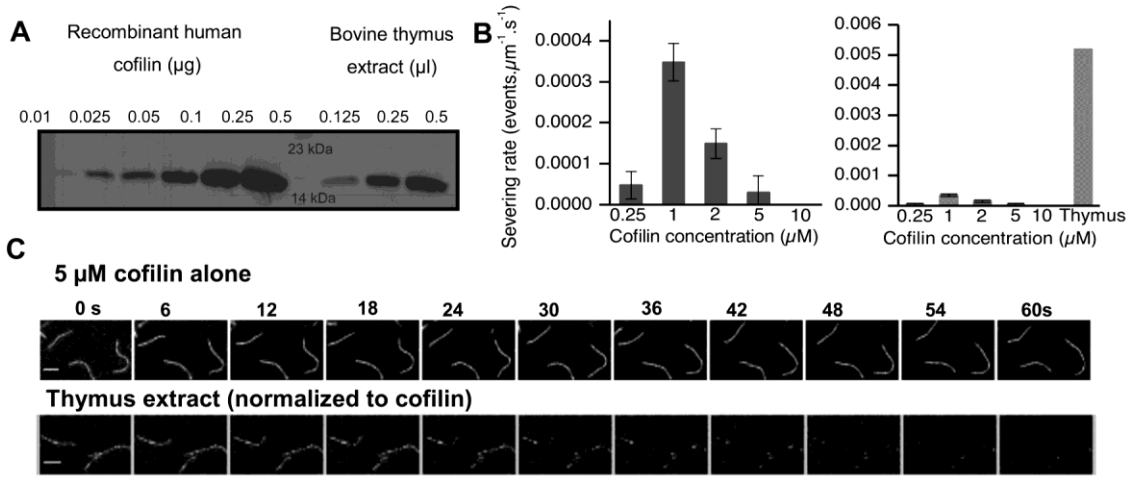

Figure 3.1 Thymus extract is more efficient at depolymerizing single actin filaments than a normalized amount of recombinant cofilin. (A) Quantitative western blot showing standard amounts of recombinant cofilin (0.01- 0.5 µg) (left lanes, increasing order) & fixed amounts of thymus extract. (B) Cofilin severs only across a narrow range of concentrations (left graph), with activity peaking at approximately 1 µM. Thymus extract is roughly 10-fold more effective at severing than the peak cofilin severing concentration (right graph) Error bars represent S.D. n= at least 2 movies. (C) Frames from time lapse movies showing that when normalized to a cofilin concentration of 5 µM, filaments are stable in the presence of pure cofilin (top panel), whereas they are disassembled within 60s with thymus extract (bottom panel). Scale bar= 2 µM.

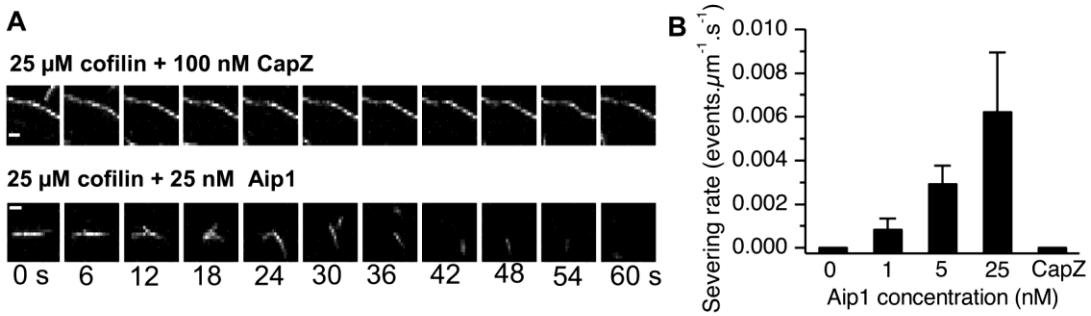

Figure 3.2 Aip1 can sever filaments saturated by cofilin. (A) Frames from time lapse movies showing the dynamics of filaments in the presence of a saturating amount of cofilin+ CapZ (upper panels) or cofilin + Aip1 (lower panels). Filaments fragment in the presence of Aip1 only, not cofilin alone or cofilin + CapZ. (B) Quantitation of severing rates of filaments in the presence of saturating amounts of cofilin. No severing events were detected in the presence of 25 mM cofilin alone or cofilin + 100 nM CapZ. Severing rates increased with increasing concentrations of Aip1 (n=3 movies, error bars represent S.D.). Scale bar= 1 µM.

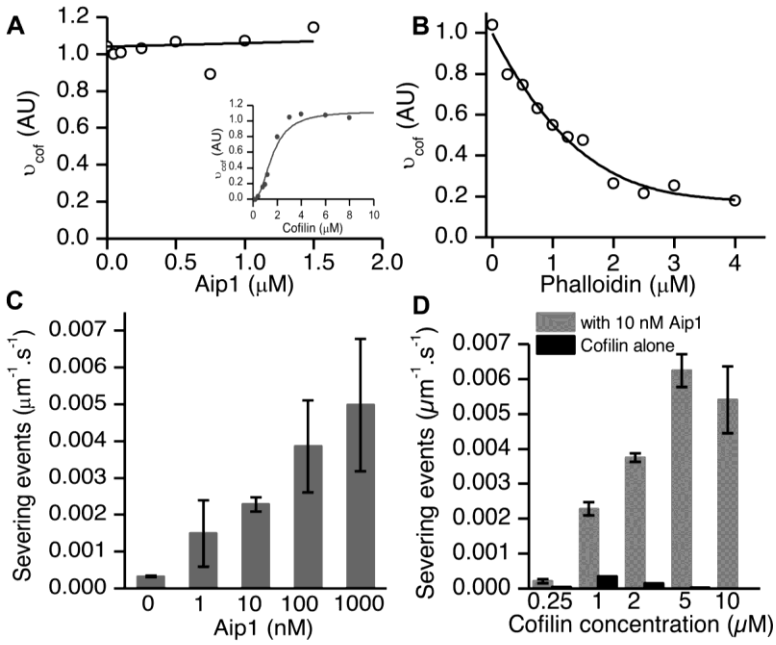

Figure 3.3 Aip1 does not displace cofilin from F-actin to sever. (A) 2 µM fluorescent pyrene-actin was treated with 3 µM cofilin corresponding to v_{cof}>0.9 and increasing concentrations of Aip1 at pH 6.8 to assay competitive binding by equilibrium fluorescence titration experiments. The graph was normalized to amount of polymer monitored by a FRET assay. Aip1 does not compete for binding by cofilin, as shown by a linear fit of the data. We carried out a binding assay to monitor cooperative binding of cofilin to fluorescently labelled pyrene actin in order to select the concentration used for graph (A) (inset). (B) Phalloidin, a known competitive inhibitor of actin, can displace cofilin from actin. (C) To validate our results from (A), we measured severing rates of actin in the presence of 1 µM cofilin that, in our single filament assays, showed maximal severing rates. Adding increasing amounts of Aip1 caused a consistent increase in severing rate. At equimolar cofilin to Aip1 ratios, the rate exceeded 10x that obtained by cofilin alone. (D) Aip1 boosts severing across a wide range

Fig 3.3 (cont.) of cofilin-actin ratios, even when it is present in sub-stoichiometric quantities (roughly 1000x less than the cofilin concentrations). Error bars represent S.D. and data from at least 2 movies was used to compute severing rates.

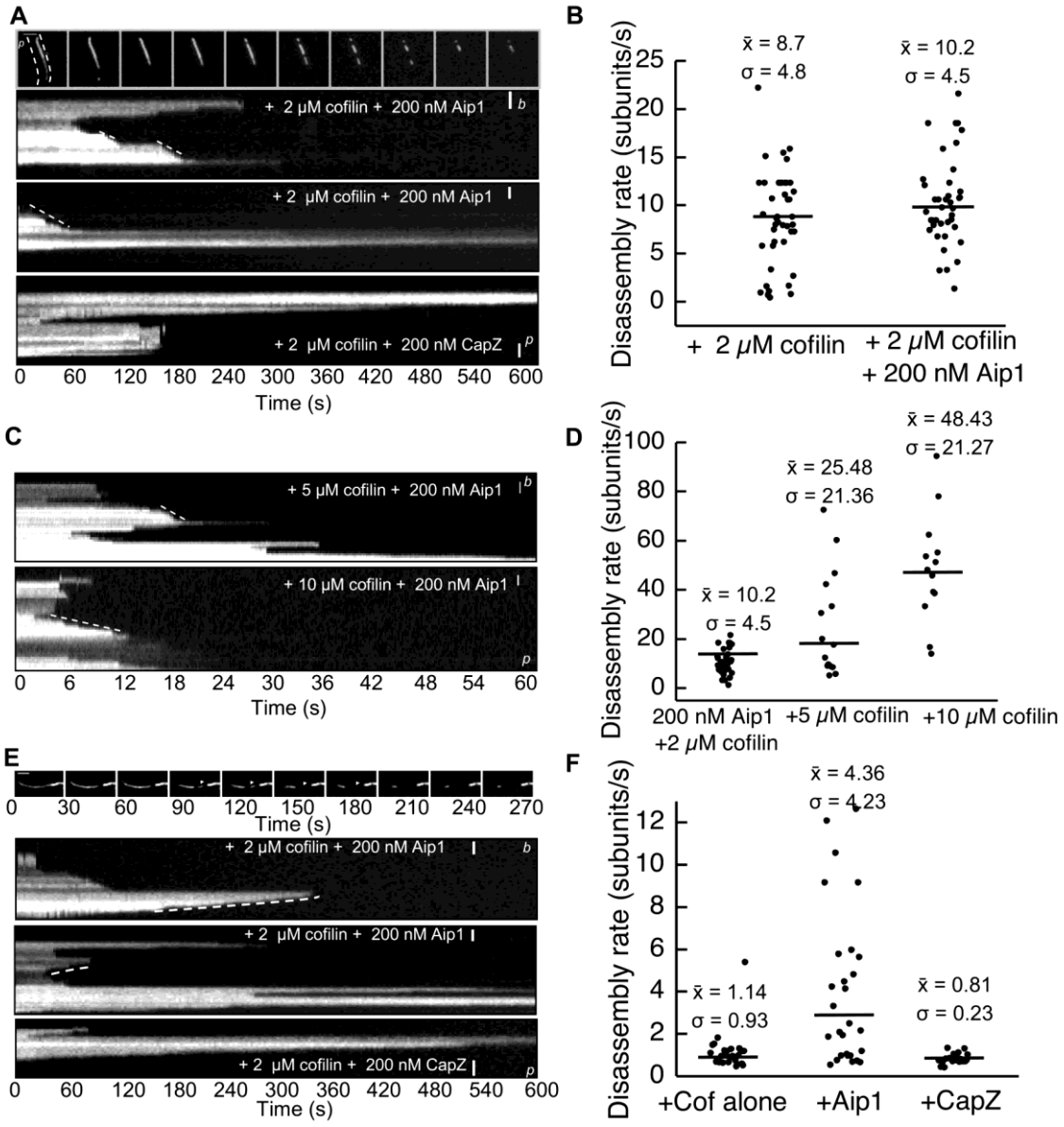

Figure 3.4 Aip1 increases rate of subunit dissociation from barbed and pointed ends. (A) Frames and resultant kymographs from a time lapse movie of polarity marked actin filaments [small letters b (barbed) and p (pointed) indicate orientation of ends] showing filaments shrinking from the barbed end in the presence of cofilin +/- Aip1, but not in the presence of CapZ. (B) Quantitation of filament depolymerization rates from (A).

Figure 3.4 (cont.) Filaments shrink at an average of 8-10 subunits in the presence or absence of Aip1. Black line represents mean of observations, n=at least 3 movies in each scenario, dots represent individual events. Mean, S.D. indicated. (C) Kymographs of polarity marked actin filaments disassembled in the presence of 5 and 10 µM cofilin and 200 nM Aip1. (D) Quantitation of rates from (C) for the barbed end shows barbed end dissociation rates roughly doubling (from 10 to 25 to 40) subunits per second for each 2x increase. in cofilin concentration. Enhanced barbed end disassembly is cofilin-dependent. (E) Frames from a time lapse movie showing filaments shrinking from the pointed end after a severing event (white arrowhead) and resultant kymographs. These indicate that filaments depolymerize at increasing rates from the pointed end in the presence of 200 nM Aip1. (F) Quantitation of rates from (E) shows a roughly 4x increase in subunit dissociation rate from 1 to 5 subunits per second in the presence of Aip1, but not in the presence of cofilin or CapZ. Scale bar= 1 µm.

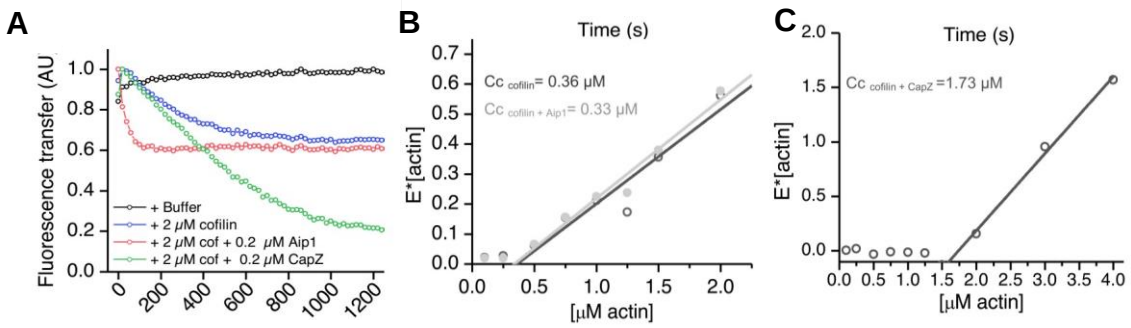

Figure 3.5 CapZ and Aip1 have differing effects on the rates and extents of cofilin-mediated disassembly. (A) CapZ and Aip1 depolymerize actin at differing rates and to different extents. Pre-polymerized actin was treated with 2 µM cofilin +/- 0.2 µM Aip1 or CapZ. Actin depolymerizes initially in the presence of cofilin alone as described (blue line). In the presence of Aip1 (pink line), the reaction proceeds roughly 6x faster, but to the same extent as cofilin alone. 0.2 µM CapZ cause the reaction to proceed at roughly the same rate as cofilin alone but almost completely to monomer. (B, C) FRET assays to determine the critical concentration (Cc) of actin as a function of cofilin, CapZ or Aip1. Fluorescence intensity in the presence of polymerizing and non-polymerizing conditions was measured at various actin concentrations and the equation [E]= Emax* [(actin-Cc)/actin] was used to fit the data. The x-intercept represents the critical concentration. In (B), the dark grey line shows the condition actin+ cofilin, and in the presence of 1 µM cofilin alone, the critical concentration was only moderately raised (from 0.2 to 0.36). The addition of 0.1 µM Aip1 (light grey) did not significantly affect the critical concentration, however (C) the addition of 0.1 µM CapZ to cofilin, raised the critical concentration over ten-fold to 1.73 µM. Thus, Aip1 and CapZ have differing effects on actin critical concentration in the presence of cofilin.

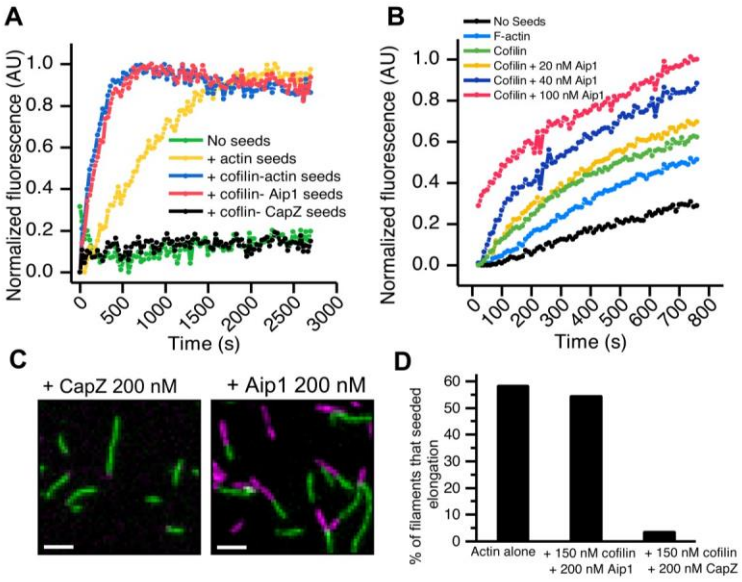

Figure 3.6 Aip1 does not cap filament ends. (A) Aip1 does not inhibit seeding of pyrene actin polymerization. Actin filaments were mixed with 2 uM cofilin +/- 0.2 uM Aip1 or CapZ for 15 minutes and then 0.25 µM total actin was used to seed polymerization of 2 µM pyrene actin. While the lag phase of unseeded actin is long (green line), actin seeds shorten the lag phase of polymerization (yellow line). Cofilin creates many severed ends that seed polymerization more efficiently whereas Aip1 does not inhibit this reaction (blue and pink lines). CapZ however inhibits the seeding reaction (black line). (B) Increasing amounts of Aip1 in the presence of cofilin produce more filament seeds for elongation. Filaments depolymerized in the presence of 20, 40 and 100 nM Aip1 (yellow, blue and pink lines respectively) and (a fixed amount) of cofilin show enhanced seeding as compared to cofilin alone seeds (green line). (C) Oregon Green 488 actin filaments elongate with Alexa 647 G-actin even in the continuous presence of 150 nM cofilin and 200 nM Aip1 (right panel), however, the presence of 200 nM CapZ inhibits this reaction (left panel). Scale bar= 2 µm.

Figure 3.6 (cont.) (D) Quantitation of the relative numbers of elongating ends in the presence of actin alone or actin and 150 nM Cofilin +/- 200 nM CapZ or Aip1.

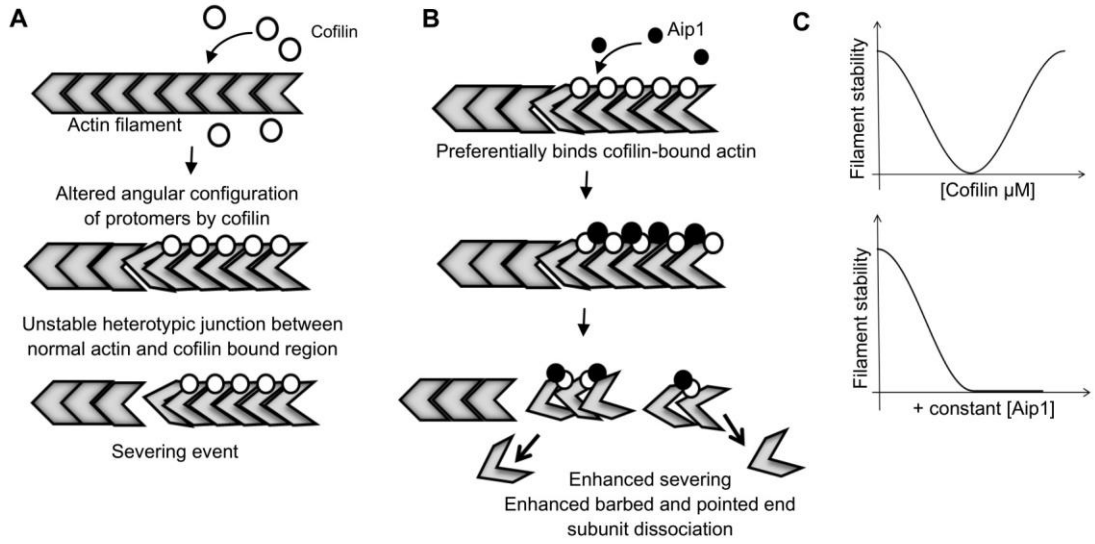

Figure 3.7 Model representing mode of Aip1 action on cofilin-actin filaments. (A) Cofilin alone binds to the filament and alters the angular configuration of actin protomers within the polymer lattice. Severing is caused due to unstable heterotypic junctions between cofilin-bound and unbound regions on the actin filament. (B) Aip1 preferentially binds stretches of actin polymer occupied with cofilin leading to enhanced severing and faster disassembly from both barbed and pointed ends. (C) In the presence of Aip1, cofilin-saturated filaments are no longer stable and can be destabilized

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

We describe an assay to report on bulk actin depolymerization in the presence of cofilin and cofilin-dependent depolymerization factors based on the phenomenon of FRET. Previous assays in the field used pyrene-actin based spectroscopy. Cofilin quenches pyrene-actin fluorescence and this precludes the use of high concentrations of cofilin in conjunction with pyrene actin, or raises the risk that results obtained in these conditions are inaccurate.

Although the FRET assay was invented in the 1980's, it has never been used in the context of cofilin-dependent actin depolymerization. We also describe the utility of this assay to supplement existing bulk actin and single filament studies in the field.

We primarily used this assay to investigate the whole filament destabilization or bursting in the presence of the triple mix of factors, namely, cofilin, coronin and Aip1. Our results concur with earlier work that shows that these factors depolymerize actin at an extremely rapid rate (Kueh et al., 2008). We found that the combination of bursting factors was able to increase the critical concentration of actin to ~1.8 μM. This partially explains how whole filament destabilization can proceed even in the presence of excess polymerizable G-actin. The bursting factors increase the critical concentration to the same extent as the combination of cofilin and capping protein (described in Chapter 3). The final products of the bursting reaction were unable to nucleate actin assembly much like the products of a severing reaction whose barbed ends

are capped (cofilin + CapZ) and unlike products of severing reactions in the presence of cofilin and actin alone (described in chapter 3).

Therefore, investigation of bursting with bulk assays raises some questions just as it answers others. The nature of the final product of the bursting reaction still remains unknown. Some possibilities are that the triple mix of factors could form a high-affinity macromolecular cap on filament ends or the final products of the reaction could be sequestered oligomers of actin. This would require detailed molecular characterization of the products of the bursting reaction.

The FRET assay also presented some interesting observations in the context of coronin and cofilin. Coronin is a protein that has many roles in cytoskeletal reorganization, however it was implicated in directly loading cofilin on actin filaments relatively recently (Brieher et al., 2006) and this observation has been fraught with controversy. In follow-up studies, no enhancement of cofilin loading by coronin was detected. In fact, coronin was shown to compete with and reduce cofilin loading on actin (Cai et al., 2007). With FRET and electron microscopy (Tang and Brieher, unpublished) we were able to reconcile these observations. Firstly, the FRET assay showed directly that coronin stabilized cofilin-actin and decreased the depolymerization rate (Brieher et al., 2006). Depolymerization with cofilin and Aip1 alone proceeded at a high initial rate however the combination of cofilin and Aip1 alone did not change the critical concentration. The triple mix of factors altered the critical concentration of actin and the stabilizing effect of coronin in the presence of cofilin was reversed when Aip1 was present.

When the reaction was carried out in the presence of fixed amounts of cofilin and Aip1 and a varying amount of coronin, we found that coronin exerted its effect when it was present in substoichiometric amounts to cofilin, consistent with unpublished electron microscopy data. At higher concentrations it served an inhibitory role to the depolymerization reaction, presumably due to its ability to compete with cofilin for binding and to stabilize actin filaments.

Although the molecular basis behind coronin's ability to increase cofilin loading on actin is being uncovered and appears to be related to the nucleotide state of the filament (Tang and Brieher, unpublished), there are specific structural and molecular details that are yet to be elucidated that will be the subject of future interesting work. For example, we do not yet know if coronin primes the filament to bind cofilin and is then itself displaced or if it stays bound to the filament once cofilin is loaded on to the filament.

A broad conceptual advancement from this thesis (that is founded in work carried out by Brieher, Kueh and Mitchison) is the discovery that Aip1 is capable of destabilizing filaments that are stabilized by the binding of either cofilin alone or cofilin and coronin. We now have a potential explanation for why cell extract can potently disassemble filaments in spite of having high concentrations of cofilin or why *Listeria* actin comet tails disassemble faster with increasing concentrations of cofilin (Rosenblatt et al., 1997). This is because in the presence of a factor such as Aip1, more cofilin translates into greater destabilization of actin. We could hypothesize that cofilin is a way for filaments to be "marked" for disassembly, and the molecule that is responsible for

disassembling them is not cofilin, but Aip1. This also puts into context some observations that cofilin's severing activity appears to be more critical for polymerization rather than depolymerization (Ghosh et al., 2004; Chen and Pollard, 2011).

Although there was previous evidence to indicate that Aip1 worked by mechanisms other than filament capping (Rodal et al., 1999; Ono et al., 2004), our work unequivocally establishes that Aip1 does not cap filament ends, thus disproving a dominant model in the field. Additional studies also report an enhancement in cofilin's severing activity by Aip1 (Chen et al., 2015; Gressin et al., 2015) and have observed Aip1's destabilization of cofilin-saturated filaments thereby validating our results. However a controversy still exists in the field regarding whether or not Aip1 severs filaments by competing with cofilin.

Recent work showed that Aip1 is able to bind filaments in the absence of cofilin with a low affinity of about 2- 3 μM and in the presence of cofilin, less Aip1 bound to the filaments (Chen et al., 2015). This led to the interpretation that cofilin and Aip1 compete for binding to actin and Aip1 potentiates severing by displacing cofilin and creating more heterotypic interfaces that support the severing reaction. However, this contradicts previous results that show that Aip1's affinity for actin is greatly increased by the presence of cofilin (Rodal et al., 1999) and is also in disagreement with our results that show that Aip1 and cofilin act synergistically and not in competition (Nadkarni and Brieher, 2014).

The displacement model of enhancing severing is popular in the field however, the evidence that Aip1 works by this mechanism is unconvincing due

to the fact that the reaction is difficult to resolve spatially and temporally. Bulk sedimentation assays to study filament binding by Aip1 and cofilin are complicated by the fact that Aip1 severs cofilin-actin into small fragments that remain in the supernatant.

The biophysics of filament severing by cofilin is the subject of intense discussion in the field. Vertebrate cofilin binds actin cooperatively (De La Cruz, 2005) altering the twist of actin filaments (McGough et al., 1997) as well as filament flexibility (McCullough et al., 2008). Filament severing is thought to occur when filaments are partially occupied with cofilin at junctions of bare and cofilin-decorated regions (Andrianantoandro and Pollard, 2006; Suarez et al., 2011). Fragmentation of filaments by cofiin has been likened to stress fracturing of nonproteinaceous materials due to the observation that most severing events occur where the critical angle of filament bending is particularly high (McCullough et al., 2011). Recently the idea that passive displacement of cofilin could drive severing has been propounded (Elam et al., 2013). However, in the presence of small molecules or actin binding proteins that displace cofilin we were unable to observe augmenting of severing rates. We also observed that filaments were able to reanneal at ‘heterotypic’ junctions. We showed that passively tethering a portion of the filament in place was an effective way of stimulating the severing reaction supporting the view that severing is a mechanically transient event. If a diffusible cofilin-dependent factor such as Aip1 can dramatically potentiate severing at such low ratios of Aip1: cofilin, we hypothesize that this must involve a mechanical deformation of the filament by the protein.

In the context of cellular depolymerization, Aip1 could be a potential switch for filaments to be converted from the cofilin-saturated stable to a rapidly depolymerizing unstable state. Loss of Aip1 appeared to reduce the available pool of monomer for polymerization (Okreglak and Drubin, 2010) consistent with its behavior in *in vitro* assays. We have discovered that Aip1 can augment rates of monomer loss from either end of the filament in addition to being a critical factor in the catastrophic disassembly or bursting behavior of actin filaments. These describe three unconventional mechanisms for disassembling actin filaments other that the widely popular filament severing model.

Unlike microtubules and certain prokaryotic actin homologs such as ParM (Mitchison and Kirschner, 1984; Garner, 2004), actin filaments are not thought to undergo dynamic instability. This is perhaps due to the fact that the difference in stability between the ADP and the ATP forms of actin is only 10-fold (in contrast to >100-fold in the case of microtubules). Thus, ATP-actin filaments can polymerize to form long filaments that are relatively stable without the action of any barbed end stabilizing factors even when nucleotide has been hydrolyzed. This may begin to explain why cells have evolved multiple factors to destabilize actin filaments.

Although we do not yet know if the mechanisms we observe *in vitro* operate within cells, it is possible that the cell uses different combinations of factors to depolymerize different kinds of arrays. This may be how actin structures of vastly differing stabilities are maintained inside cells. For example, one study that attempted to reconstitute the properties of filament networks

showed that Aip1 was particularly effective at depolymerizing both parallel and branched arrays of filaments however, cofilin alone was effective only at remodeling branched networks (Blanchoin et al., 2014).

Although the field of actin depolymerization has been galvanized by the discovery of multiple factors that can act in conjunction on actin filaments to disassemble them (Brieher et al., 2006; Normoyle and Brieher, 2012) even in the presence of excess actin polymer or polymerizable monomer, we are only just scratching the surface in our understanding of actin depolymerization. Actin filaments inside cells are actively polymerizing and densely crosslinked in a variety of arrays and subject to numerous forces. At the physical level, these forces could be responsible for structural changes that alter the affinity of molecules for actin. There is complex interplay between different molecules in binding F-actin and these could influence filament depolymerization. For example, the binding of cofilin is antagonized by tropomyosin, a protein that coats stress fibers (Bernstein and Bamburg, 1982) (see also Blanchoin et al., 2014) and this could be implicated in the extraordinary stability of these structures. Disassembly is also regulated by signaling and the cascades that control the regulation of factors other than cofilin could be the subject of future work. These are just a few directions in which the study of actin depolymerization could proceed. It is also likely that crosstalk between various cytoskeletal networks influences their stability although this area of research is virtually untouched.

However, despite a myriad of factors that influence the organization of the actin cytoskeleton and the diverse ways filament depolymerization could be executed and regulated, perhaps future work will reveal unifying principles that dictate actin disassembly and show us that the reality is not as complicated as we supposed.

APPENDIX

ACTIN FILAMENT SEVERING BY COFILIN

Introduction

Unlike microtubules and certain actin homologs such as ParM, actin filaments do not undergo the phenomenon of dynamic instability (Mitchison and Kirschner, 1985; Garner et al., 2004). ADP-actin filaments are relatively stable, with a 10-fold lower stability than ATP-actin filaments, contrary to a >100 fold difference in the case of microtubules and ParM. One way that eukaryotic actin is destabilized in order to undergo remodeling is by the action of a protein known as ADF/cofilin (Bamburg et al., 1980). Cofilin has many roles inside cells (Bravo-Cordero et al., 2013). However most pertinently, cofilin binds ADP-actin with high affinity (Blanchoin and Pollard, 1999) and severs them into smaller fragments (Maciver et al., 1991). Although cofilin-dependent severing was thought to be the dominant mode by which filaments disassembled inside cells, subsequent studies have shown it to be more important for the polymerization reaction (Ghosh et al., 2004; Chen and Pollard, 2013) due to the generation of free barbed ends that can undergo elongation.

The biophysics of filament severing is a widely debated question in the field. Most studies concur that the binding of cofilin to actin results in a change in twist of the filament (McGough et al., 1997) decrease in filament persistence length, increase in filament flexibility (McCullough et al., 2008) and weakening of lateral contacts (Galkin et al., 2011).Severing events occur at boundaries of bare and cofilin-decorated actin filaments, at substoichiometric amounts of cofilin to

actin (Andrianantoandro and Pollard, 2006) and has been likened to the fracturing of non-proteinaceous materials (Suarez et al., 2011). Due to the fact that cofilin increases the bending of filaments the critical angle of bending that is achieved by cofilin decorated filaments is higher and severing has been shown to coincide with these junctions (McCullough et al., 2011).

Mechanical tethering of filaments by proteins such as alpha actinin and heavy meromyosin has been shown to cause filament severing (Pavlov et al., 2007). Holding part of the filament in place while the rest of the filament undergoes bending movements might enable the filaments to reach a high critical angle and undergo fragmentation. However, recently the idea that passive displacement of cofilin can also drive fragmentation has gained traction in the field (Elam et al., 2013). We tested the contribution of filament tethering versus passive displacement of cofilin to severing by single filament imaging experiments.

Materials and Methods

Single filament imaging assays: These are described in Chapter 3.

Filament annealing assays: TMR-labelled filaments and Cy5 labeled filaments (described in Chapter 2) were polymerized and treated with 5x the amount of cofilin or phalloidin at pH 6.8 and 7.8. Filaments were then fixed with 0.25% glutaraldehyde for 3 mins. They were quenched with 25 mM glycine and allowed to anneal for 9 h in various combinations. Filaments were imaged on poly-L-lysine coated coverslips. N>100 filaments were analyzed from 3 separate areas of the coverslip. % Fraction of filaments that underwent annealing were quantified.

Cofilin binding assays with Pyrene actin: These are described in Chapter 3.

Results

Passive displacement of cofilin by phalloidin does not lead to severing

Substoichiometric amounts of cofilin are more effective at severing actin due to the fact that severing occurs at the junctions of cofilin-bound and unbound regions. When filaments are saturated by cofilin they are stabilized in an alternative hyper-twisted configuration. Severing by cofilin has been shown to occur best at 0.5 occupancy of the filament (McCullough et al., 2011) by cofilin. Previous work showed that severing could be induced by adding small molecules such as phalloidin that could compete off cofilin from actin. This was carried out by measuring average lengths of filaments in the presence of various concentrations of actin, cofilin and phalloidin. Since the filaments were not fixed, displacement of cofilin during dilution could potentially perturb the results of the experiment. We therefore assayed for cofilin-mediated severing by phalloidin by imaging single filaments of actin in the presence of a high initial occupancy of cofilin and increasing amounts of phalloidin. Contrary to what we expected, with increasing amounts of phalloidin and 10 µM cofilin, severing rates decreased consisently from 7*10^-5 per micron of actin to 0 severing events in the presence of 1 µM phalloidin at a ratio of 10:1 cofilin: phalloidin. In fact in live imaging assays, phalloidin was never able to increase severing rates over a wide range of concentrations (Figure A1.1).

Cofilin binding to actin is cooperative and it has been shown that small clusters of cofilin are effective at producing the bending movements/heterotypic junctions that lead to the severing reaction (Suarez et al., 2011). We

hypothesized that perhaps phalloidin was creating an overall and not a local displacement of cofilin. Phalloidin could potentially induce cofilin severing if it was present in long stretches leading to heterotypic junctions between cofilin and phalloidin bound stretches of actin filaments. We therefore prepared cofilin saturated and phalloidin saturated actin filaments at pH 6.8 and pH 7.8. We fixed these filaments with glutaraldehyde and mixed them together to test if the junctions between cofilin-bound and phalloidin-bound filaments were compatible to bind one another. At pH 7.8 there was an equal fraction of filaments that underwent annealing in spite of possessing “heterotypic” junctions as control phalloidin-phalloidin filaments (Figure A1.B). Phalloidin bound filaments on average had a longer filament lengths. This indicated that they were either stabilized or able to undergo reannealing among themselves. We did not normalize our results to filament length. At pH 6.8 there was a 10% decrease in the fraction of filaments that exhibited heterotypic junctions compared to control phalloidin-phalloidin filaments. We attribute this to the fact that phalloidin filaments were longer and on average there were fewer available for reannealing. Therefore, there did not appear to be a structural incompatibility between phalloidin bound and cofilin bound filaments.

Passive displacement of cofilin by bundling proteins

Recent work has also shown that competitive displacement of cofilin by myosin can induce severing. We also decided to revisit this observation in light of our results with phalloidin. We tested the enhancement of cofilin-mediated

severing in the presence of tropomyosin which is a well known antagonizer of cofilin mediated depolymerization (Bernstein and Bamburg, 1982).

We used pyrene-actin based spectroscopy to determine the concentration of tropomyosin that was required to displace 50% of the cofilin from actin. We chose a cofilin concentration that would give a high initial occupancy of actin (6 µM) from previous work. In the presence of this cofilin concentration, roughly 15 µM tropomyosin would give us half maximally saturated cofilin decorated actin filaments. We normalized these results to pyrene in the presence of tropomyosin alone due to the fact that tropomyosin itself altered the fluorescence of pyrene actin (Figure A2A(inset). In the presence of 15 µM tropomyosin, we did not view any enhancement in severing over the first 3 minutes of the reaction. In fact, we observed a decline of severing rates from 0.00036 events per micron per second to 0.00029 events per micron per second. This showed us that simply passive displacement of cofilin in solution does not alter filament severing rates.

Attachment is more effective in potentiating cofilin mediated severing than passive displacement

We were unable to detect any enhancement in severing in the presence of passive displacers of cofilin. However, we did detect an enhancement in severing by tropomyosin if we tethered filaments on it prior to flowing in cofilin. For this we coated coverslips with 5 µM tropomyosin, 1/3rd of the amount that was required to displace 50% cofilin from actin and flowed in polymerized

filaments. We measured the severing rate that was obtained only within the first 30 seconds of flowing in cofilin. We compared tethered filaments to unattached filaments and found a close to 4-fold enhancement of severing rates when they were attached via tropomyosin from 0.001 events per micron per second to 0.0037 (Figure A2.C).

We also looked at filament severing rates in the presence of another actin binding protein alpha-actinin. In the presence of cofilin alone, severing rates decreased with increasing concentrations of cofilin. However, if filaments were tethered to the glass in the presence of a fixed amount of alpha-actinin, increasing concentrations of cofilin lead to ever faster severing rates. Severing rates increased roughly 3x from 2 – 10 μM cofilin and were higher in every condition in the presence rather than the absence of alpha-actinin. Thus, tethering the filaments with alpha-actinin fundamentally changed the effects of cofilin on these filaments.

Discussion

We investigated “passive displacement” of cofilin as a strategy to augment severing. Recently a study reported the enhancement of severing by molecules such as phalloidin and myosin that are able to competitively displace cofilin from actin (Elam et al., 2013). These experiments were carried out by measuring average lengths of unfixed filaments in the presence of cofilin and varying concentrations of phalloidin and myosin. At 0.5 occupancy of the filament by cofilin, filament lengths were found to decrease. This has led to a popular idea in the field that knocking off cofilin from actin can lead to more heterotypic (cofilin-bound versus bare) junctions and more severing. However, our work contests this idea.

We directly assayed filament severing rates by time lapse imaging of filaments in the presence of cofilin and varying concentrations of phalloidin. In our hands, phalloidin stabilized actin filaments. In fact, we observed that with increasing phalloidin, the average number of severing events decreased.

Phalloidin, unlike cofilin, does not display any cooperativity in binding. Therefore, once it binds onto actin filaments, it does not displace a long stretch of cofilin and may not create heterotypic interfaces that can promote severing (Suarez et al. 2011). We addressed this caveat by artificially generating phalloidin versus cofilin “heterotypic interfaces”, by allowing cofilin– and phalloidin-bound actin filaments to anneal to one another. We found that these filaments were in fact, compatible to bind one another.

Indeed, the existence of heterotypic interfaces between phalloidin and cofilin-saturated actin filaments indicates that severing by cofilin could be a result of a transient incompatibility or “fracturing” as has been described (McCullough et al., 2011).

We also observed similar results in the case of actin binding proteins. We looked at severing rates of filaments in the presence or absence of a well-known competitor of cofilin-binding : tropomyosin at 50% occupancy and found no enhancement in severing rates however, if we attached filaments to coverslips coated with tropomyosin, we did obtain augmented severing rates as has been observed in the past (Pavlov et al. 2011). We obtained similar results in the case of a different actin binding protein alpha-actinin 4. The tethering of filaments fundamentally altered their behavior in the presence of cofilin. Cofilin severs best at low ratios of cofilin: actin when the filaments are in suspension and increasing levels of cofilin lead to stabilization of actin filaments. But if filaments were tethered to the surface, it was the higher concentrations of cofilin that led to more efficient disassembly. This phenomenon could be especially relevant in cells where filaments are found crosslinked in dense networks.

If tropomyosin and alpha-actinin were bound to actin at a certain site, the accessibility of the site for cofilin binding might be hindered. We wished to understand if the severing event occurred because part of the filament was immobilized or because the cofilin was simply unable to bind to the portion of the filament thus creating a ‘heterotypic junction’.

Preliminary studies suggest the former. When filaments were tethered to the glass simply by doping filaments with a low amount of biotin and attaching them via streptavidin, high concentrations of cofilin led to severing. Although more studies are required to elucidate this point more fully, this is an intuitive yet important observation for actin filament severing. If an inert tether that does not hinder cofilin-binding to actin is able to increase filament severing, this indicates that simply mechanically constraining sections of the filament is sufficient to promote a severing reaction. This may influence the way that actin network dynamics are viewed.

Figures

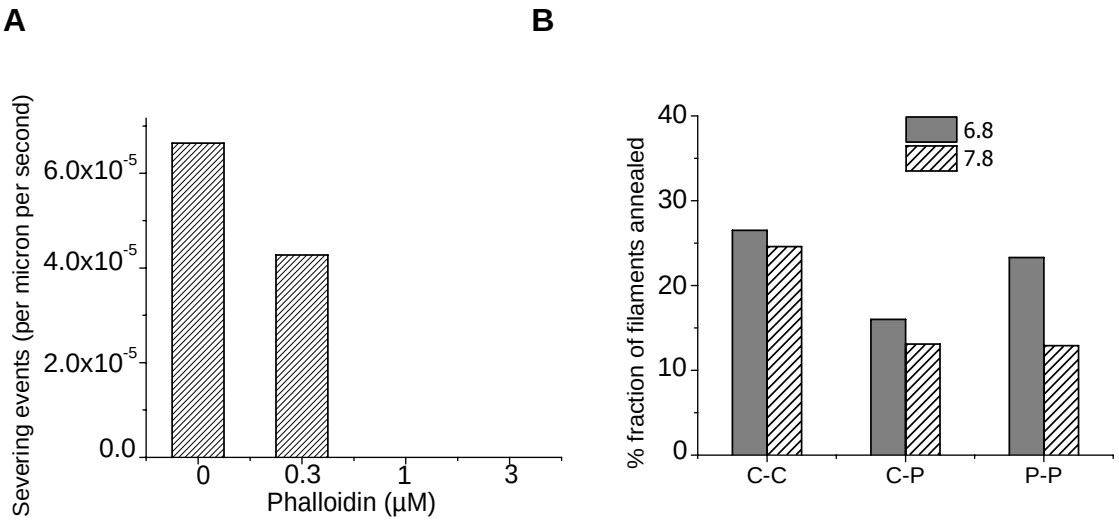

Figure A.1 Severing does not occur by passive displacement of cofilin by phalloidin. (A) Severing events were quantified with 10 µM actin and increasing concentrations of phalloidin. Rates were found to decrease from 6*10^-5 per micron to zero in the presence of 1 µM phalloidin. (B) Reannealing of cofilin-saturated or phalloidin-saturated filaments at pH 6.8 and pH 7.8. % Fraction of total filaments that underwent annealing and had heterotypic junctions were quantified.

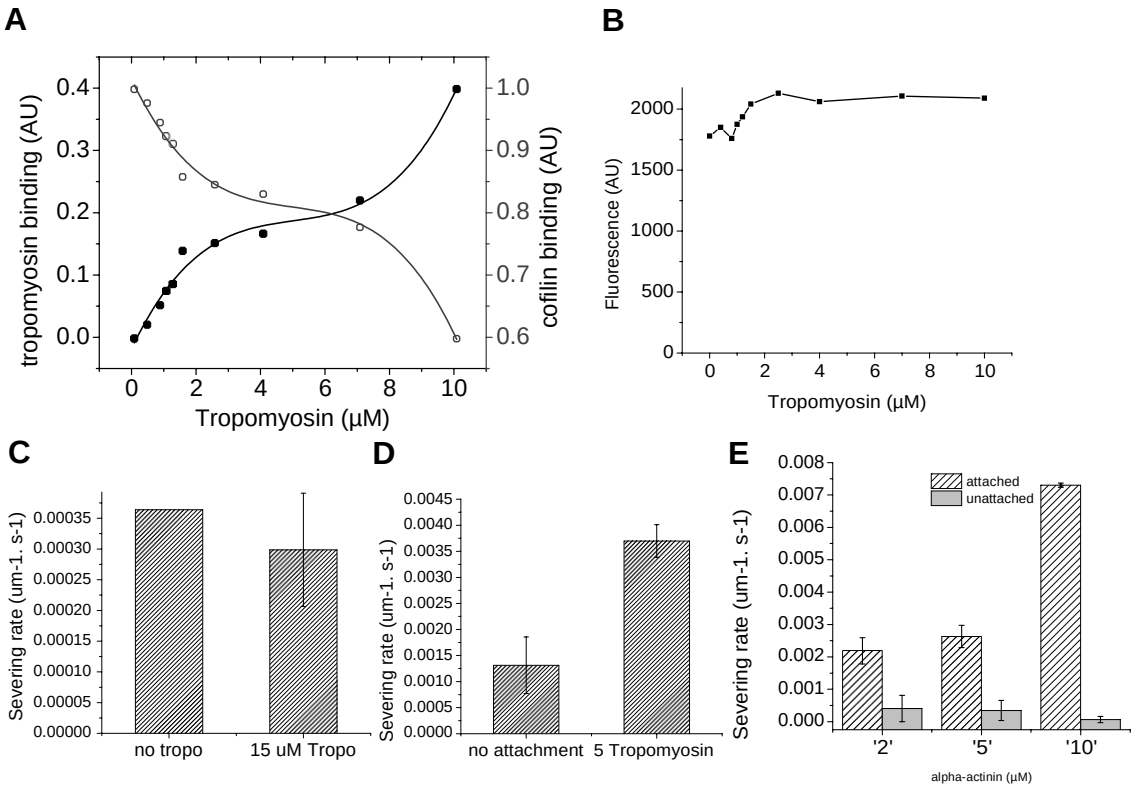

Figure A.2 Tethering filaments by actin binding proteins is more effective at augmenting severing than passive displacement. A) Displacement of cofilin from pyrene-actin filaments saturated with cofilin (6 μM cofilin, 3:1 cofilin :actin, v_{cof}>0.9) in the presence of increasing concentrations of tropomyosin showed that roughly 15 μM tropomyosin could displace 50% cofilin from actin filaments. (B) Readings were normalized to fluorescence values of pyrene actin in the presence of increasing concentrations of tropomyosin alone. (C) These concentrations of cofilin and tropomyosin were used to look at severing rates in suspension. Severing rates in the presence and absence of tropomyosin were enumerated as events per micron per second. (D) Severing events of filaments untethered or tethered by 5 μM tropomyosin were quantified in the first 30 s of the reaction. Tethering increased rates 3x.

Figure A.2 (cont.) (E) Tethering filaments by alpha actinin caused an increase in severing rates at all concentrations of cofilin. Shaded bars represent attached filaments and gray bars represent filaments in suspension without alpha actinin. In the presence of alpha actinin, increasing concentrations of cofilin do not saturate and stabilize actin filaments.

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