CYCLASE ASSOCIATED PROTEIN (CAP) AND THE PHYSIOLOGICAL DISASSEMBLY OF ACTIN

BY

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DISSERTATION

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ABSTRACT

Actin dynamics are important for both driving cellular movement and maintaining cellular structure in apparently static tissues. Actin assembly and disassembly is an energy driven cycle that is utilized by cells to generate force and organize space. Though studied to some extent by a host of fields, the study of the actin cytoskeleton itself is an area with many questions left to be answered, such as how cells disassemble actin in the presence of large concentrations of both monomeric and polymeric actin, how apparently very stable actin structures can be disassembled, and what role actin plays in the nucleus and how this role differs from cytosolic actin dynamics.

Using a biochemical reconstitution approach, we set out to find one or more factors that conferred actin disassembly activity to the known actin disassembly factors. Once identified as cyclase associated protein (CAP), we next studied the properties of this factor focusing on its interactions with actin and other actin disassembly factors including cofilin, coronin, and AIP1. We discovered that CAP has a complimentary yet synergistic relationship with cofilin, a partially redundant relationship with coronin, and that CAP can act as an independent actin disassembly factor at low pH. While CAP was a known actin interacting protein, none of these findings were known to the field before our work was published.

After an introduction and summary of the state of the field as it was when we started, Chapter 2 begins with the initial characterization of CAP and our efforts to determine its function. We soon realized that the field was mistaken about the role of CAP as an accessory protein not truly involved in actin disassembly, and we showed that CAP accelerates cofilin, coronin and AIP1-mediated actin depolymerization. We then demonstrated a partial redundancy to coronin but showed that the underlying mechanism of CAP-mediated actin disassembly was distinct from that of coronin. Next we set out to discover what the precise role of CAP was through two similar but distinct lines of experiments.

In Chapter 3 we study CAP using similar methodologies but with single actin filaments instead of the branched actin networks in the actin comet tails formed by *L monocytogenes*. This allowed us to more precisely control the experimental conditions while also giving information of single filament off-rates and allowed us to determine the interaction between CAP, cofilin and

pH. In Chapter 4 we continue to study branched actin networks formed by *L monocytogenese*, but formed under defined conditions without any cell extract. This work was designed to allow us to determine whether there might be activities of CAP which were geometrically dependent, such as any activity confined to branch points. What we found was that actin filaments built with ena/vasp-like protein (EVL) were more susceptible to CAP disassembly. Finally in Chapter 5 we offer a few concluding remarks about the state of the field and the recurrent sense of premature accomplishment that it is prone to.

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who taught me how and why to learn.

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CHAPTER 1: GENERAL INTRODUCTION

Actin polymerization and depolymerization are recognized as the processes which give rise to cellular force generation necessary for cellular movement and spatial organization[1, 2] and an important determinant in the establishment and maintenance of polarity.[3] Cellular control of the dynamic actin cycle, then, is responsible for diverse functions from directing and translocating a neutrophil toward its bacterial prey to maintaining a stable epithelial polarity in tissues from the renal tubule to the hepatic sinusoid; the inability of cells to appropriately manage these processes results in disease states ranging from systemic immunodeficiencies[4] and metastasis of cancer cells[5] in the former and polycystic kidney diseases in the latter.[6] The narrow range of appropriate activity should also be noted: immunodeficiencies result from an inability of cells to move purposefully while metastasis can be understood as the result of a failure to reign in otherwise purposeful cellular movement. Physiological actin assembly is accomplished principally by maintaining a pool of actin monomer in excess of the critical concentration, thus thermodynamically and kinetically favoring actin assembly and raising the question of just how the cell manages to disassemble actin in such an environment. This thesis engages that question and seeks to understand the cellular processes that allow and drive physiologic actin disassembly.

Understanding Actin: Thinking Forward

The study of actin dynamics has a long and venerated history of laying the scientific issues surrounding the cyclic actin process to rest – repeatedly. As new technologies emerge and important details are unearthed, the question of how actin dynamics are managed effectively by the cell is reopened for a new generation to be captivated and frustrated alike at both the cellular and molecular levels. Actin was discovered behind enemy lines, from an Allied point of view, in 1942 by Straub and Szent-Gyorgyi.[7] Actin was almost immediately seen as a complex factor, with two distinct roles identified that would later be found to underlie the actin-myosin motor: at low ionic strength actin caused contraction in the presence of myosin but at high ionic strength actin dissociates from myosin.[8] This would later be understood as the phenomenon of *rigor mortis* as it occurs when ATP is depleted and ADP•actin remains bound to myosin.[9] Actin was further proven to be involved in the utilization of ATP when actomyosin steady state ATPase

activity was found to increase during contraction, even if the respective contributions of actin and myosin were not yet understood.[10]

Due to the methods of preparation, in which actomyosin was prepared in "threads" that were seen to contract upon the addition of a boiled muscle mixture containing ATP, co-discoverer of actin Szent-Gyorgyi believed that this represented a "superprecipitation."[11] Dissenters such as Astbury and Meyerhof believed that this represented the contraction of actomyosin,[12] and with the advent of a new glycerol-extracted psoas muscle preparation they were proven correct. This was the first instance in a recurrent theme in the study of actin, as a misunderstanding of actin functionality was corrected by a new methodology that allowed a more detailed look or a more physiological experiment to be accomplished. Yet it was also perhaps unique as Szent-Gyorgyi himself was the man who developed the glycerol preparation that allowed Mg^{++}•ATP to be taken up by the intact psoas muscle, disproving his own theory of actin function when the muscle contracted.[13]

Straub also continued to contribute to the field but with a deeper mechanistic approach and it is he who discovered not only that actin itself binds ATP but that ATP hydrolysis is central to actin function, at a time when actin was largely thought to be an uninteresting foil to myosin.[14] It would be two decades before the role of actin in cell motility would be recognized, and several yet before it would reach some semblance of understanding. Cell motility was thought of as the product of pressure differences and the flow of cytoplasm between relative points of the moving cell.[15] Some of the principles of actomyosin-based contraction were applied to non-muscle cell motion even while actin was considered a skeletal muscle-specific protein,[16, 17] The importance of understanding the mechanism of cell movement was recognized early in medicine, particularly in the field of oncology,[18] yet it was apparently surprising to the field that actin could be found outside the highly regulated and repeated structure of the motor unit.

Thus with the discovery of actin in non-skeletal muscle cells and the associated implication that the motor unit was not a structural necessity dictated by the structure and function of neither actin nor myosin themselves, the wide applicability of actin and myosin appeared to dawn on the field. Actin was found in smooth muscle cells of vertebrates in 1967[19] and formed the basis of amoeboid cell movement hypotheses shortly thereafter,[20] with a young Tom Pollard describing

acanthamoeba F-actin in the Korn laboratory.[21] Actin was further recognized both for its versatility and importance when it was re-discovered in multiple non-muscle cells by Ishikawa based on myosin head decoration of actin filaments[22] and later confirmed through biochemical and immunofluorescent studies.[23, 24] The role of actin in cell movement was beginning to come to light as early as 1971,[25] but it was the work of Wegner in 1976 that detailed how actin kinetics measured in vitro might predict the *in vivo* behavior of actin to render translocation.

Wegner proposed a "head to tail" polymerization of actin[26] later referred to as actin treadmilling whereby the kinetic differences between ATP•F-actin and ADP•F-actin allow for actin to grow at one end and shrink from the other. Once actin polymerizes it quickly hydrolyzes its bound ATP, and eventually releases free inorganic phosphate.[27] The fact that free energy is released in this process means that the ends need not have the same k_{on}/k_{off} ratio, and thus can have differing critical concentrations at which the corresponding F-actin filament end will elongate. In the physiological range, this corresponds to a steady-state situation in which the barbed or plus (+) end elongates while the pointed or minus (-) end shrinks. These rates have been meticulously measured,[28-30] but it should be noted that these measurements are *in vitro*.

Just as some were pointing out that such an understanding did not appear to be adequate to explain actin dynamics *in vivo*,[31] the application of the newly developed photobleach experiment to lamellipodia by Yu-Li Wang appeared to settle the question decisively in 1985.[32] Wegner's kinetic hypothesis seemed to be demonstrated by Wang's observation that a photobleached section of actin filaments at the leading edge of fibroblasts could be seen to move centripetally while new actin appeared to form at the leading edge. Thus the actin filaments inside cells must be long enough to span from the cell edge, where they are formed, to a central location where they disassemble. Forscher and Smith appeared to corroborate this view with their work in neural growth cones.[33]

Yet it was not long before another insightful researcher using another newly developed technique would challenge this view. Julie Theriot used subtle but important differences in her approach to the problem. Utilizing photoactivatable 'caged' fluorophores attached to actin as developed in the Mitchison lab along with fast-moving goldfish keratocytes instead of Wang's fibroblasts, Theriot demonstrated that actin filaments did not span the length of the cell and that

they appeared to begin to depolymerize as soon as they had formed [1, 34, 35]. Thus actin filaments had to be either much shorter than they were thought to be or their disassembly kinetics had to be much faster than they were thought to be.

Subsequently, numerous groups began to look for factors which might increase the rate of actin disassembly. Within a few years the Bamburg group had discovered actin depolymerizing factor (ADF) in an avian system[36] and the Drubin group discovered cofilin in *S. cerevisiae.*[37] Having found them to be homologous moieties ADF and cofilin were jointly referred to simply as ADF/cofilin, henceforth cofilin.[38] Lappalainen and Drubin found cofilin to be necessary for certain processes in yeast such as endocytosis and further found that cofilin was indeed essential.[39] At this point it was recognized and accepted that physiological actin dynamics involved fast actin turnover, and that *in vitro* rates of actin disassembly were insufficient to explain physiologic function in the absence of cofilin.

Cofilin was recognized as responsible for the acceleration of actin disassembly and as part of the minimal set of factors which are necessary to recapitulate the dynamic actin cycle in a defined system,[40] and a few years later was recognized as the necessary factor responsible for actin disassembly in Xenopus oocytes.[41] Yet some uncertainty remained over exactly how cofilin was accomplishing this dynamism. Cofilin was thought by some to accelerate actin dissociation from pointed (-) ends by up to 22-fold,[42] whereas others thought that because cofilin can also increase actin assembly,[42-44] cofilin was in fact severing actin filaments in order to produce more ends from which to lose actin mass (- ends) or nucleate new growth (+ ends).[45]

Those who support the idea that cofilin accelerates the loss of actin mass from the pointed end say that cofilin functions to act on pointed ends, and that severing is merely a means to further accelerate actin disassembly by providing more - ends. However, *in cellula* experiments such as the injection of a caged cofilin moiety into cancer cells show an increase in actin assembly with increased cofilin.[46] Carlier's original 1997[42] result also found an increase in assembly *in vitro*, but the *in cellula* work raised the question "just what is the physiological function and overall effect of cofilin?" It is worth mentioning that a similar paradox arose at this time regarding the actin polymerizing protein ena/VASP family proteins (henceforth ena/VASP-like, or EVL) as Bear and colleagues found that when overexpressed EVL actually caused slowing of cell

movement [47]. It was later found that EVL accelerated actin assembly but when overexpressed EVL disrupted the actin cycle such that the imbalance caused a disruption in the motility of the cell. In terms of actin disassembly and the *in cellula* increase in cellular motility when caged cofilin is injected and activated,[46] experts in the field returned to the fundamental facts of actin, as they were understood. Wagner had hypothesized the treadmilling model over the objections of Brenner and Korn, and this model was apparently validated by Wang though tempered by Theriot and Mitchison. Whether championed or conceded it was widely accepted that the two most likely mechanistic possibilities which fell out of Wagner's equations were that either more actin monomers dissociated from the filament per unit time, or this rate remained constant but more ends were created from which more actin monomers collectively dissociated.

The Enhanced Treadmilling model, exemplified by Carlier's work showing a cofilin-mediated increase in the pointed (-) end off rate, argued for an enhanced actin off-rate within the confines of Wagner's treadmill. Opposing this view was a Severing model in which the increased overall rate of actin disassembly was accomplished not by changing the fundamental characteristics of actin kinetics such as the pointed (-) end off-rate, but rather by creating more ends from which actin could dissociate.[48] The latter had the advantage of also offering an explanation of why increased cofilin activity could increase actin polymerization as each break in the filament offered one pointed (-) end from which to lose mass but also one barbed (+) end from which to nucleate actin polymerization.[45] The difference between whether a cell experiences net polymerization or depolymerization was explained within this model by capping proteins, which would 'cap' or make inaccessible the barbed (+) end of the filament thus preventing actin elongation but allowing actin dissociation from the pointed end.[49]

While there is great power in the approach of Andrianantoandro and Pollard to use fluorescent microscopy to directly visualize the mechanism of disassembly so as interpret experiments independently of a preconceived model, their work draws perhaps its greatest importance from revealing a curious behavior of cofilin in which cofilin severs at low concentrations but loses this activity at higher concentrations[45] in sheer defiance of the principle of mass action. Due to cofilin-mediated actin filament twisting observed with electron microscopy,[50] it has been hypothesized that this phenomenon is due to a proposed mechanism of severing whereby the junction of a normo-twisted actin filament segment and a hyper-twisted segment is the site of

actin severing, thus at higher concentrations cofilin reaches maximal hypertwisting efficacy and actually stabilizes the actin filament due to a reduced frequency of effective severing sites.[45]

Such a hypothesis would offer an explanation as to why cofilin violates the principle of mass action and also how cofilin might have such different roles in different cellular situations, swinging from actin disassembly[42, 51] to phosphate release and debranching[52, 53] all the way to nucleation of actin polymerization.[46] However, this would demand that severing explain the in vivo acceleration of actin disassembly with even fewer available severing sites – the exact number dependent upon the average length of normo- and hyper-twisted filament segments in addition to the overall filament length. Neither Severing nor Enhanced Treadmilling can be precisely modeled mathematically without knowing the mean filament length inside the cell,[54] and so neither could be ruled out nor proven with the information at hand. In the early- to mid-2000s, the Severing model with its underlying treadmilling and superimposed barbed (+) end capping was considered the leading theory of the actin field.

This same line of research highlights another problem with the severing model of actin disassembly. Cofilin is more active at basic pH values and quickly loses activity at lower pH,[55] but the extent to which this is true is striking. When McGough and colleagues studied cofilin-induced changes in actin filament twist, a pH of 6.5 was perfectly adequate to stop all cofilin activity even at saturating concentrations while still allowing cofilin to bind F-actin.[50] This is just at the lower periphery of physiologic pH[56, 57] and leaves the field once again chasing the abilities of the cell to disassemble filamentous actin. The importance of pH to cofilin function has been recognized in vivo[58] but a mechanism to explain how efficient actin disassembly is achieved at a range of pH values was not readily apparent at the outset of this project.

Further experiments utilizing a perfusion chamber set-up and a ‘take a look’ approach yielded exciting results. After pioneering the perfusion chamber used to achieve the divorce of actin assembly form actin disassembly in 2004,[59] in 2006 Brieher and colleagues recognized and solved a thermodynamic problem: in the presence of physiological concentrations of free G-actin, actin monomers were thermodynamically incapable of dissociating from actin filaments and actin disassembly stalled.[60] Obviously the cell is able to circumvent this challenge as actin disassembly proceeds readily inside the cell indicating that LaChantlier’s principle is satisfied. It

was recognized that two accessory actin-binding proteins, coronin-1a (henceforth coronin) and actin-interacting protein 1 (AIP1), are together necessary and sufficient to overcome the inhibition of excess G-actin on cofilin-mediated actin disassembly[60] In a technically challenging but beautifully executed follow-up, Kueh, Mitchison and Brieher demonstrated that the addition of coronin and AIP1 to cofilin-mediated actin disassembly changed not only the rate of actin disassembly but also the mechanism.[61]

While coronin and AIP1 did not entirely dispel all cofilin-mediated actin severing, it became clear that the acceleration of actin disassembly that allowed resistance to excess free G-actin coincided with the advent of an end-dependent loss of relatively long stretches of actin filaments that appeared to dissipate into actin monomers. Under these conditions severing occurred but was not the dominant behavior of disassembling actin filaments. Even with an exposure time of 16 milliseconds, no severed filamentous fragment could be visualized after such an event.[61] These events were dubbed "bursts," though it should be noted that as the mean filament length inside cells is unknown these bursts may be capable of disassembling an entire *in vivo* actin filament in one single rate limiting step. This possibility would later be studied in more detail and referred to as whole filament destabilization (WFD), a phenomenon more in line with the results of Theriot and Mitchison and with the physics of maintaining cellular structure while also maintaining fast actin disassembly.[54]

Just as excess G-actin of the scale found inside cells inhibited cofilin-mediated actin disassembly, it was recognized by Brieher and colleagues that physiological levels of F-actin inhibited the more complete tripartite cofilin, coronin, and AIP1 disassembly mixture. This result was achieved by pre-polymerizing unlabeled (or dark) actin to be added along with the three disassembly factors to a fluorescently labeled actin substrate such as an actin comet tail assembled by *Listeria monocytogenese*. The addition of F-actin inhibited disassembly mediated by cofilin, coronin and AIP1. Thus a new problem emerged, one of a kinetic nature. Adding more coronin or AIP1 had no effect on disassembly rate and the addition of cofilin was only effective at overcoming excess F-actin inhibition at ratios of cofilin to actin so high relative to physiologic ratios as to border on the ridiculous. How, then, could the cell manage to

accomplish physiologic disassembly rates with physiologic concentrations of F-actin and actin disassembly factors? With the hypothesis that a physiological factor was missing, Brieher added cell extract to the three-protein disassembly mixture lifted the inhibition of excess F-actin and physiological concentrations of cofilin, coronin and AIP1 were again sufficient to disassemble actin and we set out to identify this additional factor through the lost art of biochemical reconstitution.

Understanding Actin: Marching Forward

Throughout this progressing history of actin and the deepening of our understanding of actin dynamics, there were several times at which both molecular and cellular actin function appeared solved.[62] Yu Li Wang classically used the recently developed technique of photobleaching[32] to describe centripetal (retrograde) flow from the lamellipodial leading edge, seen as the cellular application of Wegner's[26] proposed 'treadmilling' molecular kinetics of actin. So convincing was this visual evidence that the work of Brenner and Korn,[31] which only two years prior to Wang's seminal paper had appeared to mathematically rule out treadmilling as a serious contender to adequately explain intracellular actin dynamics, seemed forgotten.

While subsequent work appeared to show retrograde flow in effect at the tips of neural growth cones[33] and conventional wisdom held that apparent sub-cellular observations must extend to the molecular level, it is understandable that actin filaments were thought to be assembled at the cellular edge and span many micrometers to be disassembled at a more central location. It was not until the introduction of a photo-activatable actin moiety in 1991 that this view was seriously challenged when Theriot and Mitchison looked at time-lapse videos of photo-activated actin in goldfish keratocytes. Photo-activated actin filaments lost intensity over time as they remained fixed in position relative to the substrate in motile cells.[34] As confirmed in subsequent studies, the rate of actin polymerization generally matches the rate of cell movement[1] and continuous actin disassembly accompanies the retrograde flow of actin from the cell periphery[35] originally observed by Wang. Indeed, by the early 2000s it had been shown that not only was actin disassembly present at the cell edge, the previously presumed domain of exclusive polymerization, but that actin was actually both assembling and disassembling side-by-side throughout the lamellipodia.[63, 64] Continued work from the Mitchison lab across the 1990s and

2000s demonstrated with increasing precision that actin disassembly follows first order kinetics[34, 54] not mathematically compatible with treadmilling, representing an apparent validation of Brenner and Korn. In the same time frame cellular factors responsible for accelerating both actin assembly[4, 65, 66] and disassembly[34, 41, 67-69] were reported and initially characterized.

One of the most exciting aspects of the actin field has been the wealth of factors which have been recognized for their importance in actin assembly, disassembly or – sometimes frustratingly – both. Cofilin has long been recognized for its importance in actin disassembly,[41] but more recently has been appreciated for its acceleration of actin assembly.[70] Another enigma is the apparent differences in how actin dynamics are handled in one area of the cell versus others. For instance, the necessary Arp2/3 activator N-WASP has long been recognized as an important initiator of actin assembly,[4, 71] yet recently has been shown to be unnecessary for actin assembly at adherens junctions.[72] These effects may be secondary to subcellular localization of known actin assembly factors, or may be indicative of differential properties between different types of actin arrays, or both. One somewhat surprising example of this is the actin bundling protein α-actinin, which has been shown to be necessary for actin polymerization at adherens junctions.[73] It has been postulated that N-WASP is important not for initial actin polymerization at adherens junctions but for maturation from actin polymerization to a contractile network.[74] In a similar example of the subtleties that punctuate the field of actin dynamics, Ena/VASP-Like protein (EVL) clearly has a role in cellular movement[75] but overexpression has been shown to be anti-correlated to motility rates.[47] This paradox was later reconciled with the demonstration of the role of EVL as a promoter of actin *dynamics*, thus both knocking down or overexpressing EVL resulted in perturbation of the actin cycle.[76, 77]

Crucial observations of a gram-positive bacterium, *Listeria monocytogenese*, which is capable of infecting mammalian cells and utilizing the actin cytoskeleton of the host[78] in order to generate force in a manner recognized for its similarity to that of the cellular leading edge,[79] had by the year 2000 already demonstrated continuous and uniform actin disassembly along the length of its actin 'comet tail.' Importantly, actin disassembly occurs in these comet tails through a single rate limiting step with a half-life of approximately 30 seconds.[1, 54] As its pathophysiology was better understood, *Listeria* became an important tool in deciphering actin

dynamics – particularly actin disassembly. As bewildering as actin assembly mechanisms can be, actin disassembly mechanisms revealed themselves to be at least as complicated.

One epicenter of ambiguity has been coronin, a factor which has been found both to stabilize[80, 81] and to destabilize[60, 61] actin filaments. This stark difference may be related to isoform differences (i.e., coronin 1a versus 1b), but such a dramatic and diametric swing in activity is not a typical property of protein isoforms. The function of individual actin assembly or disassembly factors would be difficult to understand even in isolation, but one must consider all relevant factors in order to recapitulate the physiological conditions within the cell. These factors and the processes they are responsible for, such as axon pathfinding,[82] physiologic lamellapodia[34, 35] and pathophysiologic invadopodia,[83] are important treatment targets for a wide range of human pathophysiology but can only be understood once the underlying mechanisms of actin dynamics are deciphered. Further complicating the issue is that the list of currently known actin disassembly factors cannot recapitulate the abilities of *in vivo* actin dynamics *in vitro*, neither qualitatively nor quantitatively.

The discovery of actin depolymerizing factor / cofilin (ADF/cofilin; hereafter cofilin)[37, 38, 67] has provided new insight into the importance of actin disassembly *in vivo*. Cofilin has been found to increase the disassembly of actin[41] thereby increasing motility when monomeric actin is limiting.[42] Not surprisingly this activity was found to be essential for functions from yeast cytokinesis[84] to neuronal growth cone dynamics.[67, 85] Cofilin was discovered as an actin severing protein[36, 37] but more recent mathematical modeling indicates that in *Listeria* actin comet tail turnover severing is not compatible with observations[54] and in yeast endocytic patches severing alone is insufficient to account for actin disassembly.[86]

Thus it is of little surprise that competing theories to explain the mechanism by which cofilin accelerates actin disassembly were proposed, both based upon Wegner's 1976 treadmilling model. If cofilin accelerates actin disassembly by increasing actin mass lost from pointed ends, then in a population of filaments acceleration could either occur through an augmentation of the pointed-end dissociation rate ('Enhanced Treadmilling')[42] or by maintaining the dissociation rate but increasing the number of pointed ends within the population by breaking existing filaments into daughter filaments, each with its own pointed end ('Severing')[49].

Perhaps a far more fundamental hurdle for actin disassembly *in vivo* is the large excess of actin relative to the critical concentration. Just as this excess thermodynamically drives actin assembly, it provides a thermodynamic hurdle to actin disassembly. Though the numbers vary by cell type, mammalian non-muscle cells contain actin at concentrations approximately 100-fold in excess of the critical concentration.[51, 87] While consistent with fast actin assembly, this excess is troubling when viewed from the perspective of actin disassembly whereby there is a large excess of product. Thus by Le Chatelier's principle one can and should ask how filamentous actin can be disassembled to monomeric actin when a significant excess of monomeric actin already exists.

Chemists will point out that actin is a steady-state system with energy input from ATP hydrolysis as opposed to an equilibrium system, and thus is not subject to Le Chatelier's constraints.[88] Thus the problem is not solved, but transformed: if cellular concentrations of filamentous and monomeric actin are such that actin polymerization can be accomplished through kinetics alone but energy input is necessary to drive actin depolymerization against a steep chemical gradient, then actin disassembly factors must be acting on actin filaments to either supply free energy themselves or to allow the utilization of some of the 30.5 kJ/mole of free energy derived from ATP hydrolysis and subsequent phosphate release associated with each actin monomer just after polymerization. In all likelihood actin disassembly factors are utilizing some of the energy of ATP hydrolysis, be it at the time of ATP hydrolysis, phosphate release, or at a later time at which some of the energy of ATP hydrolysis may still be stored in the structure of the polymerized actin filament. It is possible that different actin disassembly factors or combinations of factors may be able to disassemble actin filaments differentially with respect to filament age, as we show in Chapter 3.

It is known that cofilin accelerates actin disassembly by binding preferentially to ADP-bound filamentous actin,[41, 42] but whether cofilin simply binds to ADP-F-actin as it is formed or alternatively induces formation of ADP-F-actin through its binding is difficult to distinguish. Both the addition of inorganic phosphate, which should block the stochastic release of phosphate from actin filaments, as well as the introduction of beryllium fluoride, which stabilizes the ADP-Pi-F-actin intermediate state, inhibit cofilin binding and cofilin-mediated actin disassembly.[89, 90] On longer time scales, however, cofilin induces beryllium fluoride to leave the nucleotide

pocket,[91] indicating that cofilin may indeed induce phosphate release in agreement with the mechanistic argument for the utilization of the energy of ATP hydrolysis in the destabilization of the actin filament even though these events are temporally separated – making the actin filament an arbiter of ATP-derived energy. Further, the ATP-derived difference between the ends of an actin filament is what makes this a non-equilibrium, steady-state system; each end considered separately is a system in equilibrium with its surroundings. Given that both ATP- and ADP-G-actin are capable of polymerization, though at different rates, the high concentration of assembly-competent monomeric actin still poses a thermodynamic problem laid bare by the fact that cofilin is no longer sufficient to appreciably disassemble actin when challenged with such a physiological concentration of actin monomer.[60]

Both severing and enhanced treadmilling theories rely on barbed-end capping, either to stop barbed-end elongation or to prevent post-severing filament re-annealing, or both. However, capping proteins (e.g., CapZ) were included in the work of Carlier aimed at recapitulating the cellular system with purified components and did not result in actin disassembly reaching *in vivo* rates.[40] This work utilized the dangerous infectious agent *Listeria monocytogenese*, a potential contaminant of refrigerated meats and prepared foods that grows at low temperatures (e.g. 4ºC). *Listeria* is a particularly dangerous pathogen because of its intracellular location and its ability to utilize the actin polymerization system inside cells allowing it to propulse itself inside and between cells, thus evading the typical immune response[78] and rendering physiological barriers such as the blood-brain and placental barriers of little use. The initially benign-appearing presentation adds to the potential for tragedy, but the ability of *Listeria* to utilize actin in order to propulse itself forward in cell extract leaving an actin 'comet tail' behind has made it a very useful tool both in the study of actin assembly and actin disassembly.

Utilizing the *L monocytogenese* system and perfusion chambers to experimentally separate actin assembly and disassembly (Figure 1.1), Brieher and colleagues used a biochemical reconstitution method to identify two proteins that, along with cofilin, are essential for actin disassembly activity when that activity is challenged by cellular concentrations of polymerizable monomeric actin: coronin and actin interacting protein 1 (AIP1).[60] In an effort to distinguish between the Enhanced Treadmilling and Severing models, this three-protein mixture was studied in the presence of single actin filaments and found to disassemble single actin filaments in large

sections or 'bursts.'[61] While this bursting phenomenon is not universally accepted in the field,[92] the most recent and precise mathematical modeling-based approach to determining the *in vivo* mechanism of actin disassembly concludes that the most likely mechanism is a form of bursting in which the mean filament length is no bigger than the average burst size, thus allowing entire filaments to disassemble in a single rate-limiting step.[54] Thus bursting may account for the discrepancies between the physiological system and our current understanding of actin disassembly mechanism.

Despite such encouraging results with regard to the mechanism of actin disassembly, another problem had persisted. While cofilin, coronin and AIP1 are together sufficient to deal with the issue of excess actin monomer, this three-protein mixture was insufficient to deal with the issue of excess filamentous actin. Physiological concentrations of F-actin range from 100-330uM,[87] but the actin disassembly system reconstituted by Brieher et al in 2006[60] could not withstand even modest amounts of excess F-actin added at the disassembly step of perfusion chamber experiments; *i.e.*, when even modest amounts of pre-polymerized F-actin are added to an otherwise efficacious mixture of cofilin, coronin and AIP1, this three-protein mixture is no longer sufficient to disassemble the fluorescent actin substrate. This activity does, however, exist *in vivo* as well as in cell extract. This thesis describes in detail the identification and characterization of a new factor that explains this difference between *in vitro* and *in vivo* actin dynamics, as well as its implications for our understanding of physiological actin disassembly mechanism.

CHAPTER 2: IDENTIFICATION OF CYCLASE ASSOCIATED PROTEIN AS A FOURTH ACTIN DISASSEMBLY FACTOR NECESSARY FOR PHYSIOLOGICAL ACTIN DISASSEMBLY

ABSTRACT

Cells need to exert spatiotemporal control in order to adequately respond to and/or shape their environment using cytoskeletal polymerization and depolymerization, yet cells must do so with little to virtually no spatial separation between these opposing processes. It is currently unclear how the cell manages to accomplish both fast actin assembly, driven by a concentration of polymerizable actin approximately an order of magnitude greater than the critical concentration of actin, and fast and efficient disassembly of polymerized actin. Cofilin is known to be necessary for actin severing and disassembly, and more recently coronin and AIP1 have been recognized for their roles in accomplishing fast actin disassembly in the presence of high concentrations of actin monomer, but are insufficient to explain the ability of the cell to disassemble actin efficiently in the presence of excess polymerized actin. We identify CAP as a fourth physiologically relevant actin disassembly factor and explore its mechanism. We find that CAP is partially redundant with coronin function but operates through a distinct mechanism.

INTRODUCTION

Cells organize space, generate force, and respond to extracellular conditions through actin-dependent processes that necessitate both fast actin polymerization and fast depolymerization.[93-95] *In vivo* actin turnover is fast[34, 41] with a halflife on the order of tens of seconds,[54, 64] much faster than has been typically reproduced *in vitro*.[96] ADF/Cofilin, and more specifically cofilin,[70] has been shown to be necessary to disassemble physiological branched actin arrays in cell extracts[41, 51] and actin filaments in pure solution[42] but is insufficient to reconstitute physiological actin disassembly both qualitatively in terms of the ability to function in the presence of high concentrations of actin and quantitatively in terms of observed disassembly rate. Physiological

actin disassembly is both fast and robust to high concentrations of monomeric (globular, G-) actin and polymeric (filamentous, F-) actin.

The high concentrations of polymerizable G-actin maintained by the cell (5-20 μM)[60] help drive actin assembly, but this creates a barrier to actin disassembly as free filament ends will tend to grow and not shrink.[96] Recently, coronin and actin interacting protein 1 (AIP1) were identified as factors necessary for actin depolymerization in the presence of physiological concentrations of G-actin.[60] Physiological concentrations of F-actin are also high (100-330 μM),[87] posing a stoichiometric problem in which estimated cellular concentrations of each actin disassembly factor (cofilin, 3-30 μM;[87] coronin, 1.4-40 μM;[60, 97] AIP1, 0.4-0.5 μM[60, 98]) are significantly lower than F-actin concentrations. While coronin and AIP1 are sufficient to relieve the inhibition of cofilin-mediated actin depolymerization in the presence of physiological concentrations of G-actin, the potential problems associated with physiological concentrations of F-actin have not yet been addressed.

Listeria monocytogenese utilizes cellular machinery to move through cytoplasm by assembling a branched actin network or 'comet tail' behind it.[78] Host factors are also required for comet tail disassembly, the kinetics of which are similar both in infected cells[1] and *in vitro* when treated with cell extract.[41, 54] Thus, *Listeria* comet tails offer a physiological actin substrate for the *in vitro* study of cellular mechanisms of actin filament disassembly. Importantly, comet tail assembly can be experimentally separated from disassembly, and we have taken advantage of this in the past to identify several factors necessary for actin comet tail disassembly under the physiological challenge of high actin monomer concentrations. We have now extended the *Listeria* system to identify additional factors necessary for actin disassembly when the reaction is challenged by high concentrations of actin polymer.

MATERIALS AND METHODS

Proteins and Reagents

All reagents unless otherwise noted are from Sigma-Aldrich, St. Louis MO. Rhodamine and Alexa dyes are from Invitrogen, Grand Island NY. Rabbit skeletal muscle actin, recombinant human cofilin, and bovine coronin and AIP1 were purified as described previously.[60] Actin bundling protein α-actinin-4 was recombinantly expressed and filamin was purified from chicken gizzard as described previously.[59] Human Cyclase Associated Protein (CAP) was recombinantly expressed in Rosetta E. coli (EMD) and purified using a Ni-NTA-agarose column (Qiagen). CAP expression was induced with 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 7 hours at room temperature and further purified on a monoQ column (Pharmacia Source Q) at pH 7.8, eluting at approximately 220mM NaCl.

Standard buffers consist of 5mM Tris, 0.2mM $CaCl_2$, 0.2mM ATP (pH 8.0; G Buffer) and 100mM HEPES, 50mM KCl, 1mM $MgCl_2$, 1mM EGTA, 2mM ATP (pH 7.8; Assay Buffer). When used to store proteins, 2mM β-mercaptoethanol or DTT is added. Adjustments to Assay Buffer are noted, and any version of Assay Buffer is converted to Photo Buffer by the addition of 0.2mM 6-hydroxy-2,5,7,8-teramethylchromane-2-carboxylic acid (Trolox), 0.4mg/mL Glucose Oxidase, and 2.25mg/mL glucose. Photo Buffers are used within 2 hours of initial preparation.

Protein Labeling

Cofilin was labeled with maleimide-activated tetramethyl-rhodamine (TMR) by incubation with a 2:1 molar excess of dye for 45min at room temperature in 20mM HEPES pH 7.2, 50mM KCl, 2mM TCEP. Labeling was stopped by addition of 2mM DTT, and unreacted dye was removed by centrifugation for 1 hour at 200,000g (k-factor 33.8) at 4ºC followed by dialysis against 20mM HEPES pH 7.2 Assay Buffer to remove excess dye. Actin was fluorescently labeled as described previously.[61] Briefly, actin was labeled on lysine residues by treating F-actin with N-hydroxysuccinimide-activated fluorophores at approximately stoichiometric ratios for 1 hour at room temperature before stopping the reaction with 50mM Tris Buffer and extensively dialyzing against G-Buffer with 2mM β-mercaptoethanol.

DNA Constructs

Human CAP1 cDNA was obtained from Origene and subcloned into the BamHI and SalI sites in the bacterial expression vector pET30a$^+$ (EMD) for expression in Rosetta cells (EMD) with an N-terminal 6-His tag.

Purification of CAP from bovine thymus

100g of frozen bovine calf thymus was thawed by placing it in 200ml Buffer A (5mM Tris, pH 7.4, 25mM NaCl, 1mM EGTA, 2mM $MgCl_2$, and 14mM β-mercaptoethanol) at room temperature for 30 minutes. All subsequent procedures were performed at 4ºC. The tissue was cut into small pieces and transferred to 200ml of fresh Buffer A pre-equilibrated to 4ºC and homogenized in a Waring-type blender. The homogenate was centrifuged for 30 min at 12,000g, and insoluble material was discarded. Polyethyleneimine was added to the supernatant to a final concentration of 0.05% and stirred for 30 min at 4ºC. The slurry was centrifuged at 12,000g for 30 min, and the pellet was discarded. The supernatant was centrifuged at 150,000g (k factor = 133) for 90 min. The supernatant was mixed with 100ml DE52 (Whatman), stirred for 60 min, and allowed to settle. The liquid was decanted off the beads, and the beads were resuspended in 100ml of Buffer A. The slurry was poured into a column, and the flow through was combined with the decanted solution to generate the DE52 flow through fraction. The disassembly activity flows through the column under these conditions.

1M Pipes pH 6.8 was added to the DE52 flowthrough fraction to a final concentration of 40 mM. The extract was then applied to a 70ml S HP column (GE Healthcare) equilibrated in 10 mM Pipes pH 6.8, 0.1mM DTT (Buffer B). The column was washed with 100ml of 10mM Pipes pH 6.8 and this wash was combined with the S followthrough. The pH 6.8 S flowthrough fraction was dialyzed overnight against 2 litres of 10mM MES pH 6.0, 25mM NaCl, 0.1 mM DTT (Buffer C) and centrifuged for one hour at 100,000g before being applied to a 70ml S HP column equilibrated in the same buffer. The column was eluted with a 700ml gradient to 500mM NaCl in Buffer C. Activity eluted near 180mM NaCl. Active fractions were dialyzed into Buffer B and applied to a 20ml hydroxyapatite column (BioRad) equilibrated in Buffer B. The column was eluted with a 300ml gradient to 300mM sodium phosphate. Activity eluted near 130mM sodium phosphate. Active fractions were combined, concentrated in a centrifugal

concentrating device and applied to a 16/60 Superdex S200 gel filtration column (GE Healthcare) equilibrated in Buffer A with 150mM NaCl. Active fractions eluted at apparent molecular weights between 50 and 80 KDa. Active fractions from gel filtration were applied to a 5 ml Heparin column (GE Healthcare) equilibrated in Buffer A with 150mM NaCl and eluted with a 100ml gradient to 500mM NaCl in Buffer A. Activity eluted near 300mM NaCl. At this point, only two bands remain on the gel and both were excised for identification by mass spectrometry.

F-actin Co-Sedimentation

In each experiment, either actin or CAP was held constant and the other varied; both are run together using SDS PAGE (10%). Actin was polymerized in 10mM HEPES pH 7.4 Assay Buffer such that after CAP and/or buffer were added in appropriate volumes such that 10μM actin and a given concentration of CAP were present; a similar method was used where actin was varied and CAP held constant at 3μM. In either case actin was allowed to polymerize for 1 hour before the addition of CAP, after which 20min was allowed for binding and to re-establish steady state. Of the final 40μL volume, 20μL was taken immediately (uncentrifuged, U) while the remaining 20μL was centrifuged in a Beckman TLA 100 rotor at 350,000 g (*k*-factor 8.1) for 20 min at 4°C, providing a supernatant (S) fraction and after addition of an equal volume of Assay Buffer and resuspension, a pellet (P) fraction. Equal volumes of each sample were then separated using SDS-PAGE. Densitometry of coomassie-stained bands was accomplished using NIH ImageJ software and used to derive a dissociation constant by the method of Wachsstock, Schwarz and Pollard.[99]

***L. monocytogenese* comet tail microscopy**

Listeria actin comet tails were assembled in the presence of HEK 293 cell extract in homemade perfusion chambers as described previously.[60] After rinsing, the chamber was filled with actin disassembly factors under indicated conditions, and a fluorescence timelapse sequence was acquired to obtain the fluorescence intensity decay and to derive an apparent actin k_{off} as described previously.[60] Excess F-actin, if supplied in a given experiment, was unlabeled and pre-polymerized in Assay Buffer at least 45 minutes before use. Actin disassembly factors were added to a mixture containing F-actin at indicated final concentrations, and this mixture was

applied to chambers 2 minutes thereafter. Visualization was achieved with a Zeiss 20x air objective (NA 0.8) on a Zeiss Axio-Imager M1 stand and recorded with a Hamamatsu ORCA-ER CCD camera driven with Zeiss Axiovision 4.7 software.

Cofilin Loading

Utilizing *L. monocytogenese* actin comet tails as a fluorescent actin substrate, we pre-incubated the comet tails with either CAP or coronin for 5 minutes before rinsing twice with 1.5 chamber volumes of Assay Buffer and perfusing in fluorescently labeled cofilin. After rinsing and incubation with zero-length cross-linker EDAC (100nM, 30s), chambers were rinsed twice before being perfused with Photo Buffer and visualized as described above, except with a Zeiss 40x air objective (NA 0.75), taking 5 images from each chamber (center and one from each quadrant). Images were recorded in both the cofilin and actin channels, corrected separately for background, and finally cofilin intensity was normalized to actin intensity in order to control for variance between individual chambers, fields and comet tails.

RESULTS

Cofilin, Coronin and AIP1 are Insufficient to Disassemble Actin in the Presence of Excess F-actin

Utilizing our custom perfusion chamber set-up and widefield fluorescent microscopy for screening extract fractions for actin disassembly activity, we have previously demonstrated that cofilin, coronin and AIP1 are each necessary and together are sufficient to disassemble the actin comet tails assembled by *L. monocytogenese* despite the presence of a physiological excess of G-actin.[60] However, it is also true that high concentrations of F-actin exist *in vivo.*[87] To address the question of whether cofilin, coronin and AIP1 would retain efficacy in the face of a physiological excess of F-actin, we assessed whether this ternary mixture could still disassemble fluorescent actin comet tails in the presence of unlabeled pre-polymerized F-actin. After assembling fluorescent comet tails in a perfusion chamber we perfused a limiting amount of cofilin (2μM) with saturating amounts[60] of coronin (2μM) and AIP1 (0.2μM) into the chamber along with varying concentrations of pre-polymerized F-actin. Though sufficient to disassemble

actin comet tails in the presence of G-actin, the activity of the cofilin, coronin, and AIP1 mixture was inhibited by the presence of F-actin (Figure 2.1A) in a dose-dependent manner (Figure 2.1B). A high-speed supernatant from bovine thymus extract rescued this inhibition (Figure 2.2), implying that an additional actin disassembly factor was present in the extract. This activity can be chromatographically separated from each of the three known factors in the extract, first from coronin and AIP1 by flowing through DE-52 beads and then from cofilin by flowing through an S column at physiological pH (Figure 2.3A), thus indicating that this activity is a fourth actin disassembly factor which restores actin disassembly activity under conditions of physiological F-actin (Figure 2.3B). This activity was identified as Cyclase Associated Protein (CAP; Figure 2.4).

While only one bovine CAP variant has yet been identified, there are two human isoforms. We pursued the human isoform that is more ubiquitously expressed in order to avoid any complications of tissue-specific modifications, also noting that this ubiquitous CAP isoform, CAP1, is the closer match to the identified bovine protein. There is evidence that CAP1 is largely cytosolic and CAP2 localizes chiefly to the nucleus,[100] but this result concedes that cytosolic CAP1 is broadly expressed while the nuclear localizing CAP2 is expressed in a subset of specialized tissues; indeed, CAP isoform distinction in rats appears to control expression levels in specific organs and tissues with CAP1 being the ubiquitous isoform while the two proteins share a high level of identity.[101] Therefore, we reasoned that the basic biochemical properties of each isoform will not vary appreciably and proceeded with recombinant expression of human CAP1 (hereafter 'CAP'). Recombinant human CAP scored as the factor responsible for imparting resistance to excess F-actin-mediated inhibition, as its addition to cofilin, coronin and AIP1 was necessary to allow the disassembly of the fluorescent actin substrate despite the presence of excess unlabeled F-actin (Figure 2.5A). This relief of inhibition was robust to high levels of F-actin (Figure 2.5B).

CAP has already been implicated in actin turnover dynamics and is known to accelerate the release of cofilin from its high affinity interaction with ADP•G-actin for another round of disassembly.[102-105] While CAP-mediated cofilin recycling might help explain how CAP protects actin depolymerization from high concentrations of F-actin, we noticed that CAP increased the rate of actin disassembly even in the absence of challenging F-actin (Figure 2.6). This is not

entirely consistent with our original hypothesis that CAP has activity limited to simply relieving the inhibition wrought by excess F-actin, and may instead indicate that CAP acts more directly to actively destabilize actin filaments.

CAP Partially Substitutes for Coronin Actin Disassembly Activity Through a Distinct Mechanism

We tested whether CAP could substitute for any of the other actin depolymerization factors (cofilin, coronin, or AIP1) to disassemble *Listeria* actin comet tails in the presence of excess F-actin. We found that while both cofilin and AIP1 were still necessary, in the presence of CAP comet tail disassembly was no longer coronin-dependent (Figure 2.7). We previously demonstrated coronin binds F-actin and after pre-incubation with actin comet tails excess coronin can be rinsed away, then upon addition of cofilin and AIP1 actin disassembly is accelerated in a manner similar to when all three proteins are added simultaneously.[60] To determine if CAP can similarly score in a pre-treatment assay, comet tails were assembled in perfusion chambers, incubated with CAP or Assay Buffer, and subsequently treated with cofilin, AIP1 and excess F-actin. Comet tail disassembly was then monitored as described.

We find that CAP scores in such a pre-treatment assay to disassemble actin comet tails in the presence of cofilin and AIP1 despite the presence of excess F-actin (Figure 2.8A). This result is consistent with our hypothesis that CAP is acting at the level of the actin filament and thus directly participating in actin disassembly. In *S. cerevisiae*, CAP has been shown to bind F-actin indirectly through an intermediary protein known as Abp1.[106] To determine if CAP could bind directly to F-actin, we tested whether CAP would co-sediment with F-actin in a defined system. CAP bound directly to F-actin in a sub-stoichiometric fashion as indicated by SDS PAGE stained with coomassie (Figure 2.8B). In further co-sedimentation experiments, we estimated the dissociation constant for CAP with respect to F-actin as approximately 2μM by the method of Wachsstock, Schwarz and Pollard.[99] Thus we find that like coronin, CAP binds directly to F-actin. As coronin enhances cofilin-mediated comet tail disassembly by facilitating cofilin binding to the comet tail,[60] we tested whether CAP shares this mechanistic function with coronin by assessing the ability of CAP to load fluorescently labeled cofilin onto actin comet tails. We found that unlike coronin, CAP does not act to significantly facilitate cofilin loading (Figure

2.9). Thus while CAP and coronin are each capable of binding to comet tails to increase the rate of actin depolymerization, they each act through distinct mechanisms.

At Limiting Cofilin Concentrations, CAP Does Not Accelerate Cofilin-Mediated Actin Dissassembly

We next revisited the fact that CAP is thought to augment actin disassembly by recycling ADP•G-Actin/Cofilin complexes through nucleotide exchange on the actin monomer, thus liberating cofilin for another round of actin disassembly. In order to test this hypothesis with a mechanism-independent read-out, we observed actin disassembly in the presence of increasing cofilin concentrations with or without a fixed concentration of CAP. Thus if the cofilin recycling hypothesis were the source of CAP activity, we would observe that at low concentrations of cofilin actin disassembly activity would be quickly saturated in the absence of CAP – that is, in the absence of cofilin recycling – and CAP would exert its maximal effect under such conditions. Conversely, at high concentrations of cofilin there would be a relative abundance of unreacted cofilin rendering recycling unnecessary, and thus rendering CAP quite unnecessary (Figure 2.10A). What we found, however, was just the opposite: CAP had little effect on cofilin-mediated disassembly at low concentrations of cofilin but had an increasing effect with increasing cofilin at pH 7.4 (Figure 2.10B). Taken together these results not only suggest that CAP acts directly on F-actin, but also that the efficacy of CAP is not explained by cofilin recycling.

DISCUSSION

Fast actin disassembly is necessary for cells to rapidly reorganize their actin cytoskeleton in response to both internal and external cues as well as to maintain a high concentration of actin monomer to drive fast actin assembly reactions. The importance of cofilin as the central and essential component of the actin disassembly machinery of the cell has long been recognized.[39, 41, 67, 70, 92] However, while cofilin is necessary for actin filament disassembly, it is increasingly clear that cofilin alone cannot account for actin disassembly in cells where high concentrations of

both polymerizable G-actin and F-actin, in addition to neutral pH, either favor actin assembly or limit cofilin activity. Understanding how these *in vivo* challenges are overcome in order to allow actin disassembly is essential for understanding how actin dynamics are managed and utilized effectively in cells. Our overall approach to this issue has been to design *in vitro* depolymerization assays that mimic the challenges to depolymerization found in cells and fractionate tissue extracts to identify the factors that restore fast cofilin mediated disassembly.

Originally characterized in S. cerevisiae as Srv2,[107] Cyclase Associated Protein (CAP) takes its name from its role in S. cerevisiae as a factor which co-purifies with adenylyl cyclase (AC)[108] and is a necessary mediator of the RAS signaling pathway;[109] these AC-associated functionalities map to the N-terminal domain of CAP[110] but are not conserved in mammals for lack of CAP binding sites on AC.[111] In human embryonic kidney cells, CAP has been shown to bind both cofilin and AIP1 in an actin-dependent manner, and this activity maps to the N-terminal domain.[112] *S. cerevisiae* CAP has also been shown to exist in a complex with actin but neither cofilin nor AIP1 are part of this complex,[113] yet in yeast CAP still functionally collaborates with cofilin as well as AIP1.[106] CAP interacts with actin through its C-terminal region and the loss of this region has been associated with a range of cell morphological defects in all systems studied (S. cerevisiae,[114] S. pombe,[115] plant,[116] insect,[117] protozoan[118] and mammalian cells[119]).

In mammalian non-muscle cells CAP deficiencies have been shown to mimic cofilin deficiencies,[119] which implies a role for CAP in actin disassembly. This stands in apparent contrast to yeast genetic experiments which found that CAP deficiencies could be largely overcome by overexpressing profilin,[110] a protein whose role in binding ATP•G-actin allows high concentrations of ATP•G-actin to exist at the ready for actin polymerization well above the critical concentration of actin. The rescue of CAP deficient yeast by profilin overexpression implies a role for CAP in actin assembly. This assembly/disassembly discrepancy has been hypothesized to exist because CAP is actually functioning between actin disassembly and the next round of actin assembly, affecting the cofilin/ADP•G-actin product of the actin disassembly reaction to both recycle cofilin to an unbound state and regenerate ATP•G-actin through nucleotide exchange.[106, 112] Given the evidence of a role for CAP in nucleotide exchange on actin, this theory is enticing. However, this would be thermodynamically dependent upon CAP having a much greater affinity for ADP•G-actin than does cofilin, a requirement that stands in

direct contrast to measured values. Mammalian non-muscle cofilin isoforms have affinities for ADP•G-actin of between 20 – 100nM[120] compared with the measured yeast CAP affinity for ADP•G-actin of 20nM.[121] Even allowing for affinity variances among systems, CAP appears to have no appreciable advantage in binding affinity for ADP•G-actin over cofilin. Further, even if CAP could displace cofilin from ADP•G-actin to yield a CAP-ADP•G-actin complex and free cofilin, CAP has a 100-fold stronger affinity for ADP•G-actin than for ATP•G-actin,[121] thus posing a thermodynamic problem that raises the question of how CAP-ADP•G-actin transition to CAP-ATP•G-actin and subsequent release to free ATP•G-actin is possible in the absence of energy input. One possibility is that nucleotide exchange is mediated by CAP, but is accomplished along with actin disassembly in a single mechanism which could derive energy from the depolymerization of actin.

Interestingly, the original work which established the CAP-mediated cofilin-recycling model recognized that human CAP accelerated apparent actin k_{off}, but took the further increase of apparent k_{off} in the presence of both CAP and cofilin along with nucleotide exchange results to mean that the major effect of CAP was to influence cofilin recycling.[112] The CAP-induced actin k_{off} increase, which was found not to be due to monomer sequestration, simply did not find a place in the model.[112] Since this original work in 2002, there have been several papers that demonstrate increased actin turnover in the combined presence of CAP and cofilin, but these measurements were made using various spectroscopic methods that are unable to distinguish between effects on actin assembly, disassembly, or recycling of reaction components and are only interpretable in the context of a pre-existing model.

Conveniently such a model was available after 2002[106, 112, 120] and was generally assumed to be valid, becoming the mold into which all data were poured before publication. Original experiments which painted CAP as an actin monomer sequestration factor suffered from the same assembly/disassembly ambiguity, as they relied upon venerable techniques such as falling ball viscometry[122] and pyrene- or NBD-actin polymerization assays[116, 122, 123], each of which found a reduced F-actin content in the presence of CAP but none of which could truly distinguish between a decrease in actin assembly and an increase in actin disassembly at steady state. Recently others have cited similar reasons for ambiguities in the observed function of other factors involved in actin dynamics, specifically with respect to the role of AIP1.[124, 125] We agree

and have pursued mechanistic questions through experimental methods which have the advantage of separating actin assembly from actin disassembly, and thus are interpretable in a way that can build a model from data rather than find a way to fit data into an existing model.

In trying to determine the mechanism by which CAP affects actin disassembly in a manner which is distinct from yet shares features of coronin activity, we considered several possibilities falling into two main categories. The first is a truffle hunter's view of actin dynamics hypothesizing that CAP has a unique effect on the level of the actin filament and is the focus of the next chapter (Chapter 3). This seemed an ambiguous, at times even unlikely proposition in early experiments and so we also pursued a parachutist's view of actin dynamics hypothesizing that the macromolecular geometry of an actin array dictated the mechanism of actin disassembly. The latter theory is developed in Chapter 4.

Overall, we have demonstrated that while the combination of cofilin, coronin, and AIP1 can rapidly depolymerize actin in the presence of G-actin, the activity of this three protein mixture is easily inhibited by physiologically high concentrations of F-actin in the absence of CAP. We identified CAP as a fourth factor that enhances cofilin-mediated actin depolymerization even in the presence of high concentrations of F-actin. The ability of CAP to recycle cofilin from its high affinity interaction with ADP•G-actin for another round of disassembly may still be relevant, but we have discovered a novel role for CAP in restoring fast actin disassembly under physiological conditions that is ambivalent to and easily reconciled with this recycling activity.

CHAPTER 3: CAP MECHANISTIC ELUCIDATION THROUGH SINGLE FILAMENT STUDIES

ABSTRACT

CAP is an actin severing factor at pH values too acidic to be physiologically relevant. However, at neutral and slightly basic physiological pH CAP augments cofilin function to increase cofilin-mediated severing, and at slightly acidic physiological pH at which cofilin activity is inhibited, CAP functions to rescue cofilin activity. Thus CAP appears to act as an auxiliary actin disassembly factor across the range of physiologically relevant pH values. CAP also appears to change the mechanism of actin disassembly such that in its presence even aged actin filaments stable in the presence of cofilin alone will depolymerize.

INTRODUCTION

Our perfusion chamber experiments allow us to design experiments which temporally segregate cyclical biological reactions, i.e. actin assembly and disassembly, by dictating conditions to favor actin assembly, then rinsing and instituting a different set of conditions to permit actin disassembly. It is exactly this experimental separation of actin assembly and disassembly that has allowed us to identify rather surprising functionalities of human CAP. After identifying that CAP was partially redundant with coronin in the presence of cofilin and AIP1 (Figure3A), we began to test CAP for functionalities associated with coronin such as the ability to bind F-actin and to increase cofilin loading.[60] In yeast, CAP has been shown to bind F-actin only through an intermediary protein,[106] but we find that human CAP has no such external reliance and remains functional while bound to F-actin, able to score in a pre-treat experiment in *L. monocytogenese* comet tails. Yet we were surprised to find that CAP does not facilitate cofilin loading, especially since human CAP has been shown to form a ternary complex with actin, cofilin, and AIP1 *in vivo.*[112]

At this point several salient features of human CAP were apparent to us, each of which raised certain questions about the currently accepted model of CAP function. If CAP is acting to recycle cofilin and thus acts only after actin disassembly has already occurred, what function does F-actin binding serve? Others have cited the fact that S. cerevisiae CAP binds F-actin through ABP1 as a problem when articulating the cofilin recycling model, as this necessitates invoking the hypothesized CAP-profilin "hand-off" of ATP-G-actin to complete the thermodynamic actin cycle and regenerate polymerizable ATP-G-actin.[106] We see no dependence on ABP1 or any other factor to mediate CAP binding to F-actin, nor do we see any experimental need to invoke profilin. Further, the most straight-forward hypothesis for how CAP might augment cofilin function through F-actin binding – CAP-induced cofilin loading – turns out to be false. Thus we knew that CAP must be having an effect on or at the actin filament, and in order to sort out what mechanism of action CAP might be employing we turned to single actin filaments.

If CAP were facilitating increased severing, thus increasing the number of ends from which to lose actin mass, this would be readily apparent in our direct wide-field microscopy experiments. Furthermore, differences in k_{off} rates would be apparent as well. Thus we would readily be able to visualize Severing through an increased severing rate, Treadmilling through the observation of standard k_{off} rates, Enhanced Treadmilling through the observation of enhanced or greater than standard k_{off} rates, or any other mechanism for that matter given that we are able to directly visualize the filament.

MATERIALS AND METHODS

Single actin filament microscopy

Actin filaments were assembled in perfusion chambers as described previously.[99] Briefly, actin-bundling protein filamin is adsorbed onto the glass surface before blocking the remaining glass with 10mM HEPES pH 7.2 Assay Buffer containing 10mg/mL bovine casein, 0.2% Tween 20 and 0.05% F-127 pluronic acid. A 4µM solution of Alexa-647 G-actin (20% labeled) in either 50mM Imidazole Assay Buffer at the indicated pH or 50mM MES Assay Buffer at pH 6,

is allowed to polymerize in the chamber before rinsing with Photo Buffer. Actin disassembly factors are then perfused into the chamber in Photo Buffer and actin filament disassembly is observed using a Zeiss 63x oil objective (NA 1.4) and the microscopy equipment and software described above.

To image actin polymerization off of new filament ends generated through severing reactions, disassembly is allowed to occur as described for 90s before rinsing the chambers with buffer and reperfusing with G-actin labeled with a different fluorophore. Photo Buffer is then perfused into the chamber for imaging. Filaments which had severed during the disassembly step are compared to the dual color image to identify whether new F-actin polymerization happened after a severing event; not all filaments sever and not all filaments nucleate growth in the times allotted.

To test whether CAP-mediated or CAP/cofilin-mediated severing reactions depend upon phosphate release from F-actin and thus respect the nucleotide state of F-actin, 20mM phosphate was added to Assay Buffer from stock phosphate buffers, then adjusted to the final pH value indicated (6 or 7.4).

In order to age actin filaments, perfusion chambers are left at ambient temperature in a closed humidified vessel after polymerization and rinsing, then rinsed again before depolymerization is attempted. Disassembly step proceeds as described in a manner identical to standard experiments.

Microscopy data analysis

Original movies are acquired and converted to '.tif' stacks using Zeiss Axiovision 4.7 software. In the case of *L. monocytogenese* actin comet tails, data analysis is accomplished through measuring background-corrected intensity decay over time and either displaying this normalized decay directly or using a single exponential model to plot an apparent actin k_{off} from normalized data as described previously.[60] To determine the severing rate from imaging of single filaments, we measured the total length of actin polymer in a field using a custom routine in Matlab (R2011b; The Mathworks, Inc.). Severing rates are thus reported as the number of events per

second over the time course of the experiment normalized to the starting total length of actin in micrometers.

Pyrene Fluorescence Experiments

To determine if severed actin filaments produced barbed ends capable of seeding actin polymerization, a solution of 10μM unlabeled F-actin was mixed with an equal volume of Assay Buffer containing 6μM CAP and 4μM cofilin at pH 7.4, or 6μM CAP at pH 6. After incubating at room temperature for 5 minutes, this solution was diluted 1:20 into a solution of G-actin labeled with pyrene (25% label) and Assay Buffer at pH 7.4 with or without 300nM cytochalasin D. Pyrene fluorescence was monitored in a Spectromax M2 plate reader.

RESULTS

CAP Augments Cofilin-mediated Severing

To gain more mechanistic insight into how CAP enhances cofilin-mediated actin disassembly, we used wide field fluorescence microscopy to image disassembly of single filaments as a function of CAP. Actin filaments were assembled in perfusion chambers coated with the actin bundling protein filamin, after which the assembly solution was replaced with a disassembly solution containing 2μM cofilin and increasing concentrations of CAP. We found that CAP increased the frequency of cofilin-mediated actin severing events approximately 7-fold relative to cofilin alone (Figure 3.1).

CAP Remains Active in a Pre-Treatment Severing Assay

Under these conditions at pH 7.4, CAP did not have any significant activity on its own (see below, Figure 5). This allowed us to test whether CAP would bind single actin filaments and whether its presence would significantly affect cofilin activity. In agreement with our *Listeria* comet tail assays, CAP scored in such a pre-treatment experiment in the context of single actin filaments, in which we pre-treated actin filaments with CAP and after rinsing we supplied

cofilin, resulting in quantitatively similar severing activity to that seen when adding both CAP and cofilin simultaneously (Figure 3.2). Therefore, CAP binds directly to F-actin to accelerate cofilin-mediated actin severing.

CAP- and CAP/Cofilin-Severed F-actin Nucleate Growth

AIP1 is another auxiliary factor capable of enhancing cofilin activity, and while it is thought to occlude the barbed end of cofilin-decorated filaments to prevent reannealing of severed daughter filaments,[126] it may also have additional roles in actin disassembly.[98, 125, 127] We can conclude that CAP, however, is not augmenting cofilin-mediated severing through filament end capping and blocking of reannealing because filament ends created by severing events in the presence of CAP can extend new actin polymer (Figure 3.3A). In addition, bulk pyrene-actin experiments demonstrate that the products of disassembly reactions generated by the combination of cofilin and CAP reduce the lag phase normally associated with actin polymerization, again demonstrating that the ends of the filaments are free and can seed actin assembly (Figure 3.3B). From these results we conclude that CAP acts directly on F-actin to accelerate cofilin-mediated actin filament severing.

CAP Severs F-actin at Acidic pH

Twinfilin, like CAP, was originally identified as a G-actin sequestration factor[128] but is now known to also sever F-actin at acidic pH.[129] We therefore imaged single filaments in the presence of CAP alone at varying pH and found that at acidic pH values, CAP is sufficient to sever actin filaments (Figure 3.4A). CAP severing activity at pH 6 is comparable to rates observed in the presence of CAP/cofilin basic physiological pH (Figure 3.4B). Further, actin severing reactions driven by CAP alone at pH 6 produce filament ends that can seed actin assembly reactions demonstrating that CAP does not occlude filament ends (Figure 3.5).

CAP is a pH-Sensitive Actin Disassembly Factor

Knowing that CAP is capable of severing actin filaments at acidic pH but not capable of such activity at basic pH, we titrated CAP function at increasing pH in order to probe this relationship more deeply. In contrast to cofilin, CAP activity decreases as pH increases and we found that at neutral pH CAP had lost essentially all actin disassembly activity (Figure 3.6A). Such pH-

related changes in activity are reminiscent of cofilin activity except that CAP appears to be an acidic-functioning actin disassembly factor in contrast to the basic-functioning cofilin, but both appear to be relieved of their activity at neutral pH. Because cofilin appears to rescue CAP function at basic pH, we tested whether the combination of CAP/cofilin could function at this mutual point of inactivity and found that at neutral pH CAP and cofilin rescue actin disassembly (Figure 3.6B).

CAP and Cofilin Collaborate to Yield pH-Independent Actin Disassembly

Cofilin function is highly sensitive to pH, and at neutral or acidic pH cofilin loses all severing activity although it can still bind actin.[130, 131] Cytosolic pH in many cell types is near neutral or even slightly acidic.[132] Cofilin severed actin filaments at pH 7.2 and greater, but the severing activity of cofilin alone approached background severing rates at pH 7.0 (Figure 3.7), which is well within the range of physiologically relevant intracellular pH values.[57] The addition of 3μM CAP, however, rescued cofilin-mediated actin severing activity at neutral and acidic pH values; likewise, the addition of cofilin rescued CAP-mediated severing activity at neutral and basic pH values. Extending this result to the full range of relevant pH values, we found that CAP and cofilin rescue and accelerate actin disassembly activity across the whole physiological range of cytosolic pH from 6.8 to 7.4 (Figure 3.7). CAP therefore accelerates actin severing reactions in the presence of cofilin yielding a constant, accelerated severing rate that is independent of pH.

CAP-Mediated and CAP/Cofilin-Mediated F-actin Severing Respects Nucleotide State

Binding of pure cofilin to F-actin and cofilin-mediated actin filament severing is nucleotide dependent,[90, 103, 105] and inorganic phosphate must be released from F-actin following ATP hydrolysis before cofilin can bind. We therefore tested whether CAP-dependent actin severing reactions were also dependent upon the release of inorganic phosphate from F-actin by challenging severing reactions with the inclusion of inorganic phosphate in the reaction buffer at every step of the experiment. We find that both CAP-mediated severing at acidic pH (Figure 3.8A) and CAP/cofilin-mediated severing at basic pH (Figure 3.8B) remain dependent upon phosphate release.

Modeling Physiological Actin Disassembly

At this point it clear that CAP does more than the Moriyama-Yahara model would lead us to believe. CAP is augmenting cofilin activity, but is doing so by interacting with the actin filament. This interaction is not in the context of capping filament ends as partially disassembled actin filaments are capable of seeding actin assembly. Additionally, CAP activity is both pH and nucleotide-state dependent. Even further, CAP is known to have activity as a nucleotide exchange factor on ADP•G-actin despite having a 100-fold higher affinity for ADP•G-actin than for ATP•G-actin.[121] Given the physical interaction between F-actin and CAP, it is possible that a portion of the energy derived from ATP hydrolysis on each actin monomer is available to CAP for use in one or more of its several activities. It is already thought that actin utilizes some of the energy of ATP hydrolysis to destabilize the actin filament; given the role of CAP as an actin disassembly factor, we posit that CAP could realize enough energy to accomplish the liberation of cofilin from and nucleotide exchange on ADP•G-actin as part of the disassembly mechanism (Figure 3.9).

Combination of CAP and Cofilin Disassemble "Old" F-actin

It is notable that we have found a curious capability of CAP and cofilin: aged F-actin, typically impervious to attempts at depolymerization, is readily disassembled in the presence of cofilin and CAP (Figure 3.10). This is surprising as actin disassembly is thought to be dependent on the energy derived from the hydrolysis of ATP and subsequent release of phosphate, which have half-lives of 2s and 6min, respectively.[52] Thus, this energy should no longer be available after incubation over a significant time period, consistent with a stable filament no longer capable of cofilin-mediated disassembly. However, in the presence of CAP and cofilin these aged actin filaments have sufficient energy stored in the F-actin structure to accommodate disassembly.

DISSCUSSION

We find that CAP acts as an auxiliary actin disassembly factor across the range of physiologically relevant pH values, and as an autonomous actin severing factor at acidic pH. CAP also appears to change the mechanism of actin disassembly such that in its presence even

aged actin filaments, stable in the presence of cofilin alone, will depolymerize. Interestingly, we also found that CAP in combination with cofilin was also sufficient to overcome pH-mediated barriers to actin depolymerization across the entire physiological range.

While a number of auxiliary factors act directly on the actin filament to augment cofilin mediated actin depolymerization, their associated mechanisms are distinct. Coronin for, example, enhances cofilin mediated actin disassembly by facilitating cofilin binding to F-actin[60] and possibly by acting to selectively disassemble filaments that have released inorganic phosphate.[133] AIP1 on the other hand accelerates cofilin-mediated actin filament severing by capping barbed ends of cofilin-severed filaments to block the back reaction of filament reannealing.[126, 134, 135] In addition to its role in barbed end capping, evidence also suggests that AIP1 acts more directly on the filament, further weakening F-actin to enhance cofilin-mediated severing.[125, 127, 136] We find that CAP apparently acts in a unique fashion, neither increasing cofilin loading like coronin has been shown to do nor acting to bar filament re-annealing after severing as AIP1 is proposed to do. CAP may be acting to increase cofilin function by binding to and further destabilizing actin filaments, producing a substrate more susceptible to cofilin-mediated severing. Alternatively CAP may be producing a more effective cofilin as a consequence of a CAP-cofilin interaction. The N-terminal domain of CAP has been shown to directly interact with cofilin in all systems studied,[106, 112] possibly affecting cofilin activity in such a way. However, the C-terminal actin-binding domain of CAP is sufficient to rescue morphological changes observed in CAP deficient systems,[121] which combined with our data showing that CAP is capable of directly severing actin filaments at acidic pH leads us to favor the first model: CAP is acting on the filament to make it more susceptible to cofilin action.

Cofilin-mediated actin depolymerization is pH dependent and cofilin mediated actin severing itself titrates with a pKa of 7.4.[44] Consistent with these results, we find that cofilin-mediated actin severing is undetectable at pH 7.0. Cytosolic pH varies by tissue and cell type but is typically kept within a narrow range, usually between 6.8 and 7.4 with most cells tending towards neutral or slightly acidic pH rather than pH 7.4 which is typical of extracellular space.[132, 137, 138] Therefore, cofilin-mediated actin disassembly inside cells requires either alkalinization of cytosol or the use of auxiliary depolymerization factors to overcome pH-dependent inhibition of cofilin function. Certain signaling molecules such as PDGF transiently elevate cytosolic pH and

rapid actin reorganization might be coupled to controlled changes in pH.[139] In addition, the chronically elevated cytosolic pH of cancer cells helps drive faster actin turnover dynamics to increase their metastatic potential.[70] Finally, it is increasingly evident that systems responsible for controlling cytosolic pH are coupled to the actin depolymerization machinery.[56, 140] Thus the regulation of cytosolic pH may be an important determinant of actin turnover dynamics. However, fast actin disassembly is still required in normal cells, both motile and non-motile, where cytosolic pH is often near neutral. In these cases, all cofilin-mediated actin disassembly reactions most likely require auxiliary factors such as CAP, a point supported by *in vivo* evidence that CAP insufficiencies phenocopy cofilin insufficiencies.[119] Our data demonstrating that the combination of CAP and cofilin sever actin filaments at neutral pH provide at least one mechanism for driving fast actin disassembly without having to alter cytosolic pH and incur the potentially grave consequences for many cellular reactions beyond those affecting actin.

Our identification of CAP as an actin filament severing protein should help inform interpretation of earlier results with respect to how CAP contributes to actin-dependent processes in both physiological and pathophysiological settings. For example, the observation that CAP locally controls the amount of actin at apical cell-cell adhesive junctions[117] while cofilin controls total F-actin levels throughout Drosophila epithelial cells[141] is easier to reconcile with CAP-enhanced actin filament severing than with its ability to recycle cofilin. In addition there is evidence that CAP overexpression strongly correlates with invasiveness in at least one highly metastatic cancer type. In aggressive pancreatic cancers, CAP overexpression was recognized in 100% of clinical cases studied and correlated with invasive behavior and poor prognosis.[5] Our identification of CAP as an actin depolymerizing factor provides a mechanism for understanding how CAP can locally control the stability of specific actin arrays in distinct regions of the cell and how elevated CAP activity might also contribute to the accelerated actin turnover dynamics that appear to characterize metastatic cells through the cofilin pathway.

CHAPTER 4: EVL-DEPENDENT CAP FUNCTIONALITY: THE ANTAGONISM OF CAP AND EVL

ABSTRACT

Before realizing that the efficacy of CAP is pH-dependent when in the absence of cofilin, we were puzzled by the fact that CAP was efficacious in the context of listeria actin comet tails but not in the context of single actin filaments. We reasoned that the geometrical differences between the two actin arrays may account for the difference in CAP activity. We find that CAP suppresses the ability of EVL to form listeria-dependent actin clouds and comet tails, and in reconstituted biochemical bulk actin assays CAP dose-dependently suppresses EVL-induced acceleration of actin polymerization; these experiments were done in the presence of cellular factor Arp2/3 and *Listeria monocytogenese* protein ActA,. This could be due either to a direct interference with actin assembly or an increased disassembly rate, thus we turned to our single-filament studies to experimentally separate actin assembly from disassembly.

INTRODUCTION

The fact that CAP proved efficacious to disassemble actin comet tails but not in the context of single actin filaments led us to consider two alternative hypotheses, the first of which was that listeria actin comet tails assembled in the presence of cellular extracts may retain some actin binding protein or proteins that influence the disassembly reaction in an undetermined manner, simply due to binding tenaciously enough so as not to be rinsed out during the post-assembly wash step. We assumed that sufficient rinsing would mitigate the potential effects of tenacious binders as this approach had been sufficient for the original purification of coronin and AIP1,[60] but in light of the difference between CAP efficacy in disassembling actin comet tails built in complex extract versus actin filaments assembled in pure solution we recognized that CAP inhibition was a possibility and wanted to move to a system in pure solution.

The second hypothesis involved the geometrical differences between branched actin networks and straight actin arrays built by processive elongators like formins[142, 143] and Ena/VASP-like protein (EVL).[144, 145] It has already been postulated that CAP facilitates actin disassembly indirectly through nucleotide exchange or cofilin liberation from ADP-G-actin; to discover that CAP debranched or otherwise 'primed' actin arrays for cofilin-mediated disassembly would be one possible way to reconcile others' data indicating an auxiliary role for CAP with our data indicating that CAP is an active participant in actin disassembly.

Although our parallel experiments described in Chapter 3 would prove that CAP does act on the actin filament to influence dynamics in both comet tails and single actin filaments, initial debranching experiments demonstrated an antagonistic relationship between CAP and EVL suggesting an indirect effect through branching. We realized that this interaction was potentially of considerable importance, both because of the substantial literature regarding EVL participation in *L. monocytogenese* motility[146, 147] and the substantial literature suggesting such an interaction between CAP and EVL in drosophila oocytes[148] and germline cyst follicle cells[117] but lacking mechanistic information. EVL has previously been shown to add actin processively at the barbed end creating a linear, unbranched actin filament[144] in contrast with an Arp2/3-mediated branched actin network as is present in *L. monocytogenese* actin comet tails. This is also true of the formins,[143] but EVL was chosen because of its apparent *in vivo* antagonism with CAP.[82, 117] The biochemical mechanism of this interaction was completely unknown, and we felt our work could make a contribution to the understanding of the CAP-EVL relationship, which appears to be so important for axon pathfinding,[82, 117] dendritic attachment,[117] and cell polarity.[148] Thus even if actin geometry turned out not to be an issue of utmost importance in CAP function, at least we could contribute to the understanding of CAP function in those fields of study listed.

MATERIALS AND METHODS

Proteins and Reagents

Rabbit skeletal muscle actin, recombinant human cofilin, and bovine coronin and AIP1 were purified as described previously.[51] Actin bundling proteins α-actinin-4 was recombinantly

expressed and filamin was purified from chicken gizzard as described previously.[59] Human Ena/Vasp-Like protein (EVL) and human Cyclase Associated Protein (CAP) were recombinantly expressed in Rosetta E. coli (EMD) and purified using a Ni-NTA-agarose column (Quiagen). CAP expression was induced with 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 7 hours at room temperature while EVL was induced at 0.3mM IPTG for 6 hours at 37°C and further purified by subsequent binding to a monodispersed Q column (Pharmacia Source Q) at pH 7.8, eluting at approximately 220mM NaCl. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDAC) and Tris(2-carboxyethyl)phosphine (TCEP) from Sigma-Aldrich, St. Louis MO. All labeling moieties, including rhodamine and Alexa dyes, are from Initrogen, Grand Island NY.

Standard buffers consist of 5mM Tris, 0.2mM CaCl2, 0.2mM ATP (pH 8.0; G Buffer) and 100mM HEPES, 50mM KCl, 1mM MgCl2, 1mM EGTA, 2mM ATP (pH 7.8; Assay Buffer). When used to store proteins, 2mM β-mercaptoethanol or DTT is added. Adjustments to Assay Buffer are noted, and any version of Assay Buffer is converted to Photo Buffer by the addition of 0.2mM 6-hydroxy-2,5,7,8-teramethylchromane-2-carboxylic acid (Trolox), 0.4mg/mL Glucose Oxidase (Aspergillus niger, Sigma Aldrich), and 2.25mg/mL glucose. Photo Buffers are used within 2 hours of initial preparation. Tris, HEPES, MES, and imidazole buffers as well as standard laboratory salts are from Sigma-Aldrich, St. Louis MO.

Actin Cloud Experiments

After allowing listeria to adhere within a perfusion chamber as described previously, subsequent blocking is accomplished as described except with the addition of 10mg/mL casein. EVL is then allowed to bind to listeria surface for 5min. After washout, labeled G-actin is added with Arp2/3 in the presence or absence of CAP. Actin clouds represent filamentous actin growing from the bacterial surface in the absence of symmetry breaking, and their number are dependent upon the density of listeria in a given field; this variation is minimized by using a single dilution of stock listeria for all experiments and remaining variation is dealt with by taking a large number of fields. Each field is normalized to global highest intensity field; 5 fields per chamber, 3chambers per condition. EVL 1uM, Actin 2uM, Arp2/3 300nM, CAP 3uM.

Debranching Experiments

Briefly, using the method of Weaver and Cooper et al,[149] we allowed dark actin to polymerize in the presence or absence of EVL and/or CAP before diluting into buffer containing fluorescently labeled phalloidin and applied slowly to perfusion chambers prepared as described for single filament assays. Reaction concentrations are EVL 3uM, CAP 3uM, actin 2uM, Arp2/3 100nM, ActA 300nM. Phalloidin is applied equimolar to actin after first dilution, 40nM. Final dilution was 500x, and solution was applied to perfusion chamber. Branched filaments were then visualized using widefield microscopy 3 minutes after application to allow binding filamin-coated coverglass. At least 3 chambers were used per experimental condition and 5 fields were manually counted for branchpoints from each chamber, one from the center and one from each quadrant. The number of branches were also normalized to total linear amount of F-actin in a given field.

Pyrene-actin Experiments

Experimental procedure as described in Chapter 3. When CAP is added after actin assembly, either CAP or buffer is added to bring all components to their final concentrations. Thus, no kinetic data from the polymerization phase of these experiments are used in actin polymerization analysis. When EVL is added after actin polymerization plateau, it is added simultaneously with CAP in place of some of the assay buffer used to dilute CAP stock, maintaining all concentrations of other proteins during each phase of the experiment. Actin 2uM, EVL 300nM, Arp2/3 100nM, ActA 300nM. Unless otherwise specified, CAP 2uM.

Single-Filament Off-rate

Measured through kymograph analysis as described previously.[60] Briefly, kymographs of individual filaments are obtained from experiment movies, and each one is manually analyzed for rate of disassembly while actively depolymerizing. Units are pixels per second, then converted to subunits per second but retain a certain quantum character due to the pixel-dependent analysis that lends itself well to histogram analysis. Rates are then binned using a custom MATLAB routine, and mean, median and mode are obtained. Cofilin 2uM, CAP 10uM.

RESULTS

CAP Decreases EVL-induced Actin Cloud Intensity

We set out to challenge known functions of EVL with CAP in order to find what specific mechanistic quality or qualities of EVL are antagonized. We began by probing EVL-mediated actin cloud formation,[150] in which purified Arp2/3 is supplemented with fluorescently labeled actin and EVL. Under these conditions actin 'clouds' are formed around listeria expressing the Arp2/3 activator ActA on the bacterial surface. EVL is necessary for this symmetrical cloud formation (Figure 4.1). In the absence of CAP, EVL facilitates a robust actin cloud but does not allow symmetry breaking in the absence of an actin bundling protein. With the addition of CAP, however, the EVL-mediated actin cloud is diminished to approximately half its intensity, indicating that CAP is antagonizing EVL function in the context of actin cloud formation (Figure 4.1). This result is clear in terms of the antagonistic biochemical relationship between CAP and EVL, but equivocal in terms of the underlying mechanism of action; it is possible that CAP is antagonizing EVL-dependent actin polymerization, or alternatively that polymerization is unencumbered while CAP efficiently disassembles newly-formed actin structures.

CAP Decreases Actin Branches in the Presence of EVL

We next probed another known EVL-dependent process: actin network debranching. Utilizing the method of Weaver et al,[149] we probed the ability of CAP to antagonize the increased branching effects of EVL. What we found was that while the presence of EVL substantially increased the number of actin branch points, the presence of CAP eliminated this increase in arborization (Figure 4.2). In the presence of CAP there were also far fewer total filaments, thus branching data are normalized to total actin filament length. Again, this result demonstrates the antagonistic relationship between CAP and EVL while equivocating on the point of polymerization effects versus depolymerization effects. That is, our data are consistent with either a CAP-mediated depression of EVL-induced actin branching or a CAP-mediated actin debranching effect occurring sequentially after EVL-induced actin branching. Thus we turned away from endpoint assays and focused our attention on real-time spectroscopic assays of CAP and EVL antagonism.

CAP Dose-Dependently Suppresses EVL Enhancement of Actin Polymerization

Utilizing pyrene-labeled actin polymerization assays in which monomeric actin is added to a defined mixture containing activated Arp2/3 and EVL in the presence or absence of CAP. Relative to the rate of actin polymerization in the presence of EVL alone, as CAP is titrated into the reaction mixture the rate of EVL-accelerated actin polymerization drops such that more CAP dose-dependently slows EVL-mediated actin polymerization (Figure 4.3). However, we recognized that as CAP has been shown to bind G-actin it could be argued that this decrease is not a direct antagonistic effect with respect to EVL but instead is a simple sequestration effect. To address this possibility we chose a concentration of CAP and compared its efficaciousness with respect to actin polymerization in the presence and absence of EVL.

CAP Decreases Actin Assembly Rates in an EVL-Dependent Manner

As expected the presence of EVL caused a 2 to 3-fold increase in actin polymerization rate over and above the increase attributable to the presence of activated Arp2/3 while the addition of CAP to the same mixture had no significant effect. Importantly, when CAP is added to the EVL-containing mixture, but not a similar mixture excluding EVL, CAP markedly decreases the effects of EVL and attenuates EVL-mediated acceleration of actin assembly by at least 25% (Figure 4.4A). However, this apparent actin k_{on} is a conglomerate estimate of actin nucleation and elongation from nucleated foci; a decrease in nucleation will provide fewer elongating filaments thus reducing the total observed polymerization rate (apparent k_{on}), making this difficult to distinguish from a true reduction in actin elongation rate. While this experiment cannot definitively distinguish between attenuated nucleation versus attenuated elongation, we can infer the effects of CAP on nucleation by considering the lag time – the time from experiment initiation to actin polymerization. Actin alone has a long lag time (i.e. slow nucleation), but this lag time diminishes slightly in the presence of activated Arp2/3 and significantly in the presence of activated Arp2/3 and EVL (Figure 4.4B). The addition of CAP does not significantly alter either the lag time achieved in the presence of activated Arp2/3 or the diminished lag time in the presence of EVL. Thus, it is unlikely that the effects of CAP with respect to EVL are due to alterations of actin nucleation. However, we must still differentiate

between a decreased rate of EVL-mediated actin polymerization versus an increased rate of actin depolymerization.

CAP Increases Actin Disassembly Rates in an EVL-Dependent Manner

In order to determine if CAP had any effect upon the rate of disassembly of EVL-built actin, we adjusted these experiments to include a final disassembly step after assembly had reached steady state. When buffer alone is added, a small amount of disassembly occurs due to dilution effects whether EVL was present at the time of actin polymerization or not; there is no discernible EVL-mediated assembly effect on actin disassembly. However, the addition of CAP induces a reproducible increase in actin disassembly that is significantly amplified (from 2 to 3-fold to approximately 8-fold) when the population of actin filaments are assembled in the presence of EVL (Figure 4.5). This difference can be attributed to the effects of EVL during actin assembly and not to any active disassembly on the part of EVL because if we assemble actin in the absence of EVL and supply an equal concentration along with CAP during the disassembly phase, there is no increase in the rate of disassembly (Figure 4.5).

These results appear to indicate two important points. One is that in the absence of cofilin the maximum effectiveness of CAP is EVL-dependent. The other, perhaps more important point is that it is apparently not the EVL itself that is responsible for this difference but rather it is the nature of the actin filament which it produces. It is possible that the mechanism by which CAP disassembles actin is more directly applicable to EVL-assembled actin filaments, or perhaps that some structural difference between these filaments make them more susceptible to CAP-mediated disassembly. It is also possible that EVL-mediated actin filaments engender a different, faster mechanism of actin disassembly. With this possibility in mind we turned our attention back to single-filament experiments which lend themselves to direct observation and mechanism-independent interpretations.

CAP Accelerates Evl-Built Single Actin Filament Disassembly

In order to divorce actin disassembly from actin assembly, we returned to our perfusion chambers. The original reason that we had diverted to bulk population-based spectroscopic experiments was because we saw no CAP activity with EVL-built filaments, but we have since

learned that CAP is more efficacious at lower pH values than the pH 7.4 used in those original experiments (see Chapter 3). By reducing the pH to 7.0, we retained both CAP activity and physiological relevance. When EVL-built actin filaments are washed into control buffer, they are remarkably stable; however, when washed into CAP these same EVL-built filaments readily disassemble (Figure 4.6A). When quantified, CAP appears to reproducibly increase the apparent k_{off} of actin approximately 2-fold (Figure 4.6B).

DISCUSSION

Given the curious result that CAP appeared to be efficacious when disassembling actin arrays of a branched geometry but not of an unbranched geometry we recognized the need to study this behavior as cells produce both types of actin arrays and such a bifurcation of functionality may be of great physiological relevance. We thus set up experiments to test CAP involvement in EVL-mediated actin arrays before our results in previous chapters demonstrated that pH and cofilin presence were more likely the factors which gave differential CAP activity. Nevertheless, we pursued the issue of CAP/EVL antagonism because of its apparent importance in drosophila oocytes[148] and germline cyst follicle cells.[117] CAP has even been implicated in axon pathfinding[82] but all of this work was lacking information regarding biochemical mechanism. We utilized techniques which allowed us to look very closely at CAP/EVL antagonism in both branched[146, 147] and unbranched actin arrays that were built in the presence of EVL.[144] We realized that this interaction was potentially of considerable importance, both because of the substantial literature regarding EVL participation in *L. monocytogenese* motility[146, 147] and the afore mentioned substantial literature suggesting such an interaction in drosophila but lacking mechanistic information.

The importance of CAP in the establishment of cell polarity was first appreciated in drosophila oocytes and confirmed in the budding yeast *S. cerevisiae*. The authors of this initial study found that a CAP knockout produced oocytes that bore ectopic filamentous actin accumulation, shifting from posterior to anterior during oogenesis with mature oocytes displaying ectopic filamentous actin at the apical cortex.[148] Turning to the follicle cells lining each developing drosophila germline cyst, the same group noticed that CAP still localized to the apical region of the cell and

that the absence of CAP resulted in the accumulation of filamentous actin at the apical junctions of this cuboidal epithelium.[117] Hypothesizing that CAP opposes a juctional factor that is nucleating actin assembly, the authors pursued ena, the dropsophila homologue of EVL and a known regulator of epithelial F-actin, and found that ena/EVL localizes to the follicular adherens-like junction and functions to nucleate actin assembly at these junctions. It was at these sites that CAP normally localized to, and at these sites where F-actin accumulated in the absence of CAP; additionally, the absence of both CAP and EVL resulted in no excess F-actin accumulation.[117]

The antagonism between CAP and EVL is anything but trivial. While it had been known that CAP binds Ableson (Abl) protein tyrosine kinase[117, 151] and that Abl and EVL collaborate to provide a signal important for axon pathfinding in drosophila intersegmental motor nerves,[75, 152] what was not known when Baum and Perrimon were publishing their results was that CAP is also involved in this system. CAP is expressed ubiquitously during early embryonic stages, but largely restricted to the nervous system during later stages of drosophila development and has been postulated to serve as a counter-signal to EVL, with EVL causing actin assembly and growth cone advance while CAP contributes to growth cone collapse.[82] The authors of this paper cite the ability of the C-terminal region of CAP to bind G-actin[116, 151] and correctly cite the influence of the N-terminal region of CAP and its ability to bind to cofilin and influence cofilin-mediated actin disassembly,[112] but did not yet realize the significance of their finding that a full rescue of CAP activity after knockout requires full-length CAP.[82] It has been shown in *S. cerevisiae* that the N-terminal domain regulates access to the relatively distant SH3-binding site of CAP (responsible for Abl binding) independently of adenylyl cyclase-related binding or function;[111] the latter is an important distinction because CAP does not bind adenylyl cyclase in higher eukaryotes.[153] Additionally, in human cells this same N-terminal domain of CAP was found to form an actin-dependent complex with two known actin disassembly factors, cofilin and AIP1,[112] and our own data indicate that CAP participates actively in cofilin-mediated actin disassembly. All of this implies that the relevant activity of CAP cannot be explained by suppression of actin assembly alone, but must invoke an actin disassembly activity that appears directly antagonistic to EVL.

Contemporaneously with but separate from this work in drosophila epithelium and neurons, a dichotomy was emerging around the function of EVL with respect to cell motility in mammalian cells. After discovering the drosophila homologue of EVL[154] and establishing its role as a regulator of cell migration,[75] the Gertler group attempted to build upon *Listeria monocytogenese* motility data which showed that EVL had a role in driving Arp2/3-dependent mobility[146] by demonstrating the role of EVL in mammalian cells.[155] EVL family proteins had previously been shown to localize at focal adhesions,[156] cellular leading edges,[75, 157] and at the distal tips of filopodia in neuronal growth cones,[158] thus implying a strong positive relationship with cellular motility. Yet when overexpressed in a rat fibroblast cell line, EVL family proteins crippled cellular polarity and dose-dependently slowed cellular mobility rates.[47] It was soon postulated that this difference arose from an unbalancing of a delicate cellular system, i.e. if EVL family proteins acted to provide the protrusive side of a cycle which utilizes successive protrusion and retraction in order to generate the force necessary for translocation,[77] then disrupting the balance of protrusion and retraction would cause translocation to lose efficiency and cellular speed would be expected to decrease. Unfortunately for the field, actin disassembly was not well understood at this time, resulting in the assignment of barbed-end capping as the process that yields retraction by halting actin assembly,[77, 159] a misconception which has garnered increasing skepticism over the years but remains the accepted view apparently for want of a more plausible and demonstrable mechanism.[144, 160] It is of great concern to a range of fields how EVL and CAP participate in cell motility, and the mechanistic elucidation of actin dynamics with respect to these factors would be an important contribution.

CHAPTER 5: CONCLUSIONS

Some of the most fundamental questions facing science are often cursed by the attention they receive, being thought to be of such pristine truth that to question is to curtail reason and to believe in the existing model is virtually religion. Galileo had to fight for the fact that "[Earth] does move," long before Kepler and Newton described how, and even longer before we might understand the basis by which gravitational force is transmitted over spacetime. Yet in such seemingly linear progression, in each generation there was a feeling of confidence that gravity, and thus planetary motion, was understood on the most fundamental level even as humankind was progressing from understanding gravity on Earth to planetary motion and elliptical planetary motion. When a new technique was developed, or a question was asked in a proper manner divorcing the answer from the method of asking, the debate inevitably re-ignited.

Actin was discovered in muscle cells long before its true importance and dynamism were recognized. It was thought to be entirely understood as early as 1985, only to be rediscovered again and again as new technologies allowed a deeper view of actin dynamics and its effects on cellular structure, organization, and movement. The lingering model, though built upon an early understanding which was undermined even before its own inception, would influence all future thinking until the advent of the model-independent assay of Brieher, Kueh, and Mitchison. We have now expounded upon this work through a reliance on mechanism-independent experiments such that we may describe what we see, rather than interpret data through the lens of a model such that the data agree with what we think we ought to see.

Even as Wang was publishing his famous photobleaching paper in 1985, appearing to visualize Wegner's proposed treadmilling model, Brenner and Korn were defending their contradictory findings. Yet even with scientists, it seems, a time-lapse series of pictures is worth a thousand equations. And still, every time someone put pencil to paper to resolve the biochemistry of actin with Wang's photobleaching and Wegner's treadmilling, Brenner and Korn were upheld even if unmentioned. Theriot and Mitchison described the movement of *Listeria monocytogenese* and goldfish keratocytes in the early 1990s as being consistent with first-order kinetics, which seemed inconsistent with either of the two competing models of actin disassembly: treadmilling and severing. It would take more than another decade and a substantial increase in computing

power for Kueh, Brieher and Mitchison to essentially rule out the possibility of severing through mathematical analysis.[54]

In the present work we first tried to deal with CAP within the commonly held framework, arguing within the confines of severing as an idea while raising questions about the applicability of severing as an adequate modality by which one could adequately explain actin disassembly. We then argue for the dismissal of severing as a major factor in actin dynamics, preferring instead to think of severing as a cute experimental artifact that likely does little if anything to influence *in vivo* actin dynamics even if it has been a convenient measure of certain factors' ability to affect the twist of filamentous actin. CAP appears to be influencing actin filament stability in more than one way, both at filament ends and along the length of the filament.

We have not only provided evidence that CAP accelerates actin k_{off}, but also that CAP sufficiently changes the nature of the filament to increase fragmentation. Both observations are likely related to the fact that CAP confers upon cofilin not only a pH insensitivity but also the ability to disassemble otherwise inert, aged actin filaments despite the fact that ATP has been hydrolyzed and inorganic phosphate long since released. CAP may be allowing the utilization of some energy reserved in the structure of the actin filament, perhaps hinting at another as yet unidentified role for actin in cellular homeostasis – a possible arbiter of stored energy. In any case the utilization of energy derived from ATP hydrolysis and phosphate release on filamentous actin is likely the basis of the ability of CAP to affect nucleotide exchange on actin monomers and recycle cofilin bound to ADP•G-actin.

The importance of Figure 4.5 also cannot be overlooked. Here we not only demonstrate that the effect of CAP is accentuated when actin filaments are built in the presence of EVL, but that the efficaciousness of CAP is determined during actin *assembly* and not during actin *disassembly*. EVL is capable of binding actin filament sides in order to bundle filaments, but such activity would be inconsistent with filament destabilization and further EVL was kept at concentrations low enough that we did not observe bundling in single-filament assays. Of great importance was the sequential experimental condition demonstrating that EVL-mediated augmentation of CAP depolymerization activity is seen only when EVL is present during actin assembly; EVL is inconsequential when added after actin assembly. Therefore it is apparently

not the EVL itself that is responsible for this difference in CAP activity but rather it is the nature of the actin filament array which EVL produces. This may indicate a role for hysteresis in actin dynamics, a point likely to be examined in detail by Markov-oriented biophysicists.

When considering actin dynamics, it is of paramount importance that one never lose sight of the fact that it is a cycle one is considering. Despite the observations of Theriot and Mitchison that it was the rate of actin disassembly that limited the speed by which keratocytes travel,[34] the field still found it perplexing that EVL could increase actin polymerization while decreasing cell motility.[161] Still more recently, the ability of cortactin to increase the rate of Arp2/3 activator dissociation while increasing the rate of actin network growth was found to be surprising.[162] In each instance Theriot's point that the rate of regeneration of polymerizable G-actin is of utmost importance would have been very useful to keep in mind. This is analogous to *rigor mortis* in muscle cell contraction: if the cyclic system cannot be reset and its components replenished it quickly ceases to function. Likewise, when the field considers the issue of actin dynamics solved and moves to take an exclusively signaling-based approach, a similar type of paralysis is experienced.

APPENDIX A: MATHEMATICAL MODELING OF CAP-MEDIATED ACTIN DISASSEMBLY MECHANISM

INTRODUCTION

Multiple human cell types must generate force, organize space, and respond to environmental conditions – often concurrently – through actin-dependent processes that necessitate both fast actin polymerization and fast depolymerization,[90, 93, 101] with little spatial separation between assembly and disassembly.[63, 131] In addition actin turnover *in vivo*[57, 133] is much faster than has been typically reproduced *in vitro.*[129] Several factors have been shown either to increase rates of actin disassembly[57, 60, 92, 163] or to protect actin disassembly against physiologically relevant challenges,[60, 163] or both. Recently, we identified a previously unappreciated actin disassembly factor, cyclase associated protein (CAP), a dual-domain factor which has been reported to interact with several actin binding proteins and that we have found enhances the rate of ADF/cofilin-mediated severing.[163]

While there is wide agreement that the actin severing protein cofilin is important for physiological actin disassembly, the mechanism of actin disassembly remains a source of contention.[42, 49, 54, 61, 87] As we have linked CAP to cofilin-mediated severing of actin filaments and have shown that CAP itself has severing capacity,[163] we now attempt to localize these activities to one of the two domains of CAP and track the loss of actin mass along with the actin severing rate in order to both demonstrate the mechanistic relationship between filament severing and actin disassembly as well as to localize aspects of this activity to one or both domains of CAP.

MATERIALS AND METHODS

MATLAB Analysis

Custom routines based upon our previous data and the work of Savitzky-Golay.[164] Severing events are normalized to initial actin length, as is standard in the field, as severing events are

expected to vary with available severing sites. Thus severing rates are expressed in events per initial actin length per unit time. Single filament experiments and initial analysis done as described, data is then analyzed by custom routines in MATLAB.

RESULTS

Previously, we identified two potential models of CAP-facilitated actin disassembly.[163] As our evidence indicated that CAP requires cofilin to achieve full function across the range of physiological pH values, we studied CAP domains in the context of cofilin at a standard physiological pH (7.4). It is known that the N-terminal domain of CAP directly interacts with cofilin in all systems studied,[106, 112] but that the C-terminal domain of CAP is responsible for binding actin.[165] However, it is not yet known by what mechanism pH modulates CAP activity. It is possible that this is a direct effect of pH on CAP or that pH affects cofilin or the actin filament to block CAP binding and/or function. To study these interactions, we expressed CAP N-terminal and C-terminal domains and tested their ability to sever actin filaments at physiological pH.

The severing activity of CAP varies with both pH and cofilin concentration, thus we settled on a single physiological pH of 7.4 at which to conduct experiments in the presence of a fixed concentration of 2uM cofilin, achieving consistency without sacrificing physiological relevancy. Under these conditions both the N-terminal and C-terminal domains have sub-maximal severing activity.

Observed Severing Rates Inadequate to Explain Observed Actin K_{off}

Importantly, the rates of severing activity relative to controls, as measured by visible severing events per time, do not keep pace with the relative increases in apparent actin k_{off}. Indeed, even the correlation between reduced actin mass as an apparent consequence of increases in actin severing breaks down, most notably in the case of C-terminal CAP which in the presence of cofilin increases apparent actin k_{off} while decreasing the apparent rate of severing (Figure A.1).

This result raises potentially important questions about the reliability of severing rates as a valid measure of actin mass lost from a population, as the severing rate is supposed to reflect the loss of actin mass from a population of actin filaments. Specifically, the loss of total actin filament length should be equivalent to the total rate of subunit loss from either end of the filament ($k_{+off} + k_{-off}$) multiplied by the number of filaments (N)[87]:

$$L_{lost} = N \times \left(k_{off}^{+} + k_{off}^{-}\right) \times t$$

Equation 1

We reasoned that the discrepancy between observed severing rates and actin disassembly could be partially explained by the fact that actin filaments can reanneal.[166] In this case severing events may be occurring but not productively so in terms of analysis, as it is possible they may reanneal before losing any mass form either end. In our analysis, at least one pixel (approximately 37 actin monomers) must be lost from an actin filament in order to be visually perceptible and scored as a severing event. The possibility that CAP does not accelerate actin severing at all but instead decreases the incidence of re-annealing events and thus increases the measurable severing rate has been considered, but as this would almost certainly entail filament end capping we have already demonstrated a strong case to the contrary.[163] The alternative explanation that the effect of CAP encompasses both pro-severing and anti-reannealing components is still a possibility, and so we sought a measurement method independent of pre-determined mechanism, such that any combination of known or unknown mechanistic properties could be assessed in an unbiased manner.

Modeling Acceleration of Actin Disassembly through Severing Events

The Savitzky-Golay (SG) filter[164] has been used widely both to smooth and to differentiate data without increasing noise to levels which render the acquired data uninterpretable. We observed that our data followed a binomial actin loss of mass curve better than a linear regression and no worse than higher-order polynomial fits, thus we used binomial fits of our data as a convenient method to more accurately assess the actual actin severing rate. We would like to stress that we are not stating that a binomial is the best fit for our data, but that it is good

enough to be used as a convenient method to allow a more refined data interpretation technique. Because mathematicians in the field have already reported that severing-based models such as this could not be discretely differentiated,[54] we feel justified in using an empirical binomial fit that is far more amiable to differentiation than a model attempting to account for all anticipated factors contributing to actin disassembly:

$$L_T = Ax^2 + Bx + C$$

Equation 2

Through this binomial model of total length (L_T) resolved over time, we utilized the SG method to estimate the first and second derivatives of actin mass lost per time in our experiments, the first derivative representing the apparent K_{off} of the population of actin filaments:

$$K_{off}^{App} = (L_T)' = A'x + B'$$

Equation 3

The second derivative represents the acceleration of apparent actin K_{off}, which would, based on the filament severing model, result from increasing more ends from which to lose actin mass via increased severing:

$$S^{App} = (L_T)'' = C'$$

Equation 4

We reasoned that this approach would yield a somewhat higher apparent severing rate than our visually acquired data because it would account for all disassembly mechanisms as well as missed severing events such as those which occur but are not scored due to filament reannealing.

In addition this analysis allows us to assess severing rates without having to account for such mathematical complications as shorter filaments disappearing from the field at relatively early time points, crossed filaments which can cloud certain automated measurements, and possible lag time issues which may or may not be physiological but would all have to be accounted for in a mathematical model.

Severing Rates Orders of Magnitude Greater than Observed are Necessary to Explain Data

When we appropriately normalized data to obtain equivalent units (see Methods), we observed that S-G derived severing rates matched trends seen in gross loss of actin mass (Figure A.2A) but reported apparent severing rates several orders of magnitude greater than those measured manually (Figure A.2B). That is to say that the required rate of severing to obtain actin disassembly at the rates we have observed, given the published actin dissociation constants and concept of severing accelerating apparent actin K_{off} by supplying more ends from which to shrink, is mathematically required to be several orders of magnitude greater than those severing rates we have observed using standard techniques for counting such severing events. This evidence would appear to call not only the severing rate into question but also the acceptability of severing as a plausible explanation for the increase in actin disassembly observed in the presence of CAP and cofilin, as the trends of actin disassembly with various CAP moieties agrees with the SG filter-derived severing rates and not the manually counted rates, particularly in the case of C-CAP/cofilin. Taken together our results imply that the required rate of severing is many times more than what has been observed using traditional techniques.

CAP Increases Pointed and Barbed End k_{off}

As we had been studying actin filaments at the population level but wanted to directly observe disassembly mechanism, we focused our attention back on single actin filaments. *Equation 1* states that in order for the rate of actin mass lost from a filament to increase, either the number of filament ends or the K_{off} must increase at one or both ends; we looked to see if the dissociation constants at one or both filament ends appeared to be changing, thus allowing a change in K_{off} despite a stable number of filaments. When studying a population of filaments one cannot tell these possibilities apart, but by observing filaments directly we were able to make this distinction. We observed that the K_{off} at both pointed and barbed ends of actin filaments is

elevated in the presence of CAP versus cofilin alone, with the pointed end k_{off} tripled and the barbed end raised more modestly (Figure A.3).

Calculating the actin k_{off} that would be necessary to explain the observed rates of disassembly in the absence of severing, we find that published rates for pointed end disassembly in the absence of severing or pointed end disassembly account completely for cofilin-mediated actin disassembly rates but when CAP or any combination of CAP domains and cofilin are added the actin k_{off} must increase to at least 20 subunits per second (Figure A.4). Intriguingly, this is precisely the mean total actin k_{off} with CAP plus cofilin that we found analyzing single filaments. This raises the possibility that severing may not even be necessary to account for fast actin dynamics at all, though 20 subunits per second is barely within the range found to be necessary and it is likely that other factors may still be needed. We feel those factors are very likely to be coronin and AIP1.

Severing At Calculated Rates Requires More Severing Sites Than Those That Exist

In order to better determine the nature of actin disassembly, we extended the S-G rate analysis in order to answer the question of feasibility: are there enough severing sites to allow severing to accelerate actin disassembly to the required rates? At 3.7 subunits per 10nm[50] and thus 370 subunits per um, given the dual protofilament structure of actin there will be 184 severing sites per um of F-actin. We calculated how long it would take to saturate all available sites in each of at least 3 experiments in which SG severing rate has been calculated from the observed loss of actin mass and original starting total length of F-actin recorded. We found that even when we over-estimated the number of severing sites available by not accounting for mass lost with time, therefore assuming the existence of more severing sites than were really there at all time points after initial, severing sites are saturated remarkably fast (Figure A.5). Cofiiln alone is the lone condition in which severing sites would not be saturated before completion of the experiment, but in any combination of CAP or its domains plus cofilin all severing sites would be exhausted long before the actin is observed to have completely disassembled. If the acceleration of actin disassembly is indeed a manifestation of actin severing, then a rate of severing which could achieve such acceleration should be compatible with the observed amount of actin mass remaining. We find that this is not the case.

DISCUSSION

Disagreement over the mechanism by which cofilin increases actin disassembly has been ongoing virtually since the protein was recognized for its role in actin dynamics. When cofilin was recognized as a protein which accelerates actin disassembly, the field had gained an understanding of actin as a steady-state polar polymer which gained and lost mass at each end commiserate with the appropriate kinetic constants and logically tried to fit cofilin into this framework. To accelerate the loss of actin mass from a population of actin filaments, as experiments were done with actin populations either in sprectroscopic wells[42] or microscopic perfusion chambers in the context of *L. monocytogenese* actin comet tails,[41] either cofilin had to increase the off rate of actin - thus enhancing Wagner's proposed treadmilling[167] - or if the off rate from each filament end remained the same then cofilin had to be able to create more ends by severing.[41, 87] There was also a concurrent search for factors which could enhance the population off rate to fill in the gap between rates of actin disassembly obtained with purified factors versus those observed with cell extract.[42, 60, 61, 163]

One such factor recognized relatively early was actin interacting protein 1 (AIP1), which was found to enhance the rate of cofilin-mediated actin disassembly and thus thought to increase the rate of cofilin-mediated actin severing;[127, 135] however, this mechanistic understanding was the result of a model-dependent interpretation of AIP1 effects on cofilin-induced actin dynamics and has since been called into question.[125, 126] This same protein along with another actin binding protein, coronin, whose role in actin disassembly had been ambiguous[81, 133] have since been shown to factor greatly in physiological actin disassembly. AIP1 and coronin, specifically coronin-1a, have been shown to accelerate cofilin-mediated actin disassembly and protect disassembly from otherwise inhibitory physiological conditions.[60] Further, in a study meant to distinguish between enhanced treadmiling and severing by visualizing individual actin filaments disassembling, AIP1 and coronin were found to accelerate cofilin-mediated actin disassembly through a previously unappreciated mechanism alternatively termed Bursting or Whole Filament Disassembly (WFD).[61] Most recently we and others have discovered that CAP contributes to actin disassembly[106, 163, 168] and may be an important auxiliary actin severing protein in the context of cellular pH regulation.[163] This and further studies of cofilin mechanism and function[169] argue that severing may not be the only disassembly mechanism worth consideration,

but also that actin filament severing deserves consideration in mechanistic studies of physiological actin disassembly.

However, increasingly physiological studies that employ greater mathematical precision appear to argue against the relevancy of actin filament severing. Perhaps the most comprehensive treatment of the mechanistic issue was recently published by Kueh et al, a mathematical modeling effort which utilizes fluorescent *L. monocytogenese* actin comet tails to compare models to one another and to live-cell imaging. This technique appears to render severing-based models unlikely under *in vivo* conditions.[54] Our experiments have attempted to confirm that observed increases in actin severing rates could explain the concurrent increase in the loss of actin mass over time in a population of filaments, but have instead suggested the opposite.

Through individual filament analysis in a mechanism-independent manner, we have in fact shown that observed severing rates are far too modest to explain the amount of actin mass lost per unit time. Further we sought to determine whether the rates of actin mass lost per time could be explained in the absence of severing. What we found was that the K_{off} achieved in the presence of cofilin with either full length CAP or any combination of its domains is sufficient to explain the observed off rate without invoking an increase in filament ends derived form an increased rate of severing.

While our live image series data also demonstrate non-zero severing rates and thus do not rule out the existence of actin filament severing, we demonstrate that severing is insufficient to itself account for and unnecessary to explain observed actin disassembly rates. Severing may still be of some importance *in vivo*, although recent data has argued that actin filament bursting, which is equivalent to whole filament destabilization assuming a mean filament length no larger than the mean burst size and is essentially a consistently increased actin K_{off} when averaged over time, can effectively explain physiological actin disassembly while offering a possible explanation of how actin disassembly is achieved without destroying the ability of the cell to maintain tensile strength.[54] Our own data will need to be repeated in order to decrease uncertainty by increasing *N*, and has thus been designated an Appendix chapter pending this refinement. We therefore cannot conclude whether severing may or not be irrelevant to physiological actin disassembly, but our data raise the possibility.

While the full picture of physiological actin disassembly will involve more factors – likely coronin and AIP1 – we can now distinguish between several possible models of cofilin-CAP interaction with the actin filament. We have shown that CAP interacts directly with the actin filament to increase barbed (+) and pointed (-) end K_{off} in the presence of cofilin. Interestingly, maximal CAP activity appears to be attained when the individual C- and N-terminal domains are expressed separately with no linker region and then mixed, indicating the likely element of CAP auto-inhibition, a recurrent theme in actin dynamics.[170] Because CAP regains activity at low pH, we reason that low pH also relieves this inhibition but may have other effects upon CAP activity.[163] However, because our data have pointed toward a direct CAP effect on the actin filament, and because we have found that under certain pH conditions CAP acts to disassemble actin in the absence of cofilin, there may be multiple interactions between CAP and actin, and with cofilin for that matter, which are sure to be complicated but must be elucidated in order to better understand how cells achieve and maintain spatiotemporal organization.

APPENDIX B: sNASP IS A NOVEL NUCLEAR ACTIN DISASSEMBLY FACTOR: IDENTIFICATION AND INITIAL CHARACTERIZATION

ABSTRACT

Somatic nuclear auto-antigenic sperm protein (sNASP) is a histone-binding protein with an alloimmunogenicity from which it draws its name. We identified sNASP due to its activity as an actin disassembly factor, which we discovered while trying to purify another disassembly factor with a functionally similar activity from bovine thymus. We have begun to characterize this protein using techniques already successfully employed in the study of other actin disassembly factors, and plan to use our knowledge of sNASP to elucidate the function of actin in the nucleus.

INTRODUCTION

The role of actin in the nucleus is debated, but that debate has seen an important shift in the past decade or so. In the 1970s and 1980s actin was recognized in the nucleus but was thought to be a curious interloper or a "thermodynamic wanderer" of possible, at best, consequence.[171] Since the turn of the millennium actin has been recognized as a presence, then an imported and exported entity.[171, 172] Actin has even revealed itself as the first developmentally regulated nuclear import/export substrate.[173] Part of the turn in opinion centered about the new-found roles of actin binding proteins such as Arp and WASp, and actin is now thought to function in both filamentous and globular forms.[174] We did not seek out sNASP as an actin disassembly factor, but instead found sNASP while purifying another actin disassembly factor with evidently redundant activity. As such we sought to understand sNASP from a biochemical perspective before applying this knowledge to cellular systems.

MATERIALS AND METHODS

Protein Purification and Identification

sNASP was originally identified while purifying the related protein AIP1, and the majority of its purification has been described previously.[60] Briefly, approximately 200g of bovine thymus was homongenized in a Waring blender in the presence of protease inhibitors and β-mercaptoethanol (β-ME) and centrifuged in a Sorvall SLA-3000 rotor at 7Krpm for 30min. All centrifugation and chromatography steps are performed at 4ºC. Supernatant was then centrifuged in a 45Ti Beckman preparative ultracentrifuge at 38Krpm for 90min (k factor = 186). Supernatant was applied to 50mL bed volume of DE-52 beads for 90min. Flow-through was then dialyzed against Buffer B (20mM phosphate buffer at pH 7.0, with 14.2mM β-ME) and applied to a 70 mL S column (Pharmacia). Flow-through contained both AIP1 and sNASP, and was subsequently brought gradually to 1.75M ammonium sulphate. Precipitate was cleared using a Beckman 45Ti ultracentrifuge at 38Krpm for 30min (k factor = 186) and supernatant was applied to a phenyl column which eluted a single, wide peak of activity which was pooled and dialyzed against 20mM Tris pH 8.0 (with 14.2mM β-ME). This was then applied to a Source Q column (Pharmacia; monodispersed) where two separate activities were eluted: AIP1 at its expected 150mM NaCl elution and an unknown activity at approximately 300mM NaCl which was further purified by size exclusion chromatography (S200) and determined by mass spectroscopy to be sNASP. Recombinant human sNASP was later used to confirm that this protein was responsible for the observed activity. Activity assay used is as described in Chapter 2, where fractions must substitute for AIP1 activity in the presence of cofilin at 2uM and coronin at 1uM; coronin in only supplied after the 70mL S flow-through step as this is where AIP1 (and sNASP) bifurcate from coronin.

Recombinant purification

Human somatic nuclear autoantigenic sperm protein (sNASP) was recombinantly expressed in Rosetta E. coli (EMD) using a pET30a vector and purified using a Ni-NTA-agarose column (Quiagen). sNASP expression was induced with 0.2mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 37ºC and further purified by subsequent binding to a nickel column (Qiagen), eluting at 65mM imidazole.

Polyclonal Antibody Raising

One milligram of recombinant human sNASP was purified, dialyzed against a 20mM phosphate buffer at pH 7.4 with no trace of potassium and concentrated to 1mg/mL for injection into a rabbit donor. Actual injections and serum draws were outsourced to University of Illinois Antibody Center and done as per protocol. Pre-immune serum and first serum draw are presented in Figure B.3.

RESULTS

sNASP-Mediated Actin Disassembly

After confirming that sNASP was indeed the protein responsible for the actin disassembly activity observed in bovine thymus extract, we expressed recombinant sNASP and confirmed that this protein does possess an AIP1-like activity with respect to actin disassembly. This is a novel role for the protein, which previously has been shown to bind histones and owes its name to its immunogenicity which is the cause of a small percentage of male infertility cases.[175] Through recombinant expression we both confirmed that sNASP was responsible for this unexpected actin disassembly activity as well as generated enough pure sNASP to raise a polyclonal antibody (Figure B.3). We plan to use this antibody in future pull-down assays and to inject into *Xenopus* oocytes to study giant nuclei stability.

Actin Filament Binding

As AIP1 binds barbed ends of actin filaments in the presence of cofilin,[124] we suspect that sNASP does the same. This cannot be detected in a typical sedimentation assay because of the stoichiometric problems associated with filament end- versus side-binding proteins, but by using a western blot approach we would be able to increase signal. Alternatively, we could add either AIP1 or sNASP in the presence of cofilin to sheared actin filaments, thus increasing the ratio of filament ends to filament sides, but this may create nonphysiological ends and perturb the system even more than the sedimentation assay itself is already perturbing equilibrium.

Another possibility would be to utilize fluorescently labeled sNASP and test binding to limulus arrays – parallel bundles of actin filaments formed from limulus acrosomal bundles – or single actin filaments.[61] In any case, we will test whether there is any effect on binding of sNASP versus AIP1 in the presence of coronin or CAP; if this is observed, we could then test whether this is a direct or indirect effect by utilizing nucleotide state analogs (ATP-, ADP•Pi- or ADP-actin filaments). If CAP or coronin is affecting the nucleotide state, and this is the basis of any effect on sNASP binding, then using the proper nucleotide state analog actin filament should make the presence of CAP or coronin superfluous.

Effects of Histones on Kinetics and Binding

As sNASP is a known histone-binding protein, we will determine whether histones modulate the depolymerization activity of sNASP under any physiological conditions identified above where sNASP successfully substitutes for AIP1. While sNASP has been shown to bind H1 and H3/H4 histones,[176] for our purposes a complex mixture of histones will suffice. Complex histone mixtures are readily FPLC-purified using widely published procedures.[1] Despite the ample literature describing the specific interactions between sNASP and histones,[175-178] histones have an unusually high pI and may bind non-specifically to proteins with a low pI such as sNASP and AIP1; therefore, an important control for non-specific binding with consequences on disassembly activity will be to verify that AIP1- mediated actin disassembly is not affected by the presence of histones.

Mechanism of Actin Disassembly Mediated by sNASP

Using the same single filament approach we used rather successfully for CAP, we will study the model-independent disassembly mechanism employed by sNASP under each set of conditions in which we find that sNASP replaces AIP1. If histone presence does not preclude sNASP disassembly activity, we will test to see whether histone binding alters disassembly mechanism. This would potentially illuminate the link between actin disassembly, or more broadly the actin-mediated arbitration of energy, and nuclear transcription / translation.

Sub-Cellular Specificity of Function

AIP1 is a cytosolic protein while sNASP is predominantly nuclear[175] and has histone-binding activity.[175-178] We plan to test whether sNASP functions as a nuclear AIP1 through knockdowns and cross-rescue experiments. The purpose of sNASP would appear to be to enable actin disassembly in an environment where actin monomer exists in excess, as is the case in the nucleus.[179] We will test whether AIP1 targeted to the nucleus can rescue an sNASP knockdown by subcloning AIP1 with an sNASP nuclear localization sequence (AIP1-NLS) attached. While we expect to see an accumulation of filamentous actin in the nucleus upon sNASP knockdown, AIP1-NLS should rescue to restore the nucleus to a wild-type state as viewed through fix-and-stain experiments with labeled, actin filament-binding phalloidin. As we would not expect proper expression patterns in a cell with sNASP already knocked down, sNASP perturbation must either be last in tandem treatments or it may be necessary to first make a stable AIP1-NLS cell line for sNASP knockdown.

Similarly, if sNASP did not have an NLS sequence, it would be targeted to the cytoplasm. It will be interesting to see whether sNASP lacking its NLS sequence can rescue an AIP1 knockdown phenotype. Scoring such a phenotype will be more challenging than scoring an sNASP nuclear phenotype with fix-and-stain procedures. However, as AIP1 knockdown should cause abnormalities in filamentous actin disassembly rates, we can use a photoactivation technique to study effects upon actin disassembly *in vivo*. By expressing mRFP-PAGFPactin, which is a constitutively red-labeled actin made green after photoactivation,[61] we can monitor disassembly rate and verify that kinetics proceed as single exponential decay. Thus we can score an AIP1 knockdown as a cell displaying abnormal actin depolymerization kinetics and test whether sNASP without an NLS sequence can rescue this state. A live listeria infection may also work here, but as noted above serious defects in actin dynamics may preclude listeria entry.

Xenopus giant nucleus fragility assay

Xenopus nuclear extract was found to also substitute for AIP1 in pilot experiments meant to find evidence of sNASP presence in such extracts before an antibody was raised (*Xenopus* oocyte extract courtesy of Dr. Michele Bellini Laboratory, data not shown). This data encourages us that an sNASP phenotype may reveal itself in such an oocyte extract, or that

experiments with intact oocytes may be equally revealing. It has been shown that exportin 6, known to export profilin-actin complexes,[172] when ectopically expressed leaves *Xenopus* giant oocyte nuclei in a fragile state; this is thought to be a direct result of decreased intranuclear F-actin.[173] Should we develop these observations into a microinjection assay, we would expect that sNASP injected into an intact oocyte would destabilize the nucleus, but that the microinjection of an sNASP antibody would have the opposite effect.

DISCUSSION

sNASP is predominantly nuclear, but we discovered it in an activity-based reconstitution assay while purifying AIP1 from bovine thymus from an extract designed to be principally cytoplasmic. A biphasic activity was differentially purified, one activity peak yielding AIP1 and the other yielding sNASP (Figure B.1). As sNASP revealed itself through its activity, our approach was to first characterize this activity. We knew from the very fact that sNASP was returned from our purification assay that sNASP is redundant with AIP1 under conditions of excess actin monomer when in the presence of both cofilin and coronin, and with later experiments we conformed that CAP substitutes for coronin and also allows sNASP activity in the presence of cofilin (Figure B.2). We have not yet tested whether sNASP can replace AIP1 under conditions of excess actin polymer, nor have we tested whether our polyclonal antibody raised against human sNASP will specifically recognize Xenopus sNASP. These gaps in fundamental understanding and the lack of cellular work are the reasons why sNASP is presented as an Appendix chapter.

Further, expressing a typical purification table is difficult as throughout the first several centrifugation and chromatographic steps sNASP co-purifies with AIP1, which is problematic for specific activity calculation because the two proteins share an activity in our actin disassembly assay. Indeed, it is this shared activity coupled with a differential elution from phenyl and monodispersed quaternary amine columns that allowed the identification and purification of sNASP, but our specific activity table (Table B.1) is somewhat atypical as a result.

It was previously thought that chromatin itself might provide the structural integrity that the nucleus is dependent upon, but it seems this is not the case.[173, 174] That actin has a structural role in the nucleus is likely not the whole story, as actin and its related proteins are also thought to be an active participant in transcription and other nuclear activities.[174] It is exciting that by using a biochemical reconstitution approach to the complex problem of physiological actin disassembly we have potentially opened the door to not only understanding aspects of nuclear function, but perhaps the first of many examples of how actin function is utilized *in vivo* in a diverse array of contexts that may have little to do with cellular motility.

FIGURES AND TABLES

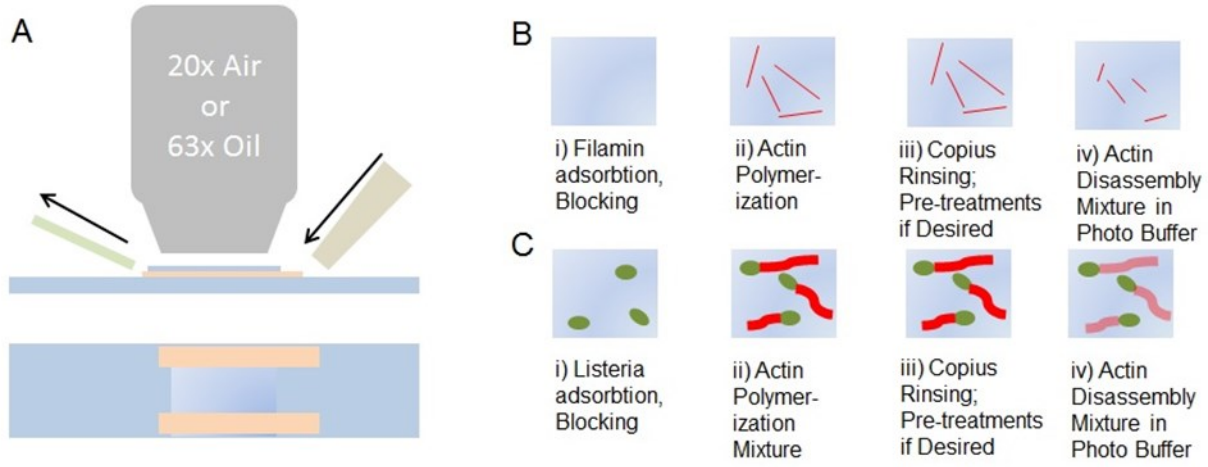


Figure 1.1: Perfusion Chamber Construction and Use in 'Comet Tail' and Single Actin Filament Assays. A) Perfusion chamber set up utilizes glass slides and coverslips separated by two strips of parafilm to form a 10-15uL channel. After heating to 65ºC, coverslip sides are gently pressed to seal and lanolin ridge is applied at the entry side. Applied buffers are 1.5-2 times the volume of the chamber and typically applied in duplicate or triplicate as described. Filter paper is used on the exit side to wick away buffer exiting the chamber as new buffer is applied by pipette on the entrance side. B) In the case of single actin filaments, filamin is allowed to absorb to the glass followed by blocking and subsequent application of G-actin diluted into polymerization buffer. C) In the case of actin comet tails, chemically killed listeria are allowed to absorb to the glass surface before blocking and application of a polymerization mixture which includes actin. B&C) After copious rinsing, disassembly mixtures are then added thus achieving the separation of actin assembly and disassembly in either experimental modality.

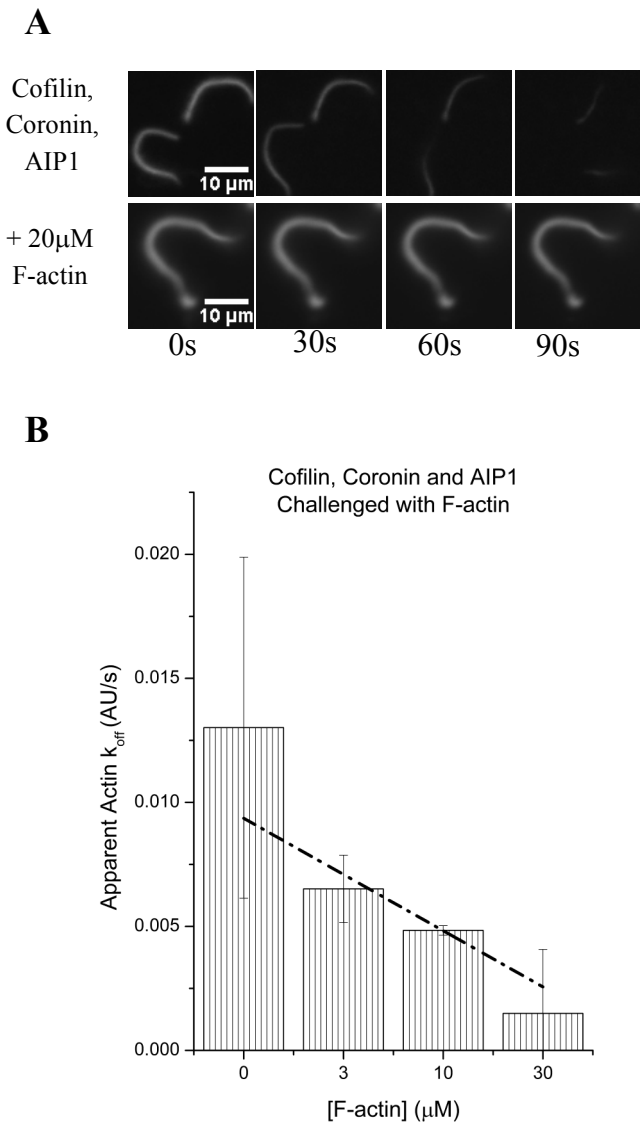


Figure 2.1: Physiological Concentrations of F-actin Inhibit Disassembly of an F-actin Substrate. A) Cofilin, coronin and AIP1 readily disassemble a fluorescently labeled actin substrate (*L. monocytogenese* comet tails) on the required cellular time scale of tens of seconds (top series), but is inhibited when challenged with a physiological excess of F-actin (bottom series). B) The inhibitory action of excess F-actin is dose-dependent, with apparent k_{off} of actin from labeled comet tails decreasing with increased F-actin challenge (intensity normalized and expressed as AU/s). Cofilin 2uM, Coronin 2uM, AIP1 0.2uM; bar graphs display mean +/- SD.

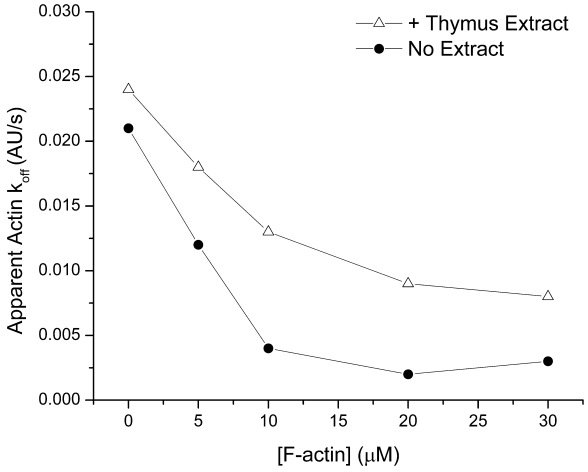


Figure 2.2: An Unknown Factor Contained in a High-Speed Supernatant of Bovine Thymus Extract Relieves F-actin Mediated Inhibition: Actin disassembly activity of this same mixture is markedly sensitive to increasing concentrations of F-actin, nearing complete inhibition by 10uM F-actin (circles). The addition of a high-speed supernatant derived from thymus extract, however, protected against this inhibition (triangles). Experiments were done in the presence of cofilin (2uM), coronin (2uM) and AIP1 (0.2uM), with pre-polymerized F-actin at the concentrations indicated. Bars indicate mean +/- SD.

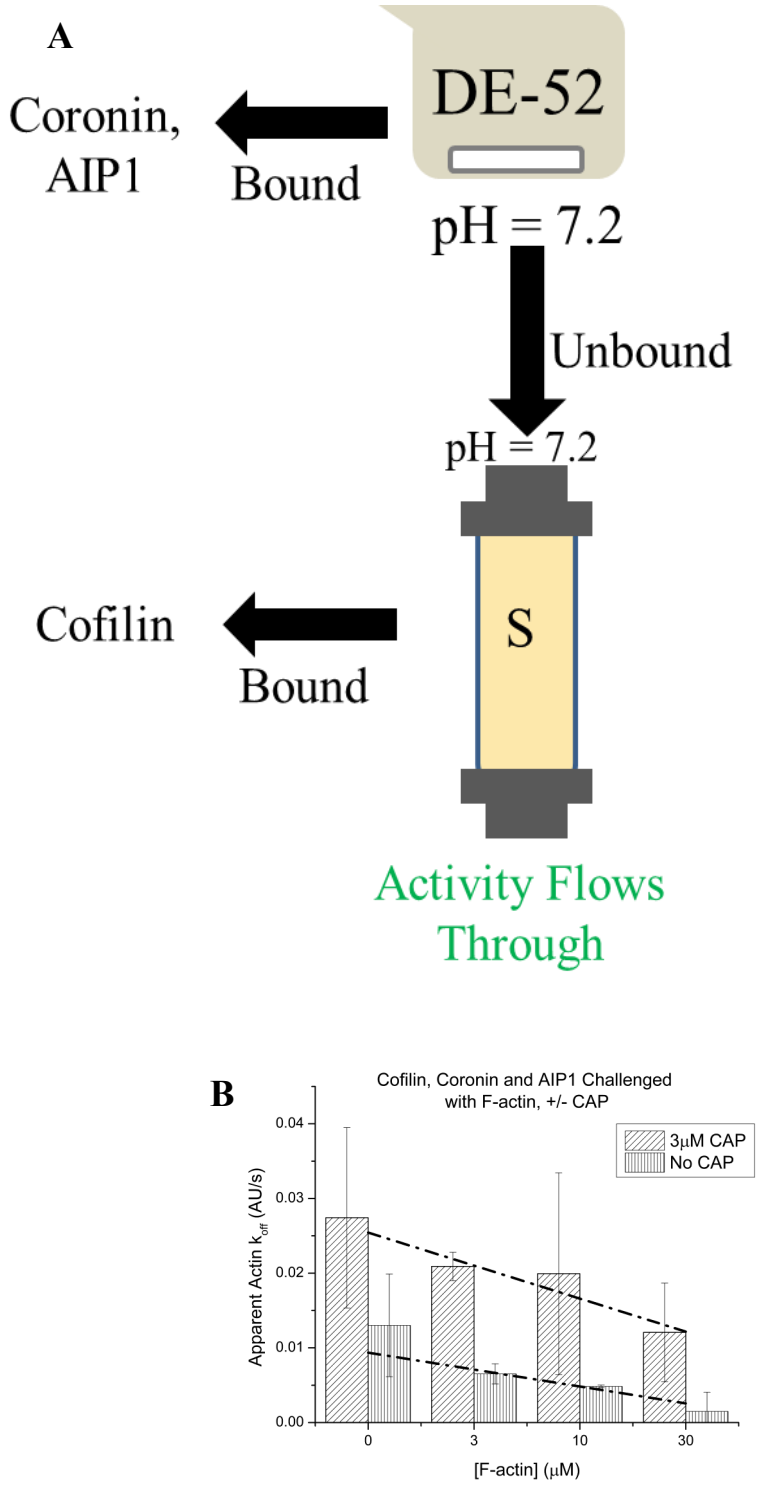


Figure 2.3: Fourth Actin Disassembly Factor Is Chromatographically Isolated. A) The factor responsible for disinhibition despite excess F-actin presence can be separated from each of the other three known factors, first from coronin and AIP1 by flowing through DE52 and then from cofilin by flowing through an S column at physiological pH. B) This factor restores activity lost when disassembly is challenged with excess F-actin. Cofilin 2uM, coronin (2uM), AIP1 (200nM), and where noted, CAP (3uM); bars indicate mean normalized actin fluorescence intensity resolved over time of at least 3 independent experiments, +/- S.D.

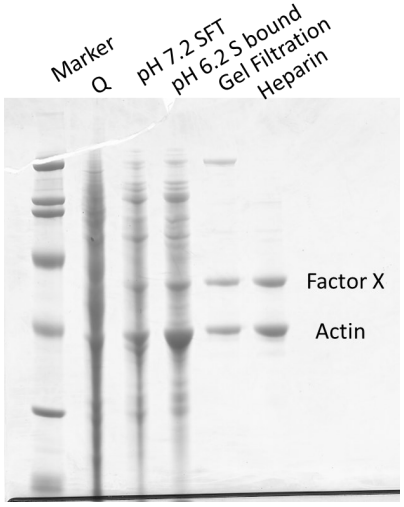


Figure 2.4: Unknown Factor Isolated and Identified as Cyclase Associated Protein (CAP). SDS PAGE summarizing purification of activity from bovine thymus extract. The activity separated from all three known factors, and subsequently purified to two bands. One band was identified as CAP (57KDa), the other as actin (43 KDa). Proteins were identified using MALDI-TOF mass spectrometry.

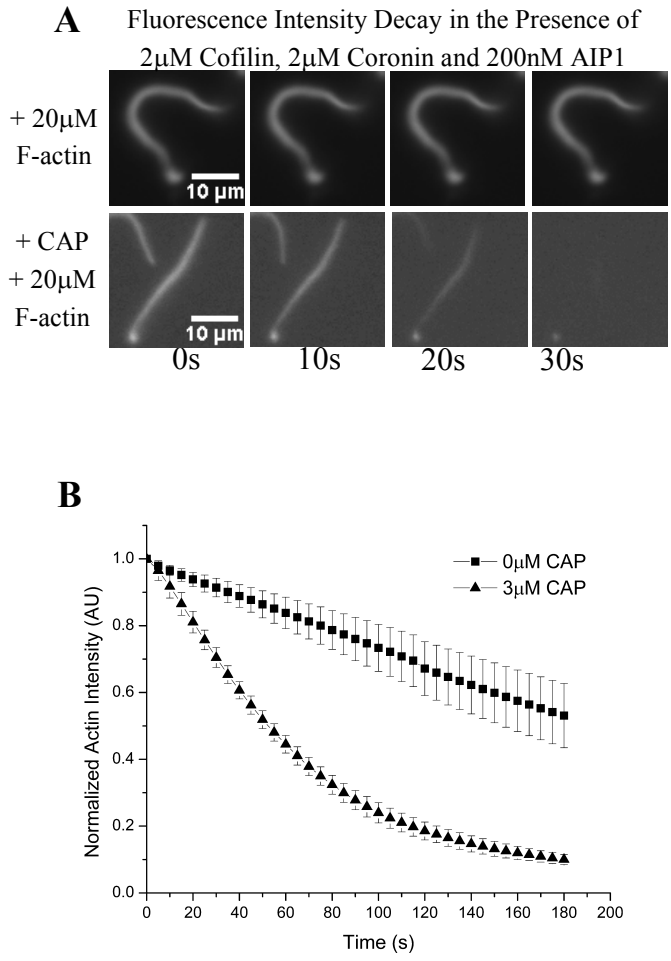


Figure 2.5: Recombinant CAP Scores in Actin Disassembly Assay Confirming that CAP is the 57KDa Factor. A) Excess F-actin (20uM) inhibits the cofilin, coronin, and AIP1 mixture (top series) but recombinant CAP confers resistance to this inhibition (bottom series). B) Quantification of CAP-mediated resistance to inhibition induced by excess (30uM) F-actin. In the absence of CAP (squares), actin disassembly is greatly attenuated, but when CAP is added to the disassembly mixture (triangles) inhibition is lifted and efficient actin depolymerization is restored. Kinetic data display mean normalized actin fluorescence intensity resolved over time of at least 3 independent experiments, +/- S.D.

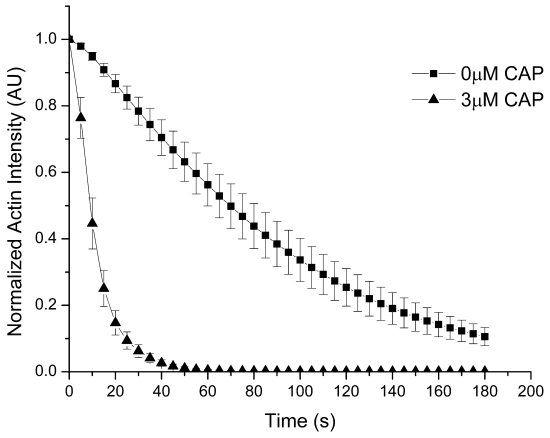


Figure 2.6: CAP Accelerates F-actin Disassembly Even in the Absence of Inhibition. In the absence of excess F-actin, CAP (triangles) markedly increases actin disassembly activity over the tripartite disassembly mixture alone (cofilin, coronin, and AIP1; squares). All experiments are done in the presence of cofilin (2uM), coronin (2uM), AIP1 (200nM), and where noted, CAP (3uM); displayed for each condition is the mean normalized actin fluorescence intensity resolved over time of at least 3 independent experiments, +/- S.D.

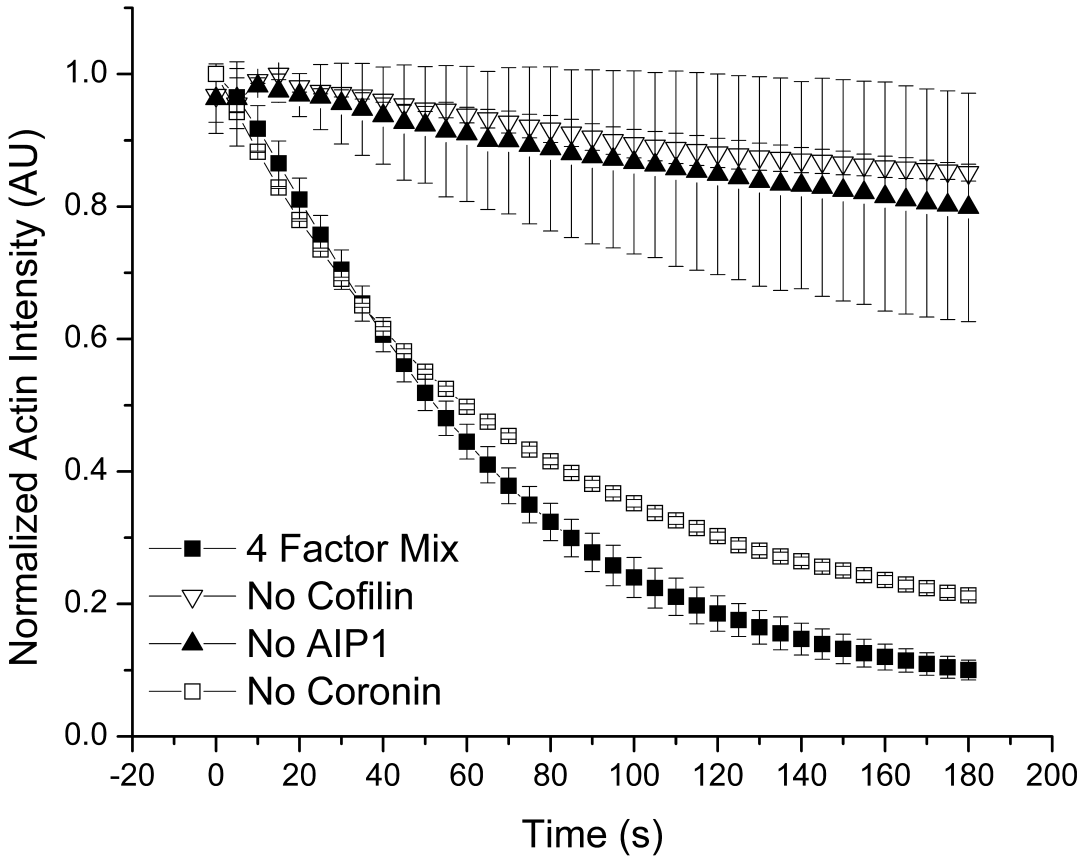


Figure 2.7: CAP is Partially Redundant with but Mechanistically Distinct From Coronin. A) Testing the 4-factor actin disassembly mixture of cofilin, coronin, AIP1 and CAP for redundancy between CAP and each of the other factors in the presence of excess F-actin, we found that in the absence of coronin (open squares) activity was virtually unchanged (vs 4-factor mix, closed squares). Cofilin (inverted triangles) and AIP1 (closed triangles) remain necessary as their absence greatly attenuates actin depolymerization. Displayed for each condition is the mean normalized actin fluorescence intensity resolved over time of at least 3 independent experiments, +/- S.D.

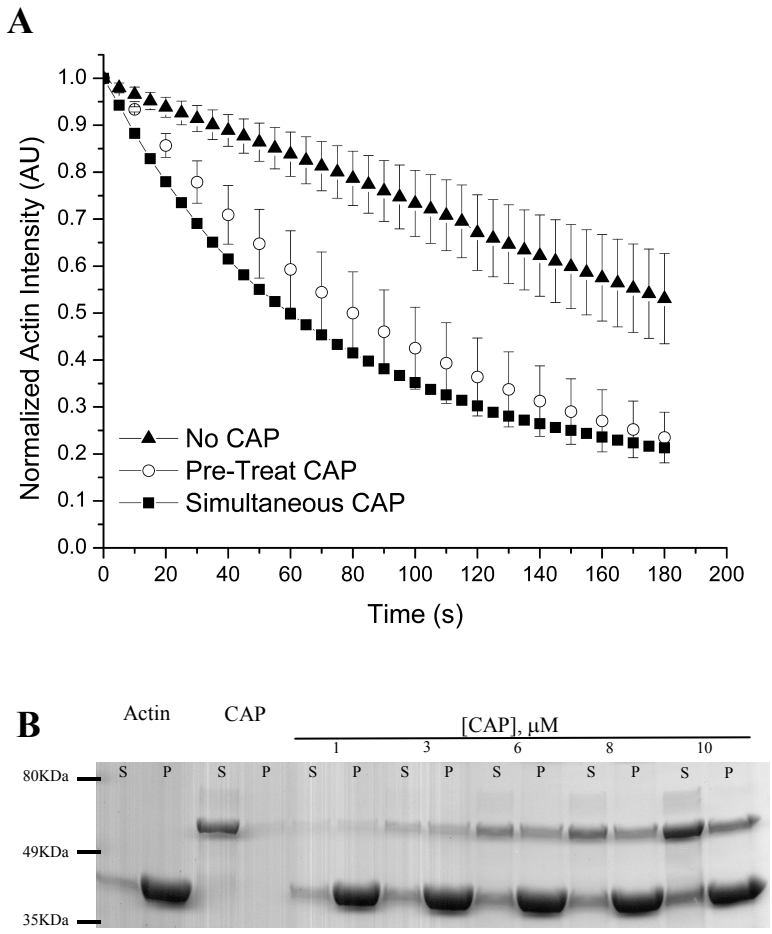


Figure 2.8: CAP Functionally and Directly Binds to F-actin. A) CAP scores in a pre-treatment assay (open circles), with activity similar to when CAP is added simultaneously with cofilin and AIP1 (squares). Triangles represent cofilin and AIP1 control. Experiments under challenge of excess F-actin at 30uM. B) CAP, like coronin, directly binds F-actin as demonstrated in this coomassie-stained SDS-PAGE gel showing supernatnant (S) and pellet (P) fractions after sedimentation of 10uM F-actin with an indicated amount of CAP. 6-His-CAP is the upper band (~56KDa) and actin is the lower band (43KDa). Unless noted otherwise, 2uM cofilin, 2uM coronin, 3uM CAP, 200nM AIP1.

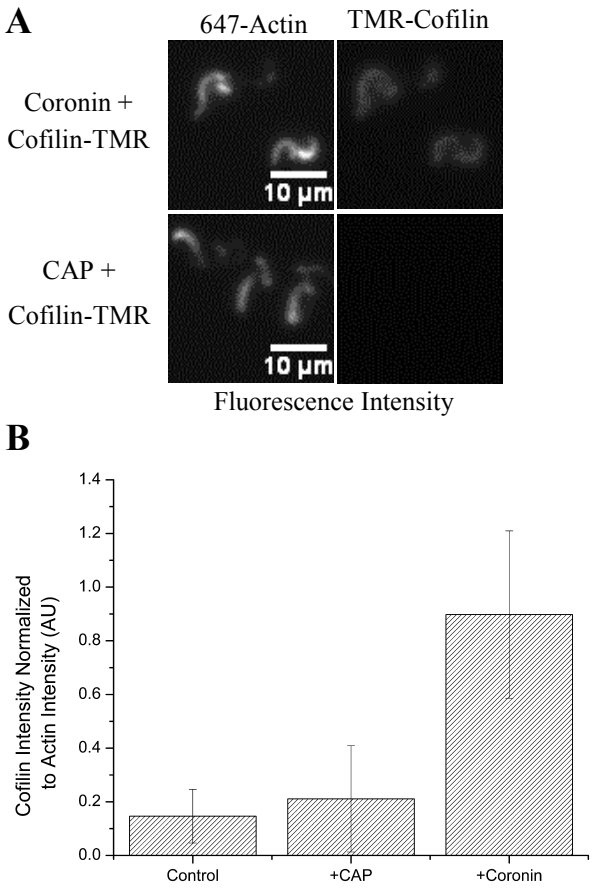


Figure 2.9: CAP is Mechanistically Distinct from Coronin. A) While coronin significantly increases cofilin loading (upper series), CAP does not (bottom series). B) Cofilin intensity is normalized to actin intensity in each image; bars represent mean of 10 data points per condition +/- SD. All experiments utilize *L. monocyto-genese* actin comet tails assembled as described. Kinetic data (A) are challenged with excess F-actin at 30uM. Unless noted otherwise, 2uM cofilin, 2uM coronin, 2uM CAP, 200nM AIP1.

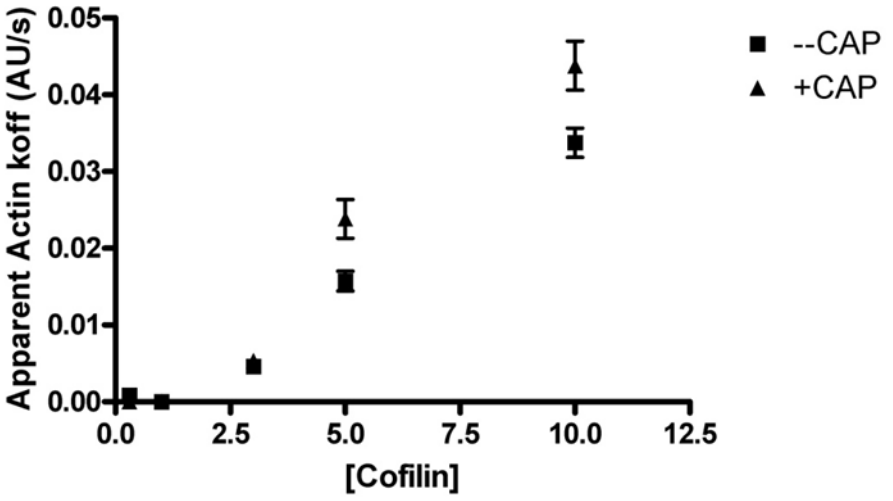
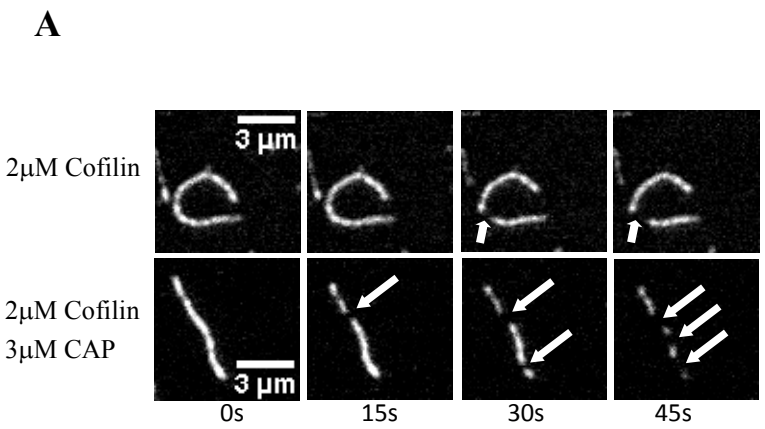


Figure 2.10: CAP Augments Cofilin-Mediated Actin Disassembly in a Manner Distinct From Recycling. Expected recycling behavior of CAP with respect to cofilin should be significant when cofilin concentrations are low and become obsolete when cofilin concentration is high. However, we observed that as we increased cofilin concentrations in the absence (squares) or presence (triangles) of 2uM CAP and found that instead CAP had an increased effect at elevated cofilin concentrations (5-10μM) with no appreciable effect at low cofilin concentrations (up to and including 3μM). These findings are inconsistent with a recycling mechanism of cofilin activity augmentation in the presence of CAP.



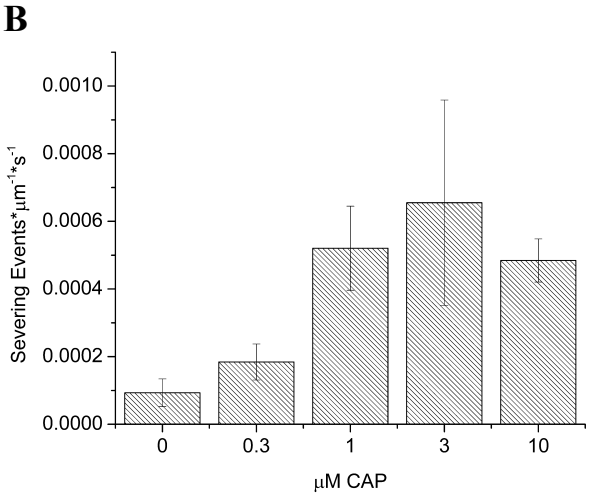


Figure 3.1: CAP Augments Cofilin-Mediated Severing at Physiologically Basic pH. A) Cofilin severs actin filaments as expected (upper series), and with the addition of CAP both proteins sever actin filaments at an elevated rate (bottom series). Arrows indicate severing events. B) Quantification of actin severing activity with increasing concentrations of CAP in the presence of 2uM cofilin at pH 7.4. C) CAP scores in a pre-treatment assay with single actin filaments, resulting in activity quantitatively similar to that seen when adding both CAP and cofilin simultaneously. Direct visualization of single actin filaments in the presence of cofilin (A, upper series) or cofilin and CAP (A, bottom series); severing events and overall disassembly increase in the presence of CAP. CAP accelerates cofilin-mediated severing rates in a dose-dependent manner saturating at 1-3uM. Cofilin 2uM; bar graphs indicate mean values of at least three experiments, +/- SD.

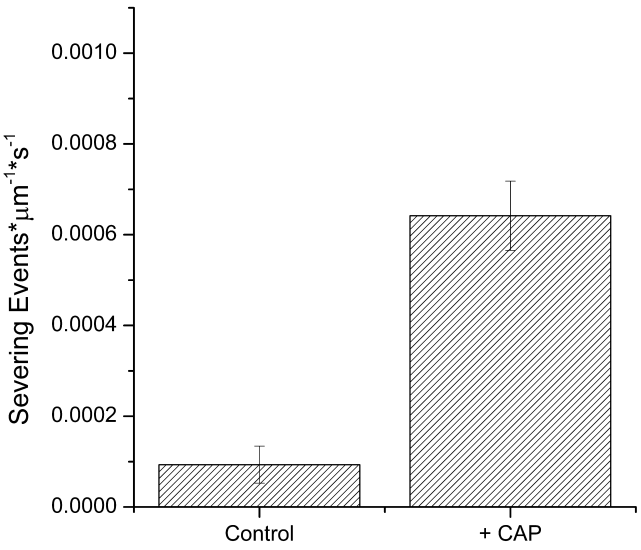
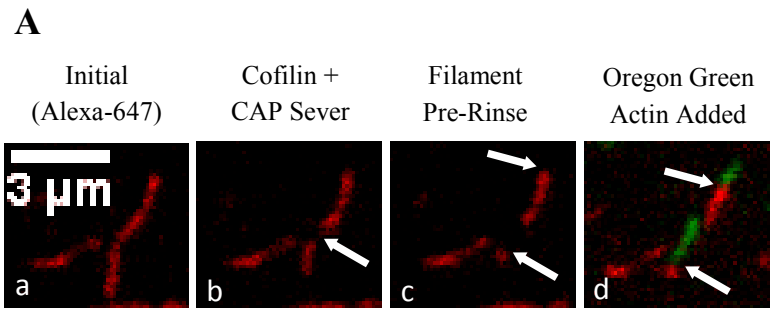


Figure 3.2: CAP Activity Remains after Washout in Pre-Treatment Assay. Similar to listeria comet tail experiments, CAP was allowed to bind to single actin filaments before being rinsed away and cofilin then applied. Severing events occurred at rates consistent with maximal CAP concentration applied simultaneously with cofilin. CAP 3uM, Cofilin 2uM; bar graphs indicate mean values of at least three experiments, +/- SD.



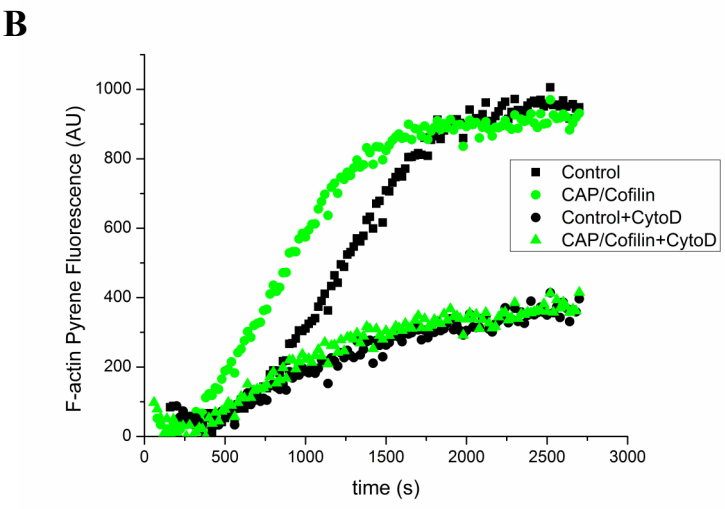


Figure 3.3: Ends Created by CAP/Cofilin-Mediated Severing are Uncapped. A) To Alexa-647-labeled actin filaments (*a*; pseudo-colored red) we added CAP and cofilin and tracked severing for 90s. Image (*b*) demonstrates a severing event at 45s (arrow), producing a severed barbed end (*c*, lower arrow) and an unsevered barbed end (*c*, upper arrow). After rinsing and supplying Oregon Green-labeled G-actin, both the severed and unsevered barbed ends display the ability to nucleate new growth (*d*). B) Unlabeled CAP/cofilin-severed filaments or unlabeled control filaments seeded pyrene-actin growth, with CAP/cofilin-induced severing producing a decreased lag (green circles) relative to untreated control (squares), consistent with a greater number of nucleating ends. The addition of barbed end capping drug CytoD gave similar decreases in actin assembly rates in both control F-actin nucleated and CAP/cofilin-treated F-actin nucleated experiments (black circles and green triangles, respectively), consistent with a process dependent upon free barbed ends. Traces from one representative experiment shown.

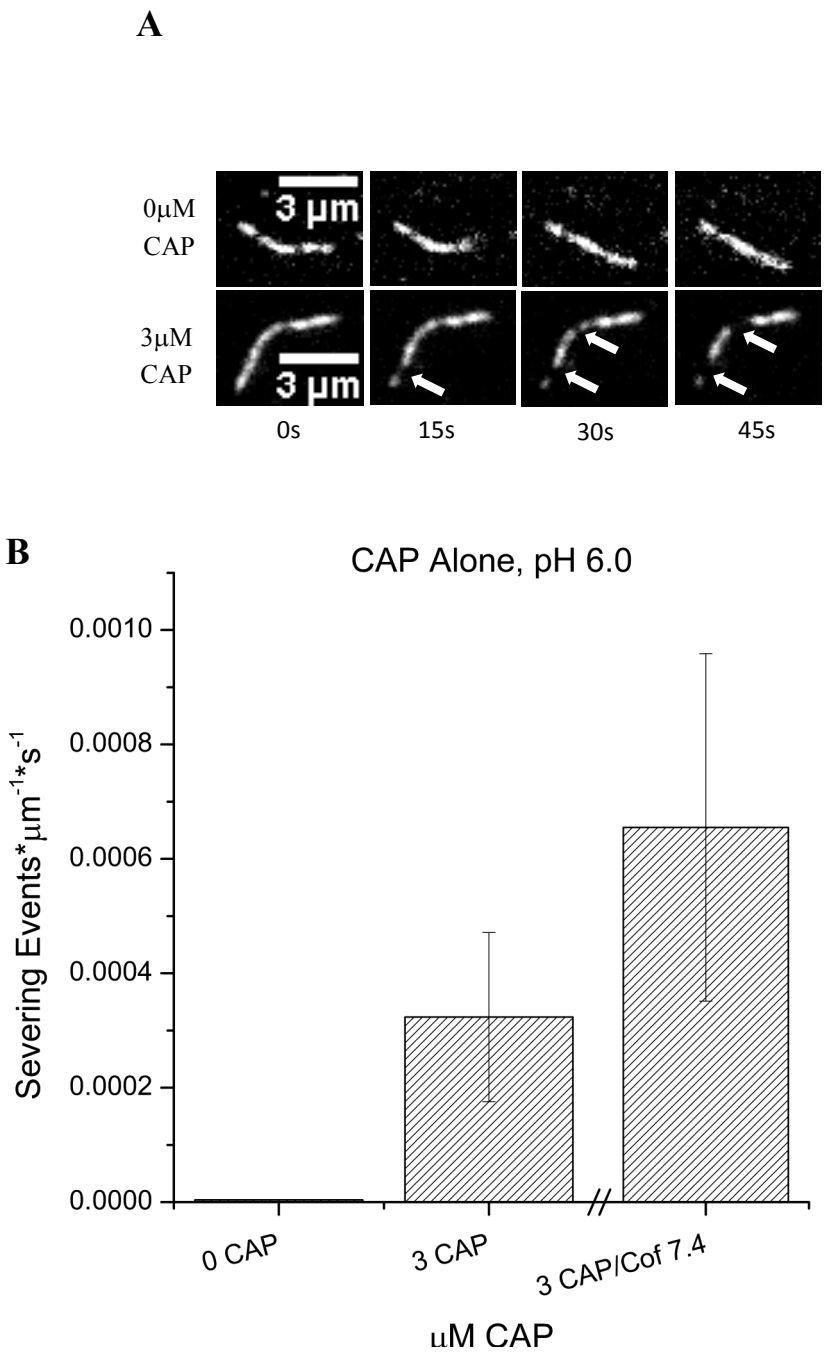


Figure 3.4: CAP Alone is Sufficient to Sever Actin at Acidic pH. A) Timelapsed images showing single actin filaments diluted into Assay Buffer at pH 6 (top series) or into 3uM CAP at pH 6 (bottom series). Arrows indicate severing events. B) CAP scores as an actin severing factor at non-physiological acidic pH, at rates comparable to CAP/Cofilin-mediated severing activity at physiologic pH. Bar graphs display mean of at least 3 experiments +/- SD; 2uM cofilin, 3uM CAP.

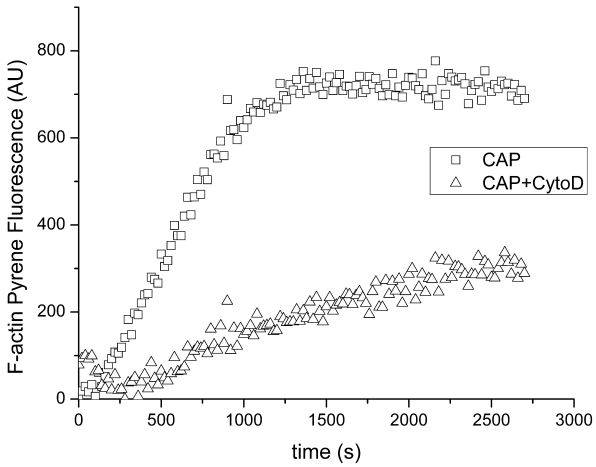


Figure 3.5: Ends Created by Acidic CAP-Mediated Severing are Uncapped. Pyrene-actin polymerization seeded with CAP-severed F-actin treated at pH 6 (squares). Polymerization is inhibited by the addition of the barbed end-capping drug CytoD (triangles), indicating that CAP-severed F-actin provides free barbed ends. Traces from one representative experiment are shown. Neither cofilin nor CAP are alone sufficient to sever F-actin, but in combination the two proteins sever F-actin at an accelerated rate. 2uM CAP, 300nM CytoD.

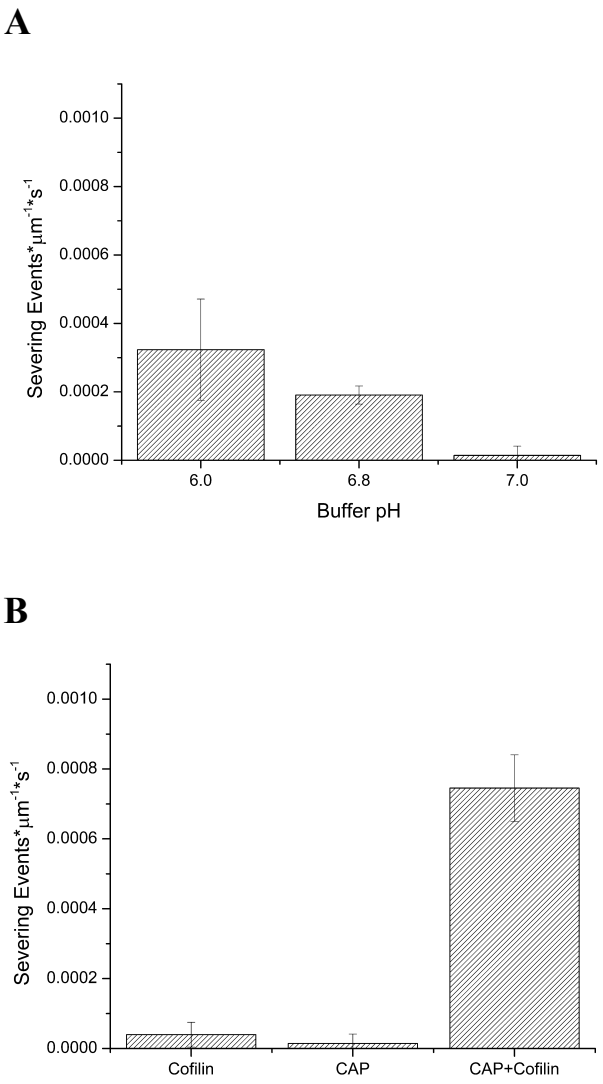


Figure 3.6: CAP Severing Activity is pH-Dependent but is Rescued by Cofilin. A) CAP-mediated severing is pH-sensitive and is no longer observed at neutral pH. B) Quantification of filament severing rates at pH 7 in the presence of cofilin alone, CAP alone or cofilin plus CAP in combination. Neither cofilin nor CAP alone are sufficient to sever F-actin, but in combination the two proteins sever F-actin at an accelerated rate. Bar graphs display mean of at least 3 experiments +/- SD; 2uM cofilin, 3uM CAP.

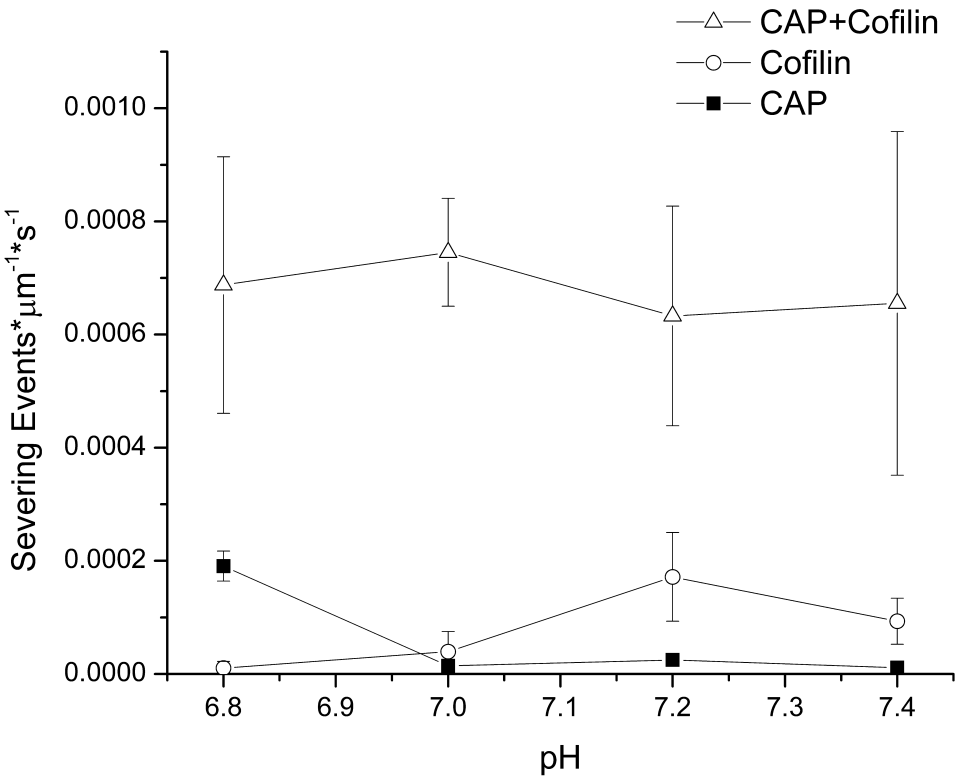


Figure 3.7: The Combination of CAP and Cofilin yield pH-Independent Actin Severing. Across the physiological range of pH values, CAP alone (squares) and cofilin alone (circles) each have individually limited actin severing activities and each is pH dependent. When added together, however, the two have a severing activity at least 10-fold higher than either can achieve independently over this pH range (triangles). 3uM CAP, 2uM cofilin; each data point represents the mean of at least three experiments +/- SD.

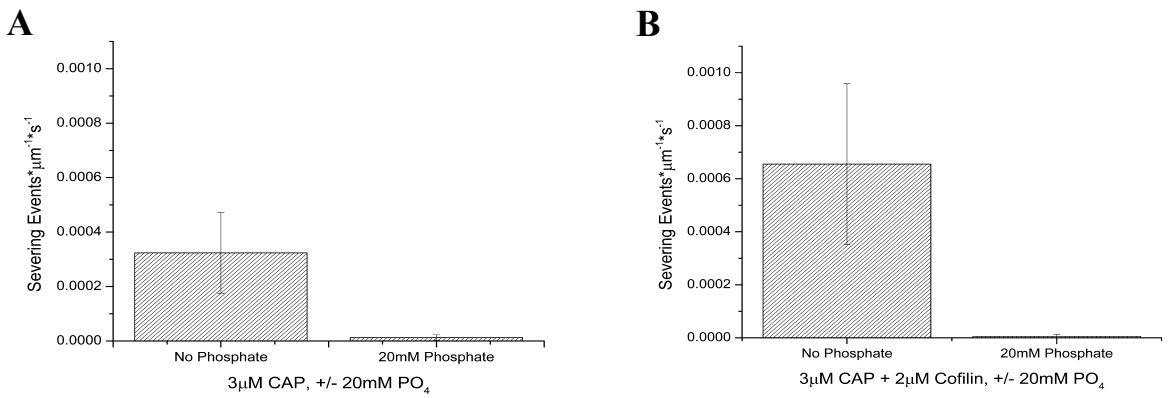


Figure 3.8: CAP-Mediated and CAP/Cofilin-Mediated Actin Severing is Inhibited by Inorganic Phosphate. A) CAP severing activity at pH 6 in the absence (left bar) and the presence (right bar) of 20mM inorganic phosphate. B) Cofilin- and CAP-mediated severing at pH 7.4 in the absence (left bar) and the presence (right bar) of 20mM inorganic phosphate. Both CAP-mediated severing at acidic pH and CAP/cofilin-mediated severing at basic pH are severely inhibited by excess phosphate. 3uM CAP, 2uM cofilin; each data point represents the mean of at least three experiments +/- SD.

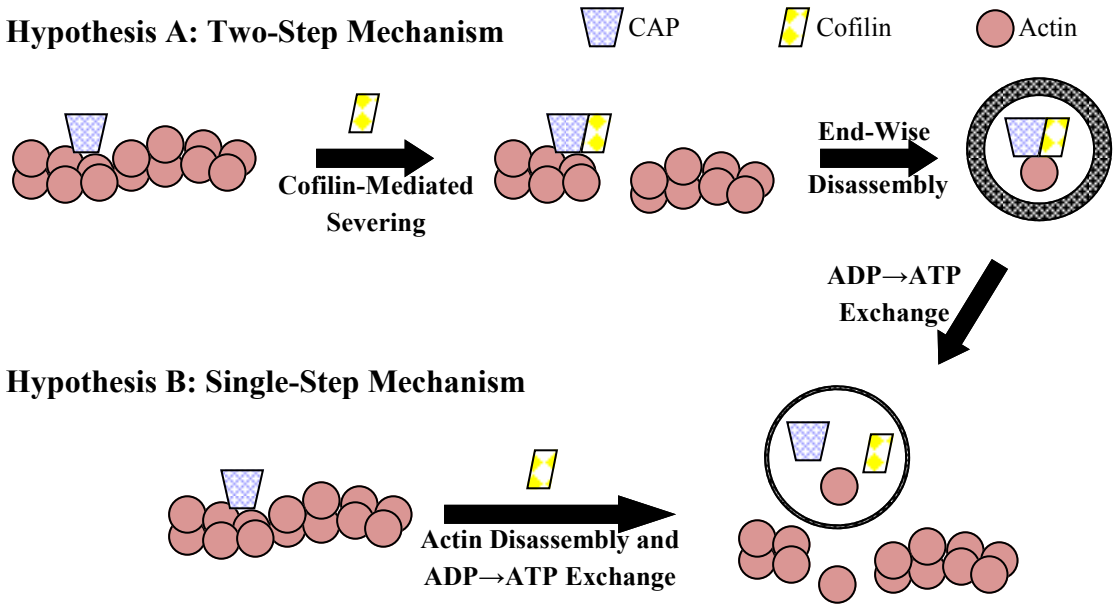


Figure 3.9: Model: Two-Step vs Single-Step Mechanism. Hypothesis A is essentially a synthesis of existing literature, in which after severing CAP acts to liberate cofilin for the next round of actin disassembly. Under this hypothesis, severing would occur cofilin-dependently and CAP would function after the severing event as a nucleotide exchange factor in an essentially separate reaction. For reasons including our own results and a more careful reading of the existing literature, we favor Hypothesis B: CAP acts in a capacity to engage with cofilin as an actin severing protein in a reaction whose end product is liberated ATP•G-actin and free cofilin in a first-order reaction.

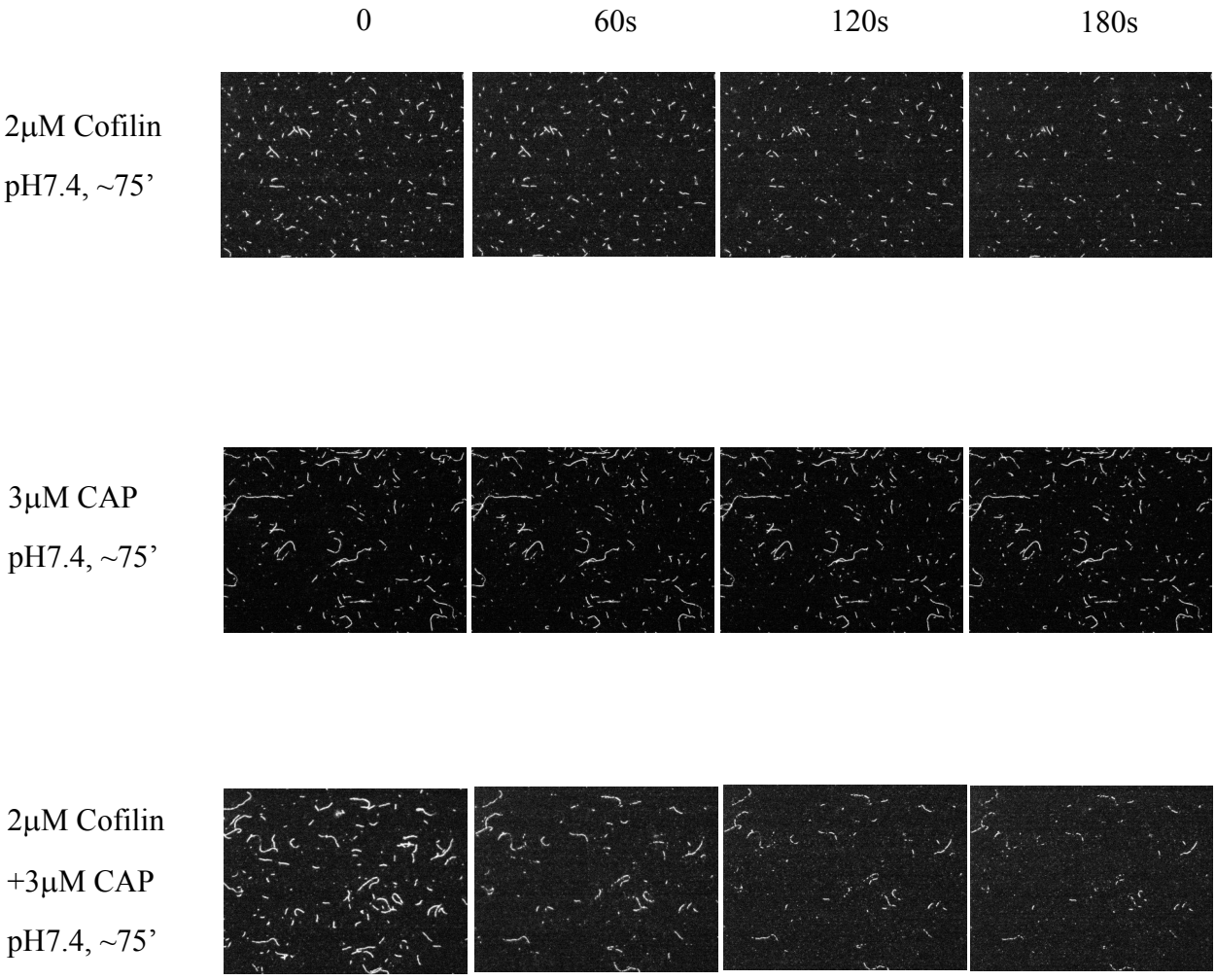


Figure 3.10: CAP and Cofilin Together Depolymerize Aged Actin Filaments. A) We find that neither cofilin (upper series) nor CAP (middle series) alone are capable of disassembling aged filaments, consistent with the notion that aged F-actin is intrinsically stable as a result of ATP hydrolysis (t1/2 = 2s) and subsequent phosphate release (t1/2 = 6min) having already happened and the energy derived from these processes unavailable. However, we find that the combination of CAP and cofilin are sufficient to disassemble aged actin filaments (bottom series), indicating that ADPF-actin filaments may represent a species of actin physiologically utilized for purposes requiring increased stability, and that CAP and cofilin may be the cellular means of turning over such a stable actin array. B) Quantification. Cofilin 2uM, CAP 3uM; actin assembled as described, allowed to age in Assay Buffer for 75 min in chamber before experiment.

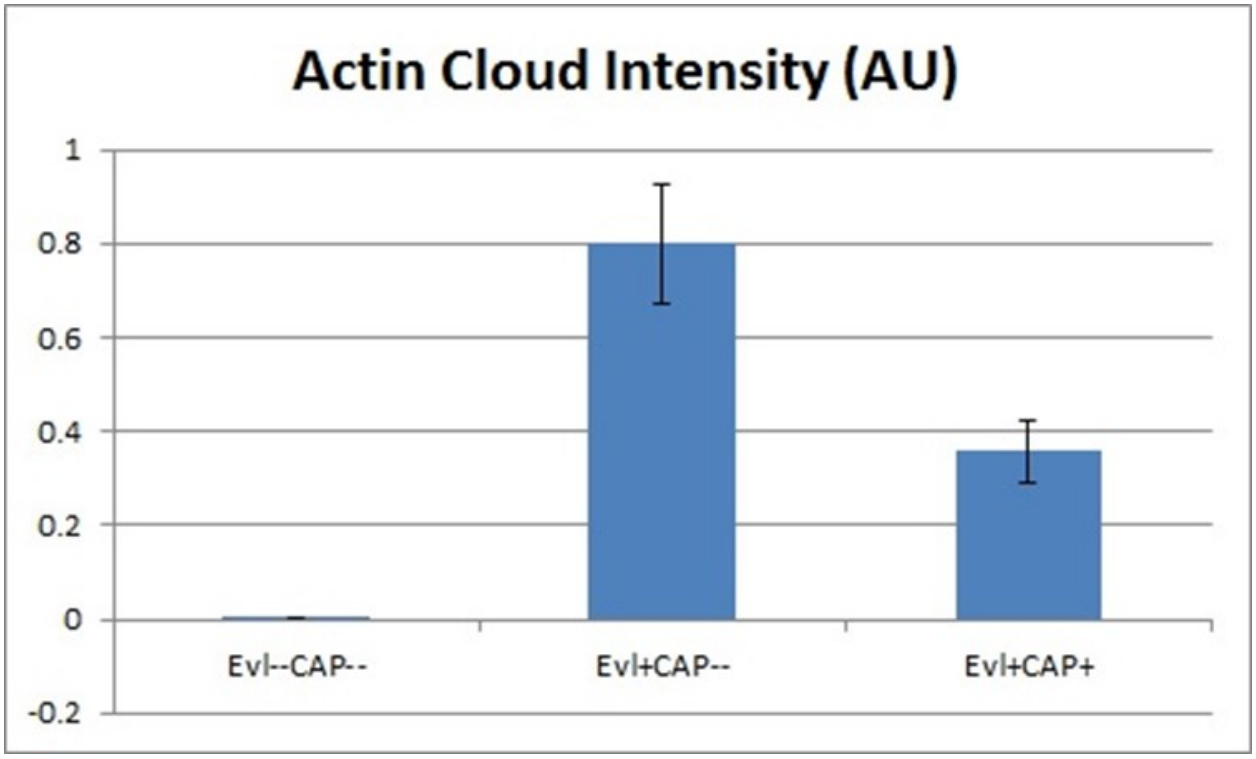


Figure 4.1: CAP Attenuates EVL-Dependent Actin Clouds. Using Listeria adhered to perfusion chambers as described, we noticed that branched actin cloud formation is EVL-dependent (left vs middle bar). The addition of CAP in the actin polymerization phase of the experiment attenuates but does not abolish actin cloud formation (right bar). EVL 1uM in pretreatment, Arp2/3 300nM, actin 2uM, CAP 3uM; each bar indicates mean of 3 experiments, 5 fields per experiment, +/- SD.

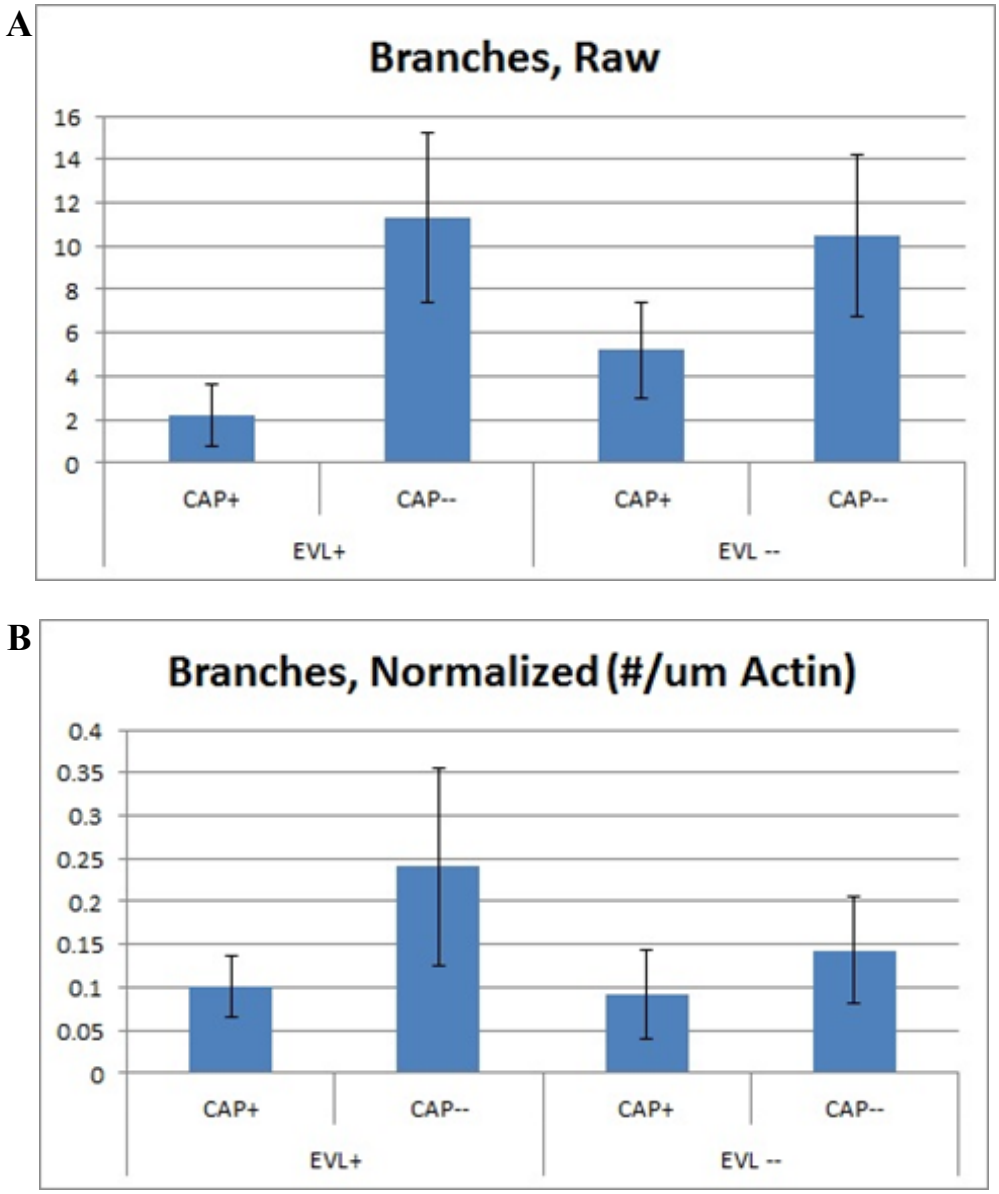


Figure 4.2: CAP Attenuates EVL-Dependent Actin Branching. Utilizing the actin branching assay of Weaver et al, we applied CAP to EVL-built actin arrays in solution and visualized to count branches directly. A) We found that in the presence of CAP there were fewer actin branches per field (bar 1 versus 2, 3 versus 4), but we also noticed that there was a paucity of actin in the presence of CAP. B) In order to control for the apparent amount of actin, we normalized the data in (A) to linear actin and found that branches per actin length per field revealed an increase in branching in the presence of EVL alone (bar 2 versus 4), but that CAP abolished this increase (bar 1 versus 3). EVL 3uM, CAP 3uM; each bar indicates mean of 3 experiments, 5 fields per experiment, +/- SD.

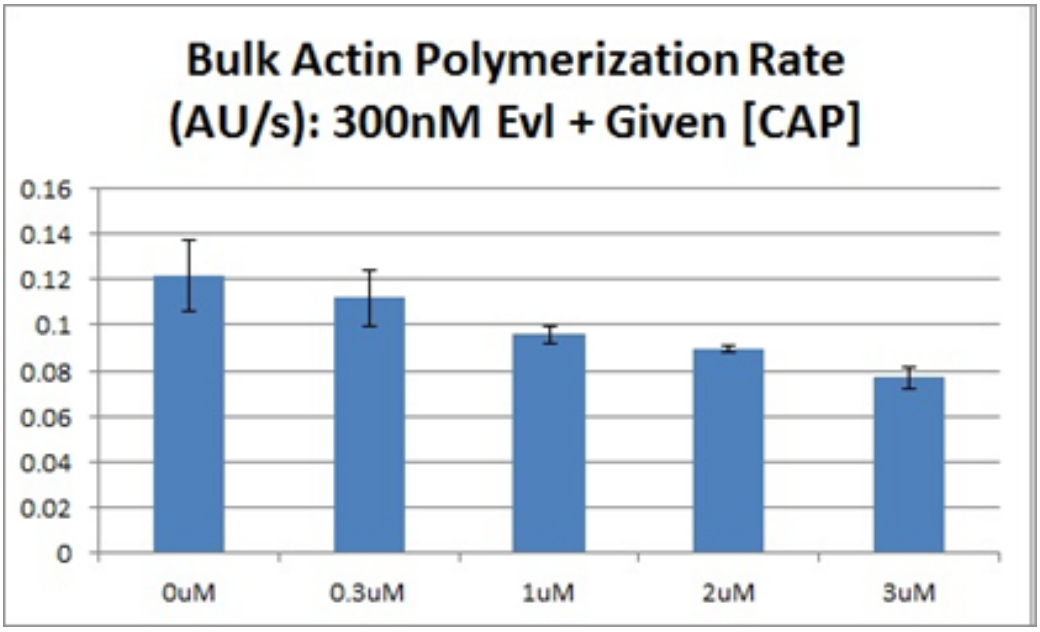


Figure 4.3: CAP Dose-Dependently Suppresses EVL-mediated Enhancement of Actin Polymerization. EVL is an established actin polymerization factor, and we utilized pyrene-labeled actin to spectroscopically measure the polymerization rate in the presence of increasing concentrations of CAP. We find that CAP dose-dependently decreases the actin polymerization rate. Bars represent the mean of at least 3 experiments +/- SD; EVL 300nM, actin 2uM, Arp2/3 500nM, ActA 200nM.

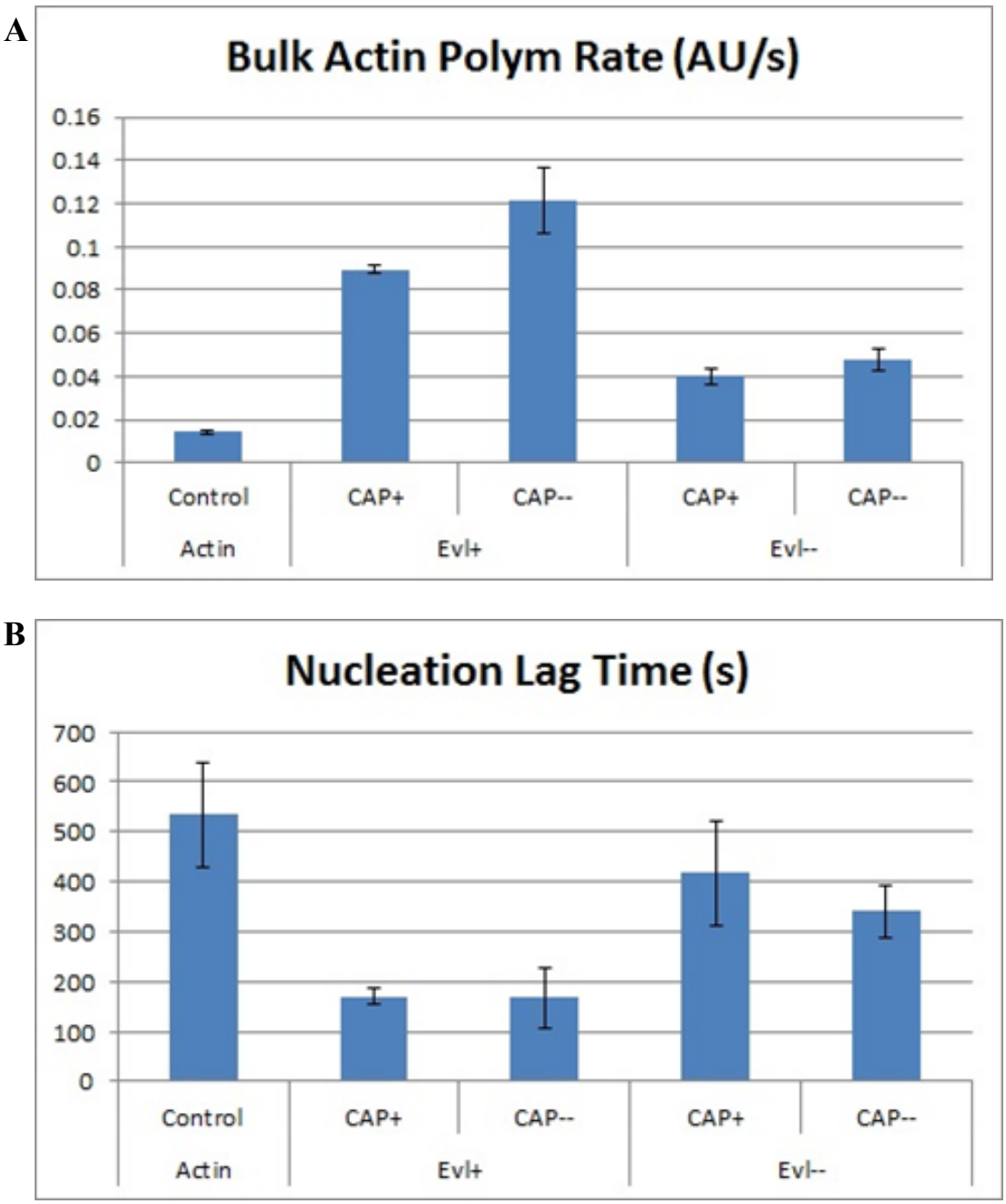


Figure 4.4: CAP EVL-Dependently Decreases Bulk Actin Polymerization Rates. A) EVL increases the rate of actin polymerization in the presence of Arp2/3 and ActA approximately 3-fold (rightmost bar vs middle bar), and the three factors accelerate actin assembly approximately 10-fold (leftmost bar actin control vs middle bar). CAP minimally decreases actin assembly rate in the absence of EVL (bar 4 versus 5), but significantly diminishes the acceleration associated with EVL presence (bar 2 versus 3). B) In order to separate actin nucleation from elongation, we measured actin lag in each condition and found that while EVL (bars 2&3 from left, +EVL) reduces lag time (vs bar 1, actin alone; vs bars 4&5, -EVL). Bars represent the mean of at least 3 experiments +/- SD; EVL 300nM, actin 5uM; Arp2/3 500nM and ActA 200nM in all but actin alone. EVL-dependent effect on actin polymerization (A) and lag time (B) can be visualized by comparing middle bar (+EVL) and rightmost bar (-EVL), both in the absence of CAP but presence of Arp2/3 and ActA.

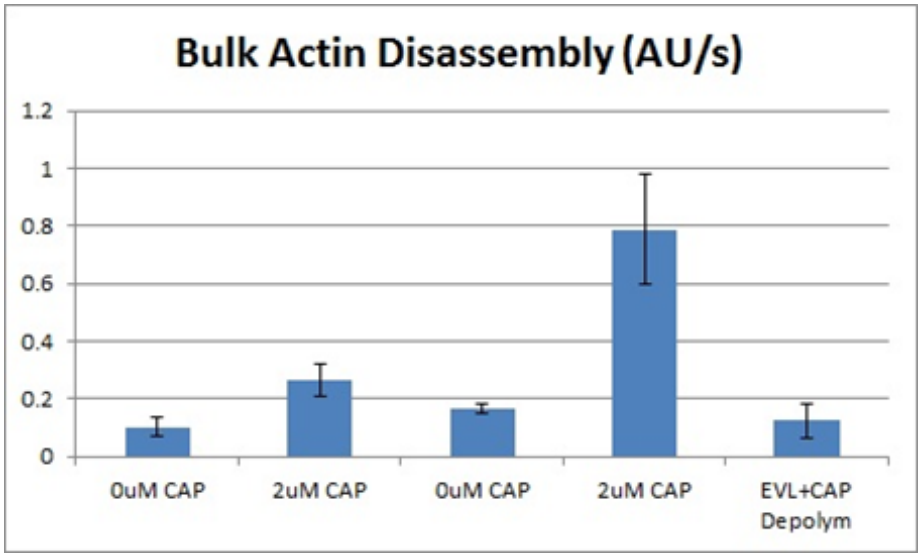


Figure 4.5: CAP EVL-Dependently Increases Actin Disassembly Rates. In order to experimentally separate actin assembly and disassembly using spectroscopy, we polymerized actin in the presence (bars 2&3 from left) or absence of EVL and watched actin disassembly rates after adding buffer or 2uM CAP. While CAP modestly increased observed actin k_{off} in the absence of EVL (bar 2 vs 1), the effect of CAP is apparent (bar 4 vs 3) and is intensified 3-4 fold (bar 4 vs 2) when EVL is present during actin polymerization. In order to better determine whether this EVL-dependent effect is due to EVL action during the polymerization phase or depolymerization phase of the experiment, we left EVL out of the mixture and added EVL along with CAP at outset of depolymerization phase. EVL and CAP, when added together after actin assembly, had no significant effect on actin disassembly (bar 5). Bars represent the mean of at least 3 experiments +/- SD; EVL 300nM, actin 5uM, Arp2/3 500nM, ActA 200nM.

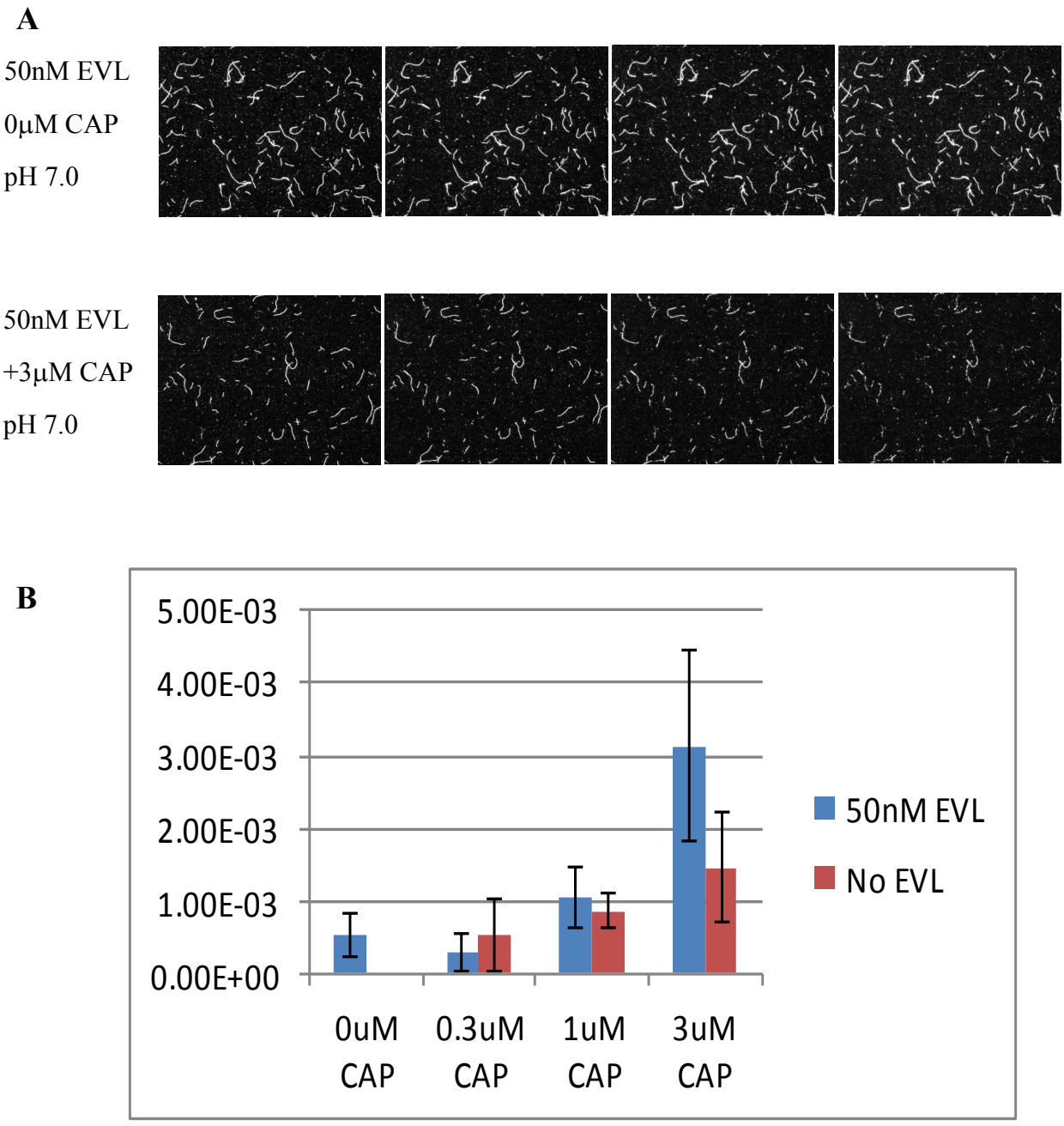


Figure 4.6: CAP Increases Actin k_{off} of EVL-Built Single Filaments. Single filament assays using small amounts of EVL similar to the method of Hansen and Mullins were conducted as described. Filaments were assembled using G-actin concentrations just above the critical concentration and small amounts of EVL in order to have a maximized population of filaments that do not bundle. A) Filaments were assembled, rinsed, then exposed to Assay Buffer in the absence (upper series) or presence (bottom series) of CAP and actin disassembly was measured (normalized AU/s). B) Rates were quantified with increasing CAP concentrations and demonstrated an increased actin koff in actin filaments built in the presence (right bar each pair) versus absence (left bar) of EVL. Bars represent the mean of at least 3 experiments +/- SD; EVL 50nM, actin 600nM, CAP 3uM.

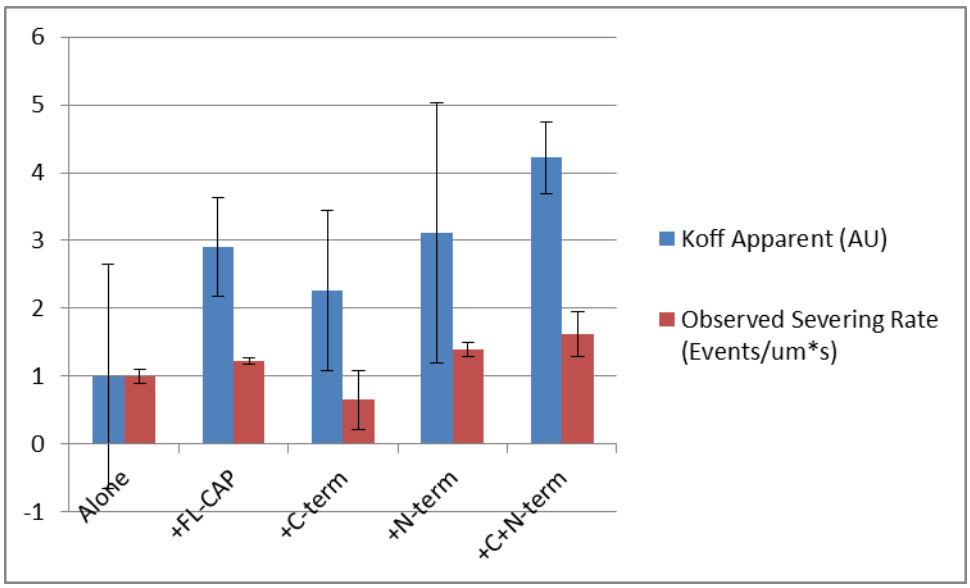


Figure A.1: CAP Full-Length and Domains Discordantly Increase Actin koff versus Severing. Having observed that CAP/Cofilin – mediated loss of actin mass appears outpace the increase in severing, we set out to quantify both as we mapped CAP activity with respect to its C- and N-terminal domains. Normalizing to values for cofilin alone, severing rates rise by a maximum of 50% while koff increases 300-400%. Bars represent the mean of at least 3 experiments +/- SD.

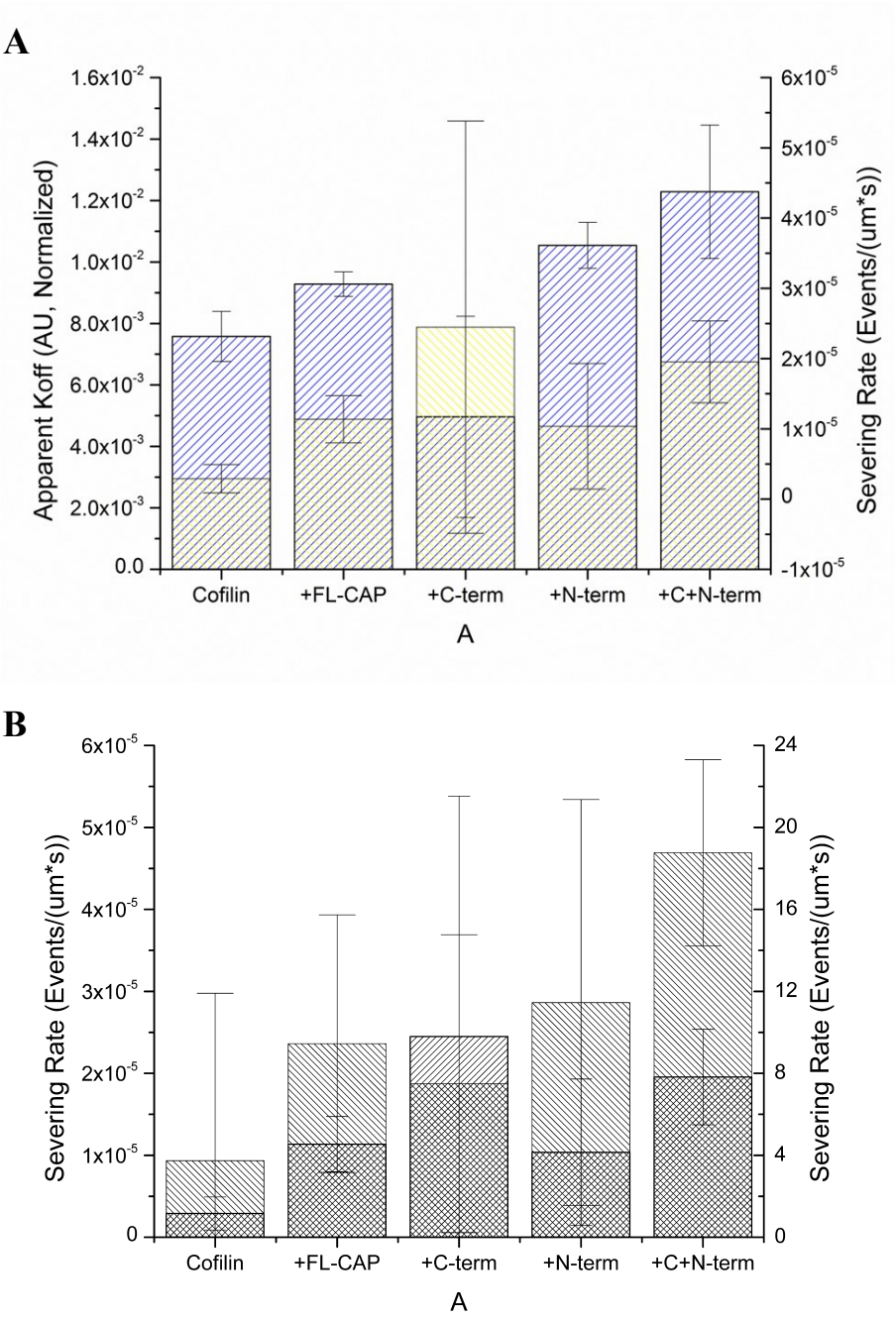


Figure A.2: Mathematical Modeling of Severing using Savitzky-Golay Derivation Reveals Inadequacy. We derived severing rates using the mechanism-independent Savitzky-Golay (SG) method to assign a value to actual severing rates in several conditions. A) We found that while our calculated severing rates matched observed loss of actin mass trends across conditions, the severing rate necessary to account for the entire increase in apparent actin k_{off} (B) is several orders of magnitude greater than observed severing rates. In (A), y-axis left, observed normalized loss of actin mass (AU/s); y-axis right, manually counted severing events per second normalized to total starting actin length (events*um^{-1}*s^{-1}). In (B), y-axis left, manually counted severing events per second normalized to total actin length (events*um^{-1}*s^{-1}); y-axis right, calculated severing events from observed loss of actin mass. Bars represent mean of at least 3 experiments, +/- SD.

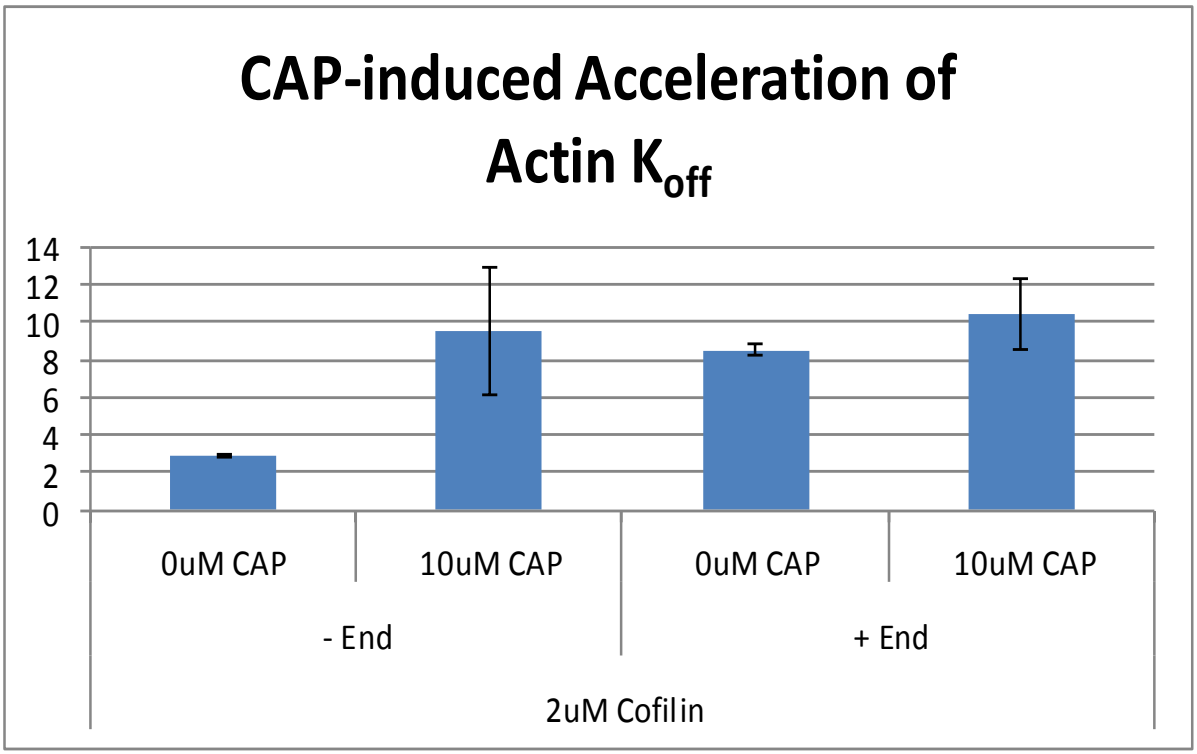


Figure A.3: CAP Increases Actin K_{off} at Both Filament Ends. Presence of CAP results in increase of end-dependent actin koff, representing an alternative mechanism to severing to explain increased loss of actin mass. Combined koff in the presence of cofilin alone is 10sub/s, in agreement with the published off rates, while in the presence of CAP the combined koff is approximately 20sub/s. Bars represent at least 3 experiments, with 12-20 filaments analyzed per experiment; mean +/- SD; cofilin 2uM, CAP 10uM.

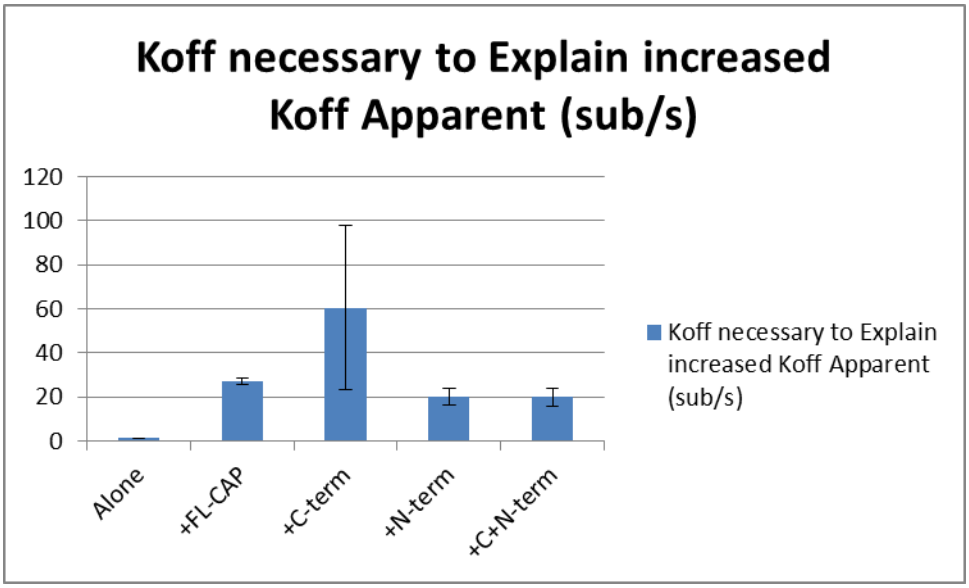


Figure A.4: CAP-Mediated Increases in Actin koff are Barely Sufficient to Explain Increased Loss of Actin Mass. We found the total subunit loss in the absence of CAP is approximately 10sub/s, assuming continuous disassembly without pauses. This is more than the rate necessary to explain apparent k_{off} in the presence of 2uM cofilin alone (leftmost bar). In the presence of full-length CAP, we found that the mean actin k_{off} is approximately 20sub/s, in agreement with the lowest part of the ranges we find for each of our CAP domain conditions (bars 2-5). This indicates that observed increases in actin k_{off} in the presence of CAP and cofilin could indeed account for observed increases in actin disassembly without appealing to severing as a major mechanism or perhaps at all, although it also unlikely that CAP and cofilin are sufficient as the sole physiological means of actin disassembly. Bars represent mean +/- SD, calculated from experiments with at least 3 repetitions.

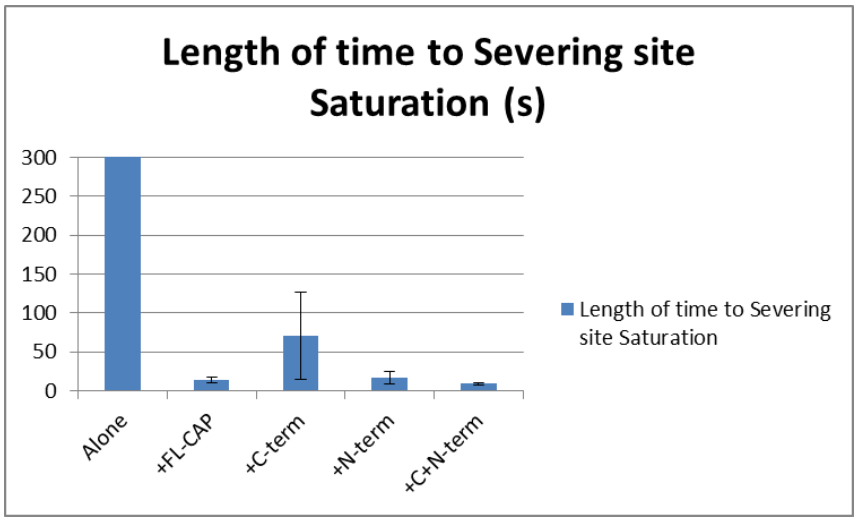


Figure A.5: Severing Requires More Sites than are Physically Available to Explain Actin k_{off} Increases. Recognizing that severing must occur at the union of two actin subunits, we calculated the number of available sites per unit length of F-actin (184 sites per um). Using this value we calculated the time it would take to have used all available sites at the severing rates we calculated using Savitzky-Golay derivation. We found that while severing sites would remain available during experiments using cofilin alone, actin disassembly in the presence of any combination of cofilin plus CAP or its individual domains would saturate all possible severing sites within approximately 10-15s, with the possible exception of cofilin/C-terminal CAP, long before actin mass has been lost to an equivalent extent. Bars represent mean +/- SD, calculated from experiments with at least 3 repetitions.

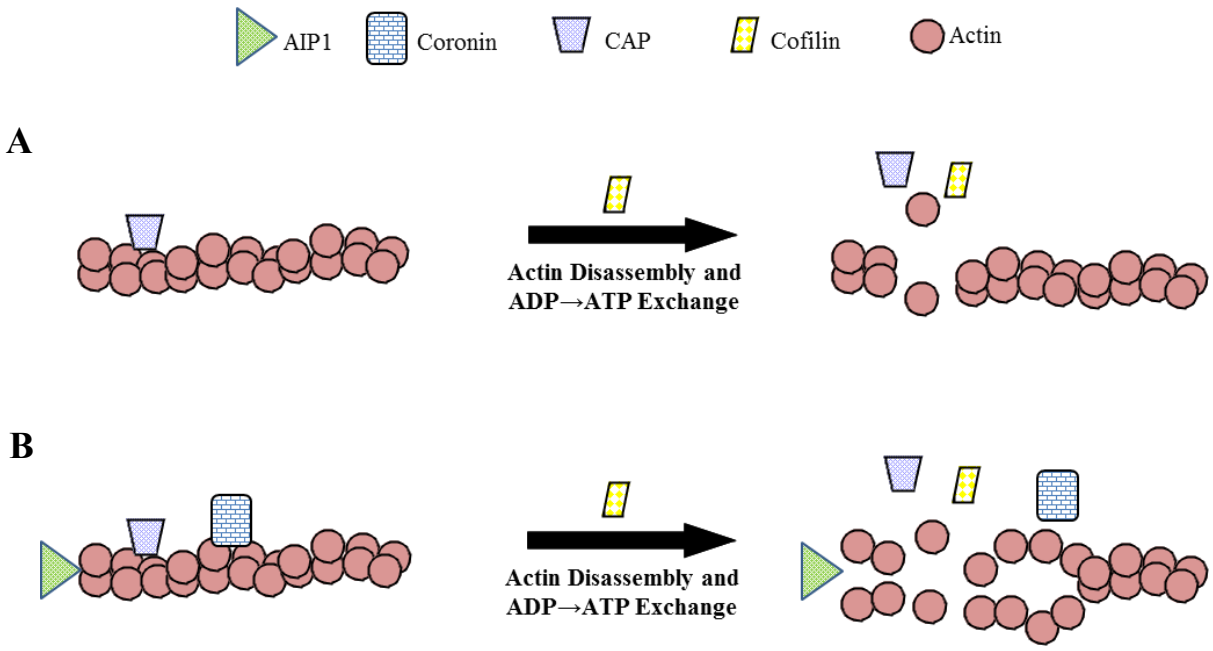


Figure A.6: Model of Actin Disassembly in the Presence of Known Actin Disassembly Factors. We conclude that while any mechanism may not be entirely excluded from playing at least a small part in physiological actin disassembly, treadmilling and severing simply do not reconcile with data and mathematical analysis. We favor a single rate-limiting step mechanism which is end-dependent, robust to allow severing or other minor mechanisms to occur simultaneously, does not depend upon filament end capping, and allows for simultaneous nucleotide exchange on G-actin. Essentially this is our earlier proposed model (A) except that at existing or newly created ends filaments lose significant numbers of actin subunits in a single rate-limiting step (B).

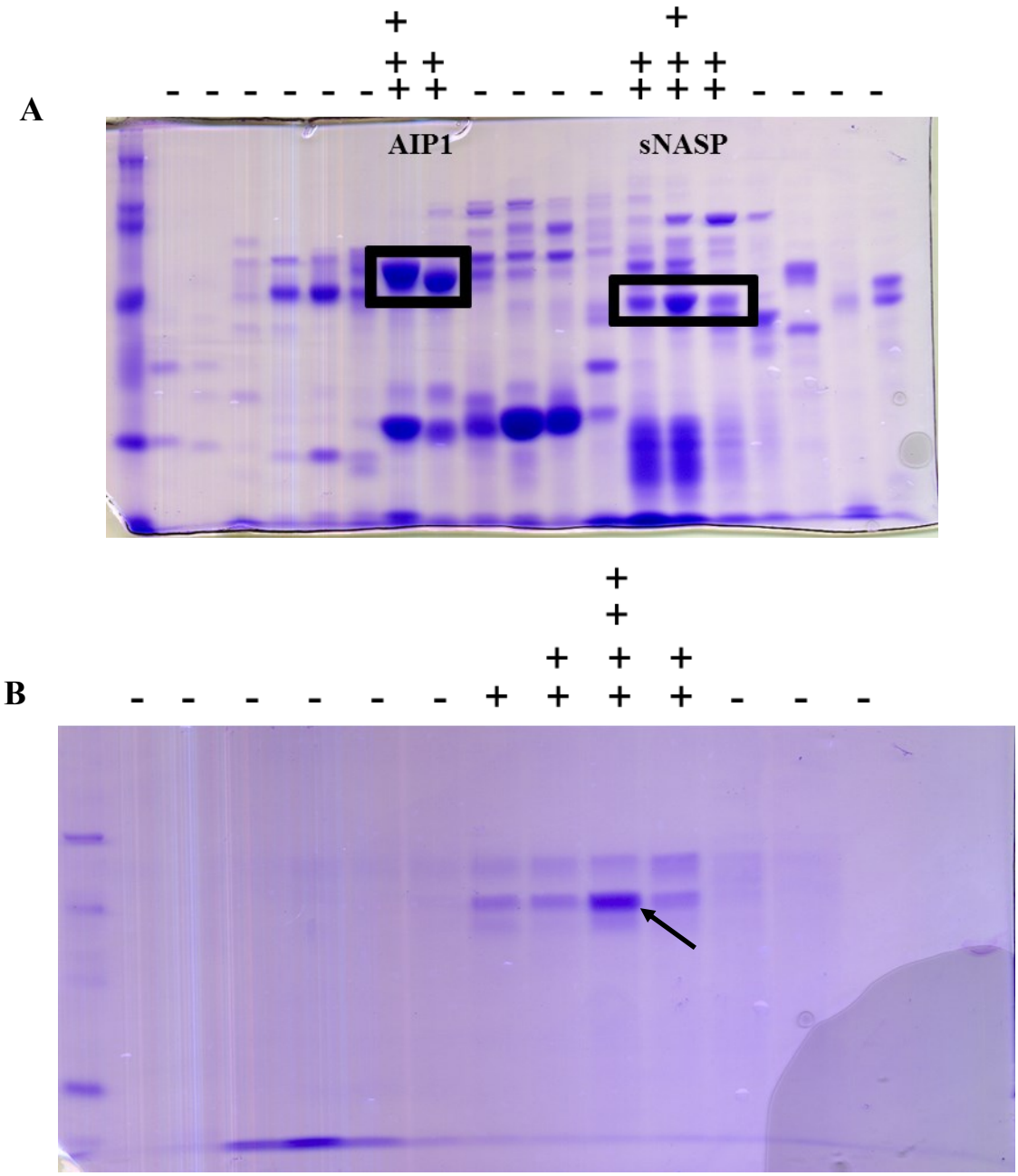


Figure B.1: sNASP Purified as AIP1-like Activity by Biochemical Reconstitution. A) During protein purification described earlier to isolate AIP1, a biphasic activity was noted at the phenyl column elution step. Two sets of fractions with AIP1 activity were subsequently isolated from the Mono-Q step of purification in an effort to separate the two apparent foci of activity. B) This unknown activity was pooled, dialyzed appropriately and eluted as a single activity off of a hydroxyapatite column. The corresponding band was identified by mass spectrometry as somatic nuclear anti-sperm protein (sNASP).

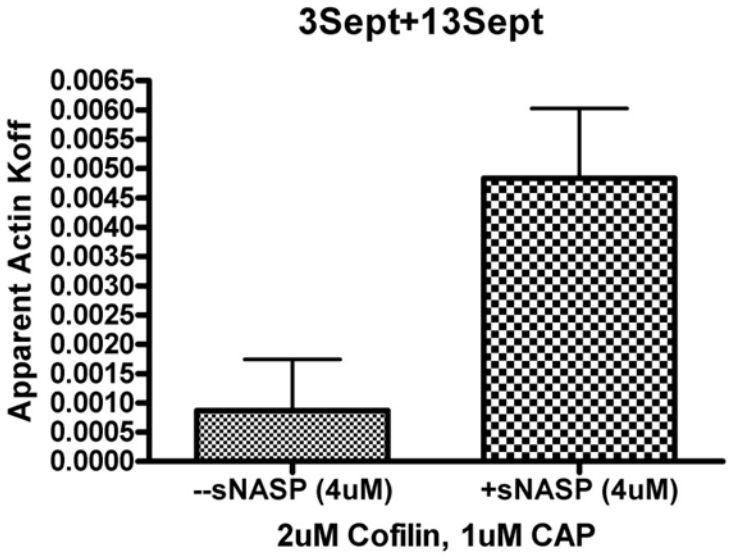


Figure B.2: sNASP is Sufficient to Rescue AIP1 Activity. In the presence of 2mM cofilin and 1mM CAP but no AIP1, recombinant sNSAP rescues actin disassembly activity. Actin disassembly activity augmented 3-5 fold over control, measured as loss of actin mass per time. Thus sNASP is a novel actin disassembly protein with a functional overlap with AIP1. This may define an AIP1-like functionality withon the nucleus, but neither this nor the possibility of post-translational modifications have yet been addressed.

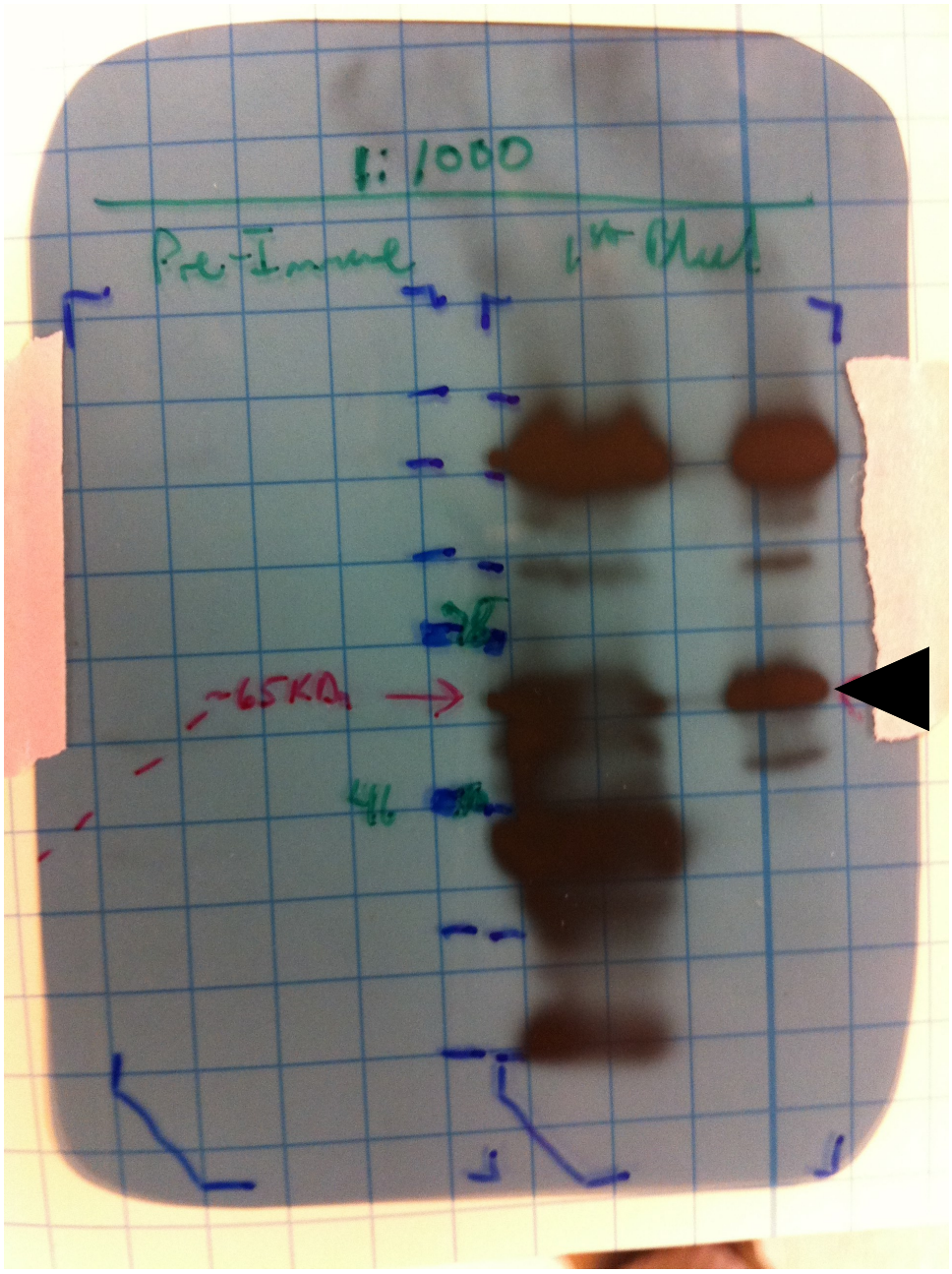


Figure B.3: Western Blot demonstrating specificity of sNASP Antibody. We raised a polyclonal antibody against recombinant human sNASP and demonstrate its affinity against human sNASP using HEK293 cell extract. Pre-immune rabbit serum is used on left side of blot, first bleed serum is used on right side, each at a 1:1000 dilution. sNASP is known to run unexpectedly high on gel electrophoresis (O'Rand 1992)., and is shown here running at approximately the expected 65KDa level (black arrowhead). A second band likely representing a dimer is also seen. Gel filtration will be employed to delineate the size of this bind and differentiate an sNASP dimer from the splice variant testicular nuclear auto-antigenic sperm protein (tNASP).

	DE-52 Flow-Through	70mL S Flow-through	70mL S Flow-Through + Coronin	Active Fractions Eluted from 70mL-Phenyl	S200 Active Fractions
Protein (mg)	8.10	3.85	3.85	0.166	0.084
Activity (U)	1.60	None	2.5	0.42	0.50
Specific Activity (U/mg)	198	None	649	2530	5950

Table B.1: Purification of sNASP: We purified sNASP as described in Material and Methods section. It should be noted that AIP1 co-purifies with sNASP through the 70mL phenyl elution step and activity is thus a conglomerate of the contribution of both sNASP and AIP1. It should also be noted that the second and third columns differ only by the addition of coronin, demonstrating that both AIP1 and sNASP share this dependence. Phenyl column activity units are subject to possible inaccuracy due to desalting method used; samples were spun through size exclusion beads and not dialyzed, and as there is no independent verification that all ammonium sulphate was retained in the beads there is a possibility that these activity readings are artificially low. This table will be reproduced afresh before journal publication.

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