Unveiling Srv2/CAP's Mechanism in Regulating Actin Dynamics

Kristin Little
Under the Supervision of
Bruce Goode, PhD
Faisal Chaudhry, PhD

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Brandeis University
Department of Biology
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Abstract

The ability of a cell to rapidly remodel its actin cytoskeleton is critical for many of its cellular functions. Actin exists in monomeric and filamentous forms and cycles between these forms in a process known as actin turnover. ATP-bound actin monomers polymerize into a filament, and as the filament ages, become ADP bound. When actin filaments are depolymerized by ADF/cofilin, which binds actin monomers and inhibits nucleotide exchange, a pool of cofilin-bound ADP-actin monomers is rapidly produced. Therefore, cells require additional factors to promote actin recycling, converting ADP-actin back to assembly-competent ATP-actin. Previous work in our lab showed that a critical player in recycling is Srv2/CAP; however, its mechanism is still not understood. Srv2/CAP has four separate functional domains, each with different binding partners and proposed functions. However, the functional relationship between these domains in the process of actin recycling is unclear. In my thesis work, I performed a genetic and biochemical dissection of Srv2/CAP and unexpectedly found that the two halves of the protein can function efficiently *in trans* to promote recycling. However, when I bisected the protein at a different boundary, the two halves were no longer functional. These biochemical results were mirrored *in vivo* when I tested the ability of the fragments to complement *SRV2* function in yeast. These data suggest that specific domains of Srv2/CAP must be physically linked to function, while others do not, which important implications for Srv2/CAP mechanism.

Introduction

Actin turnover: Remodeling the actin cytoskeleton

Cells require the ability to adapt and respond to rapidly changing environmental cues, which relies in part on a cell's ability to maintain a dynamic actin cytoskeleton. Actin is a highly conserved and ubiquitous protein that has the ability to polymerize into readily remodeled filamentous networks. Actin function lies at the heart of several critical processes such as maintenance of cell structure, endocytosis (Schafer, 2002), cell motility (Mitchison and Cramer, 1996), cytokinesis (Glotzer, 1997), (Glotzer, 2001), intracellular vesicle transport (Goode et al., 2000), and polarized growth, especially during cellular development (Moseley and Goode, 2006), (Goode et al., 2000), (Kilmartin and Adams, 1984), (Jacinto, 2003). Remodeling actin filaments necessitates the rapid turnover of actin monomers, coordinated by interactions with numerous highly conserved actin binding proteins. Actin monomer turnover is the process by which monomers dissociate from the filament ends and then are recycled back into assembly-competent form, enabling them to be used for new rounds of assembly (Moseley and Goode, 2006). These events are important for every living eukaryotic organism. My research project addresses this process in the context of the model organism *Saccharomyces cerevisiae (S. cerevisiae)*. This organism has proved useful for studying the actin cytoskeleton due to its distinct and easily visualized actin structures and its genetic and biochemical tractability.

Actin filaments possess an inherent polarity, with net assembly at one end (barbed end) and net disassembly at the opposite end (pointed end). Thus, at steady state, actin subunits "treadmill." Assembly competent ATP-bound actin monomers associate readily on the barbed, or fast growing end of the filament (Pollard, 1986). After addition to the filament, very rapid hydrolysis of the actin-bound ATP occurs, generating ADP+P_i-bound actin. Then, in a slower

step, the inorganic phosphate is released, yielding ADP-actin. Thus, the filament exists as a mosaic of actin subunit, mostly in ADP+P_i or ADP-bound form. The conversion from an ATP- to ADP-bound state alters actin's properties and promotes depolymerization (Korn et al., 1987). The pointed end is the older end and more enriched in ADP-actin than the barbed end. ADP-actin that dissociates from the pointed end must subsequently be recharged with ATP to become assembly competent. The completion of this actin turnover cycle requires the coordinated functions of several actin binding proteins, which promote first the rapid disassembly of actin filaments and then the rapid recycling of the monomers to ATP-bound form.

ADF/cofilin and profilin: Recycling actin monomers

One of the central players of this process is ADF/cofilin, a small, highly conserved protein with high affinity for both filamentous and monomeric ADP-actin. ADF/cofilin promotes rapid actin turnover by actively stimulating filament disassembly (Lappalainen and Drubin, 1997), most likely by physically twisting and fragmenting the filament (McGough et al., 1997). Once dissociated, ADF/cofilin maintains a strong affinity for ADP-actin monomers (Carlier et al., 1997) and completely blocks nucleotide exchange (Hayden et al., 1993). Thus, other factors are needed to promote the conversion of ADF/cofilin-bound ADP-actin monomers to ATP-bound actin monomers, and thus recharge actin for new rounds of assembly.

Another small, highly conserved protein that helps regulate actin turnover is profilin, which has properties that are almost opposite to those of ADF/cofilin, binding strongly to ATP-actin monomers and directing addition of ATP-actin monomers to the barbed end of the filament. Profilin has very low affinity for ADP-actin (Vinson et al., 1998) and consequently cannot compete with cofilin for binding ADP-actin monomers (Blanchoin and Pollard, 1998).

Furthermore, profilin is not sufficient to stimulate nucleotide exchange on cofilin-bound ADP-actin monomers, suggesting that another factor must be involved (Balcer et al., 2003), (Quintero-Monzon et al., 2009). The actin turnover model thus necessitates a middle-man to function between cofilin and profilin; this middle-man must be able to hand off monomers from cofilin to profilin, while promoting nucleotide exchange on actin, restoring it to the ATP-bound state.

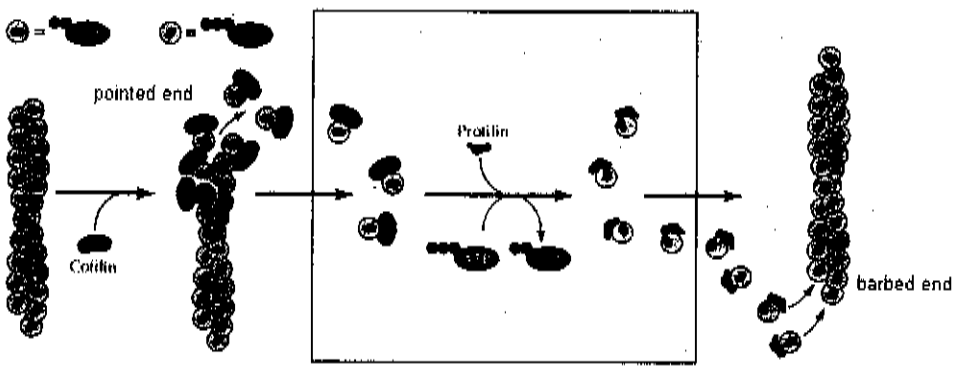


Figure 1.1--- ADF/cofilin severs actin filaments and profilin directs actin polymerization

ADF/cofilin severs the ADP-actin rich pointed (or -) end of actin filaments and sequesters ADP-actin monomers. Profilin sequesters ATP-actin monomers and directs polymerization at the barbed (or +) end of the actin filament. A separate factor must be present for the highlighted step to exchange ADP for ATP on the actin monomer and to enable the hand-off of the monomer from cofilin to profilin. (Cooper, 2000)

Srv2/CAP: The middleman of actin turnover

The *S. cerevisiae* Srv2 protein (also known as CAP (Cyclase-Associated Protein) in other organisms) was originally discovered as a gene required for Ras-activated adenylate cyclase activity due to its loss suppressing defects caused by an activated Ras-val^{19} allele. This implicated Srv2/CAP in cell signaling events, and since then Srv2/CAP has been shown to bind directly to adenylyl cyclase (Shima et al., 2000). Srv2/CAP was also demonstrated to have additional important functions critical for normal cell morphology and growth (Fedor-Chaiken et

al., 1990). It was discovered that the N-terminus of the protein was sufficient for its signaling activity, but the entire protein was critical for maintaining proper cell morphology and responses to nutrient extremes (Gerst et al., 1991). Somewhat unexpectedly, genetic studies indicated a functional relationship between Srv2/CAP and profilin, an important actin binding protein. Over-expression of profilin was found to partially suppress the morphological and nutritional defects associated with loss of the C-terminus, suggesting that the C-terminus of Srv2/CAP and profilin may operate in the same pathway (Vojtek et al., 1991). Actin monomer binding capability was later demonstrated for the C-terminus, linking it directly to cytoskeleton regulation (Freeman et al., 1995). Since then it has become apparent that this seemingly secondary actin regulatory function is conserved across all species. On the other hand, CAP does not interact with adenylate cyclase in many species, thus its actin associated functions appear to be more universal and central to its *in vivo* role (Hubberstey and Mottillo, 2002).

Srv2/CAP has the expected properties required of an actin turnover middle-man; it physically interacts with both cofilin and profilin, competitively binds ADP-actin monomers with very high affinity (K_d= 20nM) while having very low affinity for ATP-actin monomers (K_d= 2 μM), and promotes nucleotide exchange on actin (Chaudhry et al., 2007), (Moriyama and Yahara, 2002), (Mattila et al., 2004), (Quintero-Monzon et al., 2009), (Bertling et al., 2007). Its ability to carry out so many complex functions is reflected in its large size and its multi-domain structure. Srv2/CAP is 57.5 kDa and oligomerizes to form pentameric or hexameric actin-CAP complexes (Balcer et al., 2003). The protein consists of an N-terminal coiled coil domain that mediates oligomerization, followed by a dimeric helical folded domain, two polyproline motifs flanking an actin-monomer binding WH2 (Wasp Homolgy-2) domain, and a C-terminal β-sheet (Quintero-Monzon et al., 2009). Much work has been done in our lab to determine the

interactions of each domain and individually asses how each domain contributes to the overall function of the protein *in vitro* and *in vivo*.

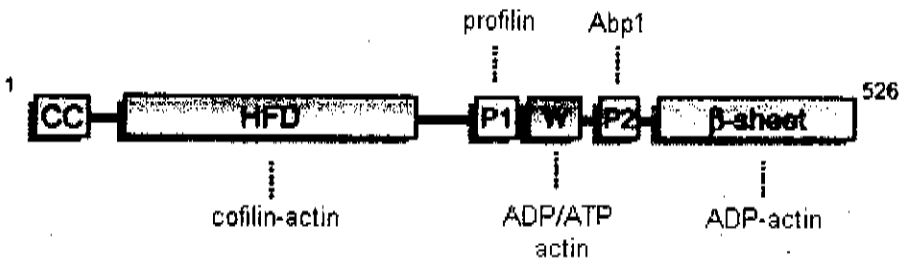


Figure 1.2--- Domains of Srv2/CAP and their known binding partners
Srv2/CAP is a multi-domain protein with multiple known binding partners. The N-terminal helical folded domain (HFD) binds ADF/cofilin-actin complexes, the first polyproline repeat (P1) binds profilin, the WH2 domain (W) binds ADP and ATP-bound actin monomers, the second polyproline repeat (P2) binds Abp1, and the β-sheet binds ADP-actin monomers. (Modified from Chaudhry et al, 2010)

The C-terminal half of Srv2/CAP has a high affinity for ADP-actin monomers (K_d = 18 nM) and could potentially compete with cofilin for ADP-actin binding (Mattila et al., 2004). It was thought this binding of the actin monomer to the C-terminus would remove ADF/cofilin's inhibition of nucleotide exchange and thus enable recycling the monomer. However, it now appears that the N-terminal half is also involved. Early on, it was discovered that the N-terminus of the human homolog of CAP could bind ADF/cofilin-actin complexes, but not ADF/cofilin or actin alone (Moriyama and Yahara, 2002). Our lab recently demonstrated that both halves of the protein must work together to effectively convert ADF/cofilin bound ADP-actin complexes to ATP-actin monomers (Quintero-Monzon et al., 2009). This is demonstrated in purified systems where addition of Srv2/CAP to ADF/cofilin and actin relieves the inhibitory effects of cofilin on nucleotide exchange (Chaudhry et al., 2007), (Moriyama and Yahara, 2002), (Mattila et al.,

2004). Thus, interaction of Srv2/CAP with ADF/cofilin-bound actin monomer has been shown to be important in recharging monomers into an assembly-competent form. This suggests that Srv2/CAP may not actually out-compete ADF/cofilin for binding ADP-actin, but instead directly weakens ADF/cofilin-actin interactions to promote more rapid dissociation of ADF/cofilin (Chaudhry et al., 2010).

While the interaction between Srv2/CAP and ADF/cofilin has been well documented, the precise mechanisms by which nucleotide exchange and hand-off of the ATP-actin monomer to profilin occur remain elusive. Recently, a critical role for the WH2 domain of Srv2/CAP in promoting nucleotide exchange was revealed (Chaudhry et al., 2010). Though the WH2 domain alone failed to promote nucleotide exchange on actin, this domain was crucial for full-length Srv2/CAP's ability to do so. The most critical sequence of this domain is a conserved LKKV motif, which binds actin monomers. Mutation of this motif led to strong defects in cell growth, morphology, and actin polymerization. Interestingly, high concentrations of a wild type (WT)-WH2 peptide could rescue the defective biochemical activity of a full length Srv2/CAP protein mutated at the LKKV motif, suggesting that the WH2 peptide can function *in trans* with full-length Srv2 protein to promote nucleotide exchange (Chaudhry et al., 2010).

The polyproline regions of Srv2/CAP also play important roles in actin turnover events. The first polyproline repeat may play a role in actin turnover through its direct physical interaction with profilin, allowing binding of both profilin and actin monomers (Lambrechts et al., 1997), (Drees et al., 2001). Mutation of this profilin-binding motif in Srv2/CAP caused a statistically significant increase in cell size compared to WT cells, but no obvious defects in cell growth. This suggests that while the Srv2/CAP-profilin interaction is not crucial, it may support an ancillary role in regulation the actin cytoskeleton. One model suggests that binding of profilin

to Srv2/CAP facilitates the transfer of profiln to ATP-actin monomers, which have low affinity for Srv2/CAP (Bertling et al., 2007). In addition, genetic interactions between Srv2/CAP and profilin further support a functional link between these two proteins (Vojtek et al., 1991). The second polyproline motif in localizes Srv2/CAP binds to the SH3 domain of Abp1 (Actin-binding protein) and is critical for Srv2/CAP localization to actin patches. Mutations at this site do not impact cell growth, suggesting that localization to patches is not critical for Srv2/CAP function (Balcer et al., 2003).

The current model of actin recycling still leaves us with many unanswered questions. Though the precise mechanism is not yet known, Srv2/CAP synergizes with ADF/cofilin to promote nucleotide exchange on the actin monomer. The N-terminal helical folding domain binds to ADF/cofilin-actin complexes. The C-terminal β-sheet and WH2 domains have the potential to out-compete ADF/cofilin for binding the ADP-actin monomer and are sufficient to promote nucleotide exchange. However, recent data suggest all three domains are required for optimal actin monomer recycling (Quintero-Monzon et al., 2009), (Chaudhry et al., 2010). Thus, it is known that the N-terminus, β-sheet and the WH2 domain are critical for Srv2/CAP function, but much remains to be learned about their functional relationships.

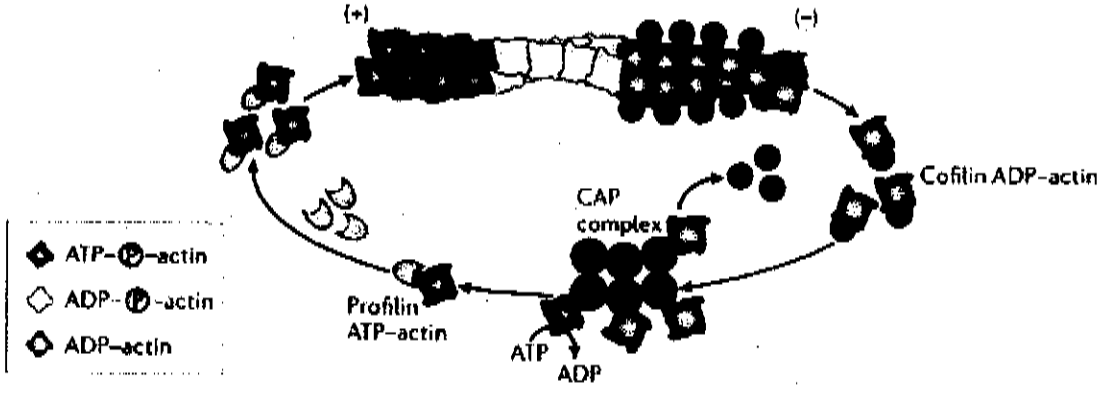


Figure 1.3 --- Coordinated activities of ADF/cofilin, profilin, and Srv2/CAP promote actin turnover
ADF/cofilin severs the actin filament at the pointed end and binds ADP-actin monomers. Srv2/CAP promotes nucleotide exchange on the actin monomer and passes the ATP-charged actin monomer to profilin. Profilin-bound ATP-actin monomers add to the fast growing barbed end of the filament (Modified from Baum, et al 2006)

Continuing genetic and biochemical dissection will shed new light on the functional relationships of the protein's domains, and how these relationships contribute to Srv2/CAP mechanism. Specifically, I wanted to learn more about the functional relationships of N-terminus, the WH2 domain, and the C-terminal β-sheet. For my thesis project, I investigated whether Srv2/CAP, when bisected at two different points, could carry out its function. Quite simply, the observation or absence of *in trans* synergy of two fragments will demonstrate whether certain domains must be physically linked for proper function. Understanding the functional relationship of the Srv2/CAP's several domains will lend insight into the mechanism of this ubiquitous and critical protein.

Results

In vitro analysis of *in trans* activity of Srv2/CAP

Previously, it was thought that only the C-terminus of Srv2/CAP functioned to recycle actin monomers. Revelation of the critical role of the N-terminus of Srv2/CAP in promoting actin turnover (Quintero-Monzon et al., 2009) suggested that both the N-terminal and C-terminal domains of Srv2/CAP function to promote actin-monomer recycling. The next step was to further investigate this potential mechanism with two sets of Srv2/CAP halves to learn more about the functional relationships amongst the protein's several domains. Specifically, I am investigating the functional relationships of the N-terminal helical folded domain, the WH2 domain, and the β-Sheet. For simplicity, the N will be referred to as N, the central region containing the WH2 domain W, and the β-Sheet B. Since the role of the N region in actin turnover still is undefined, I wanted to look further into the role of the N-terminus to determine whether any sites beside the known cofilin-actin binding site are important in actin regulation.

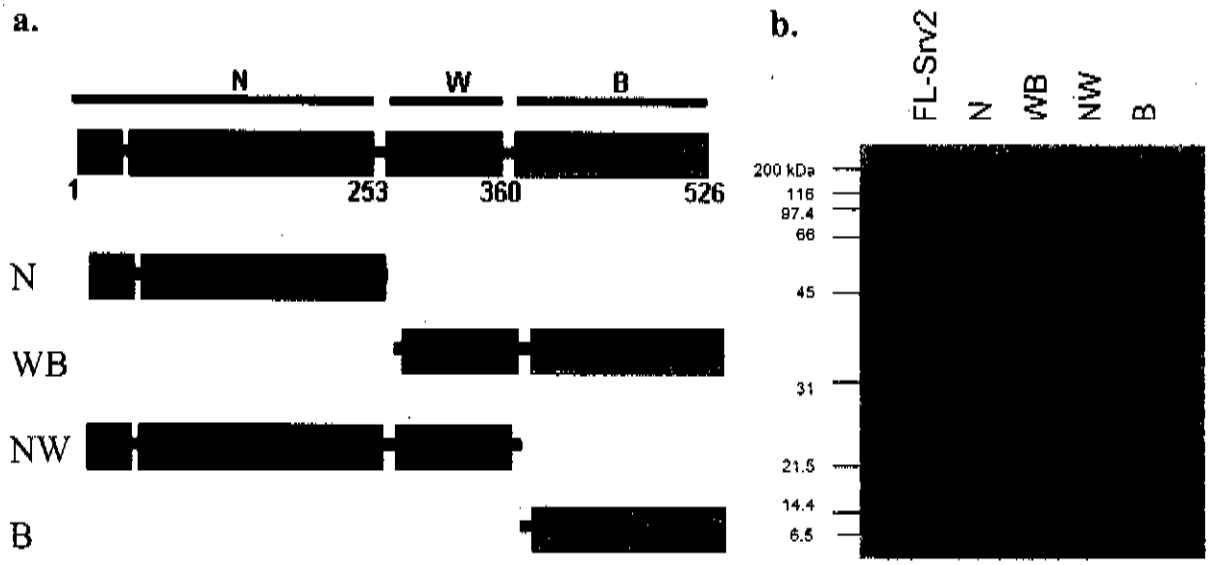


Figure 2.1--- Purified Srv2/CAP truncations
a. A comparison of the domains contained within each Srv2 truncation studied. FL-Srv2 is a 526 amino acid protein. N contains aa 1-259, WB aa 253-526, NW aa 1-359, B aa 360-526. Abbreviations: CC= coiled coil, HFD = helical folded domain, P = polyproline repeat, WH2= Wasp Homology-2 domain, Di = dimerization domain
b. Coomassie stained 12.5% acrylamide gel of full length and truncated Srv2/CAP proteins (boxed in red to distinguish from break-down products and contaminants)

6-His tagged Srv2/CAP fragments N, WB, NW, and B, along with full length Srv2/CAP were purified as described (Quintero-Monzon et al., 2009), (Mattila et al., 2004). One of Srv2/CAP's most critical roles is promoting nucleotide exchange on cofilin-bound ADP actin monomers; thus, measuring the ability of Srv2/CAP fragments to carry out this function is critical to assay protein function. A nucleotide exchange assay was carried out to measure the ability to promote nucleotide exchange on ADF/cofilin-bound ADP-actin monomers. As expected, nucleotide exchange is inhibited in control reactions containing only ADF/cofilin. Further addition of full length Srv2/CAP to the ADF/cofilin-bound ADP-actin strongly promotes nucleotide exchange. WB alone could promote nucleotide exchange at a small fraction of the rate of FL-Srv2; none of the other fragments could promote nucleotide exchange at all. Combined, however, the N and WB could synergize to carry out this function, albeit at a rate approximately

half that of FL-Srv2 (Figure 2.2A). These results suggest the potential for an *in trans* mechanism of these two halves of Srv2/CAP. The NW and B fragments, however, could not synergize at all to carry out this function, suggesting these fragments cannot work *in trans* to promote nucleotide exchange (Figure 2.2B). One clear implication of these data is that function requires physical linkage of B and W but not N and W.

Mutational analysis has shown that the N and WB regions of Srv2/CAP are required to facilitate actin turnover (Quintero-Monzon et al., 2009), however, these fragments were not tested. The ability of N and WB to promote actin turnover together *in vitro* would suggest these fragments might be capable of functioning *in trans*. To test this function, an inorganic phosphate release assay was used. This assay measures the dissociation of inorganic phosphate from aging filamentous ADP-Pi actin, as a way to monitor actin turnover rates. Both the termini separately show an insufficient ability to promote actin turnover, suggesting that both fragments are required to carry out this function biochemically. Combined, the two fragments promoted actin turnover at a greater rate than one would expect from an additive effect of their activities (Figure 2.2C). These results suggest that the two fragments can synergize *in vitro,* and that the fragments remarkably work *in trans*. This assay was not performed on the NW and B fragments due to protein availability, but should be performed in the future to better understand NW and B function.

Actin polymerization can occur spontaneously unless bound to another protein in such a way to prevent actin-actin binding. Profilin and full length Srv2/CAP, which both regulate actin polymerization *in vivo,* inhibit spontaneous assembly of actin by binding in such a fashion to actin monomers. To assess G-actin binding and test the ability of the Srv2/CAP fragments to inhibit spontaneous nucleation of actin monomers, an assay measuring spontaneous pyrene actin

assembly was utilized. Actin can spontaneously assemble without other protein factors, and inhibition of this spontaneous nucleation can indicate actin-monomer sequestering capability. B and NW both contain actin-binding domains; due to their K_d values, actin should nearly completely occupy these fragments at a concentration of 4 μM. Each peptide slightly inhibited spontaneous nucleation, but not nearly as well as full length Srv2/CAP, suggesting that the binding of either NW or B is not sufficient to prevent spontaneous actin polymerization. Interestingly, the presence of both NW and B were not able to work synergistically to inhibit spontaneous nucleation (Figure 2.3D). This suggests that when separated, the NW and B regions do not bind actin either in the same way or tightly as FL-Srv2/CAP. The abilities of the N and BW fragments to suppress spontaneous assembly will be tested soon.

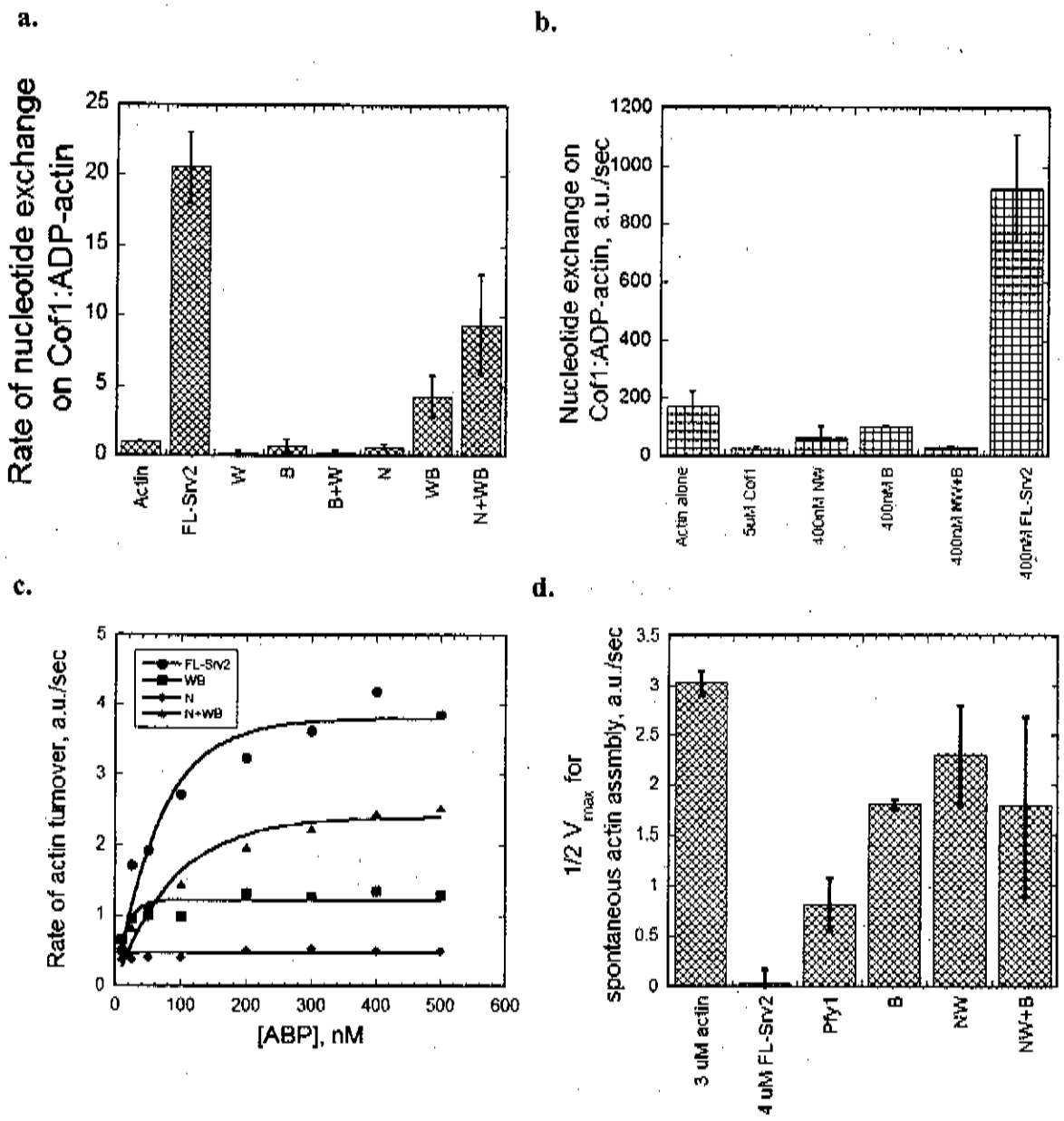


Figure 2.2--- *In trans* biochemical activity of Srv2/CAP fragments

a. Comparison of the effects of FL-Srv2/CAP, W, B, N, WB, and N+WB (400nM) on nucleotide exchange on 2μM cofilin-bound actin (F. Chaudhry, unpublished).

b. Comparison of the effects of FL-Srv2/CAP, NW, B, and NW+B (400nM) on nucleotide exchange on 2μM cofilin-bound actin

c. Comparison of effects of FL-Srv2/CAP, N, WB, and N+WB (0-500nM) on steady state actin turnover, measured by P_i release (F. Chaudhry, unpublished).

d. 3 μM monomeric actin (5% pyrene labeled) was allowed to polymerize in the presence of FL-Srv2/CAP, Srv2/CAP truncations NW and B, and profilin and the rates (½ V_{max}) of actin polymerization inhibition were graphed (control)

***In vivo* analysis of *in trans* activity of Srv2/CAP**

Next, I analyzed the functions of the fragments *in vivo*. I constructed mutant strains expressing integrated copies of N, WB, NW, and B fragments under the control of *SRV2* promoter and in a background lacking the endogenous *SRV2*. To test the abilities of these fragments to work *in trans*, I also integrated the N and C-termini, and the NW fragment and β-sheet at separate loci in *srv2Δ* cells. All of the fragments had an N-terminal 6-His tag.

I wanted to determine whether these truncation mutants showed growth defects, and whether Srv2/CAP halves functioning *in trans* could restore wild-type growth. Serial dilutions of log phase cultures were plated on YPD medium and allowed to grow at 25°, 30°, 24°, or 37°C. This assay reveals growth deficiencies and mutant phenotypes that are not otherwise observed at 25°C. *SRV2* cells are able to grow well at elevated temperatures, while *srv2Δ* cells show serious temperature sensitivity (TS) at 37°C. My *N* and *WB* strains were each mildly TS, and my *NW* and *B* strains were severely TS. Surprisingly, the *NW* cells show a worse growth phenotype than the *N*. Interestingly, the *N+WB* cells showed wild-type growth at elevated temperatures, while the *NW + B cells* were as severely TS as the *NW* or *B cells* (Figure 2.3). These *in vivo* data support my biochemical data showing that separate N- and C-termini can work together *in trans* to restore nearly WT phenotype, but the NW and B fragments cannot.

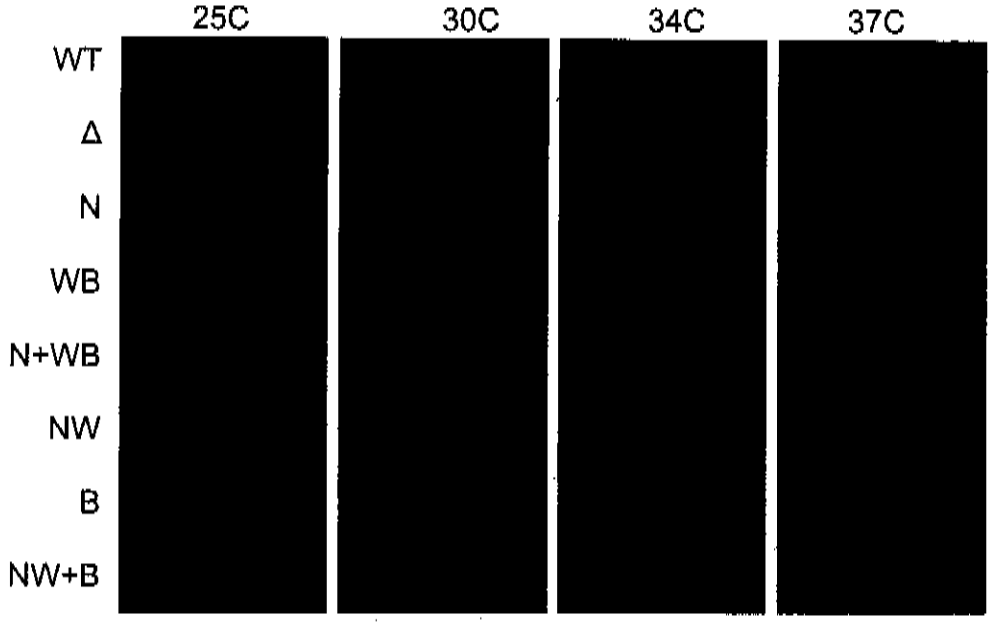


Figure 2.3--- Growth phenotypes
Cells were grown to an $OD_{600} = 0.3$ in YPD medium and a 1:10 serial dilution of each culture was plated on YPD and grown for two days at 25°, 30°, 24°, or 37°C.

Since Srv2/CAP plays such an important role in maintaining a dynamic actin cytoskeleton, it was important to analyze the actin networks in each of my truncation mutants. Loss of function mutations in the helical folded domain, WH2, or β-sheet domains of Srv2/CAP result in serious actin cytoskeleton defects (Quintero-Monzon et al., 2009), (Chaudhry et al., 2010), (Mattila et al., 2004). I wanted to determine whether the Srv2/CAP halves could synergize to maintain the integrity of the actin cytoskeleton. These mutant strains were stained with Alexa Phalloidin to visualize the actin cytoskeleton. Healthy wild-type cells show distinct actin cables running longitudinally through the mother cell and clear polarization of actin patches. In contrast, *srv2Δ* cells show a round, enlarged cell phenotype, few distinct cables, and depolarized actin patches (Figure 2.4). All of my mutant strains showed some level of actin morphology defects, though not to the same extent as the *srv2Δ* cells. All of the cells showed reduction of actin cables and more delocalized actin patches. The *NW* and B strains showed the most severe

phenotypes, with almost no visible actin cables and severe delocalization of actin patches. The *N* and *WB* strains showed a decrease in actin cables and some delocalization of actin patches, but not as severe (Figure 2.4). Interestingly, the *N* cells showed less actin defect than the *NW* cells.

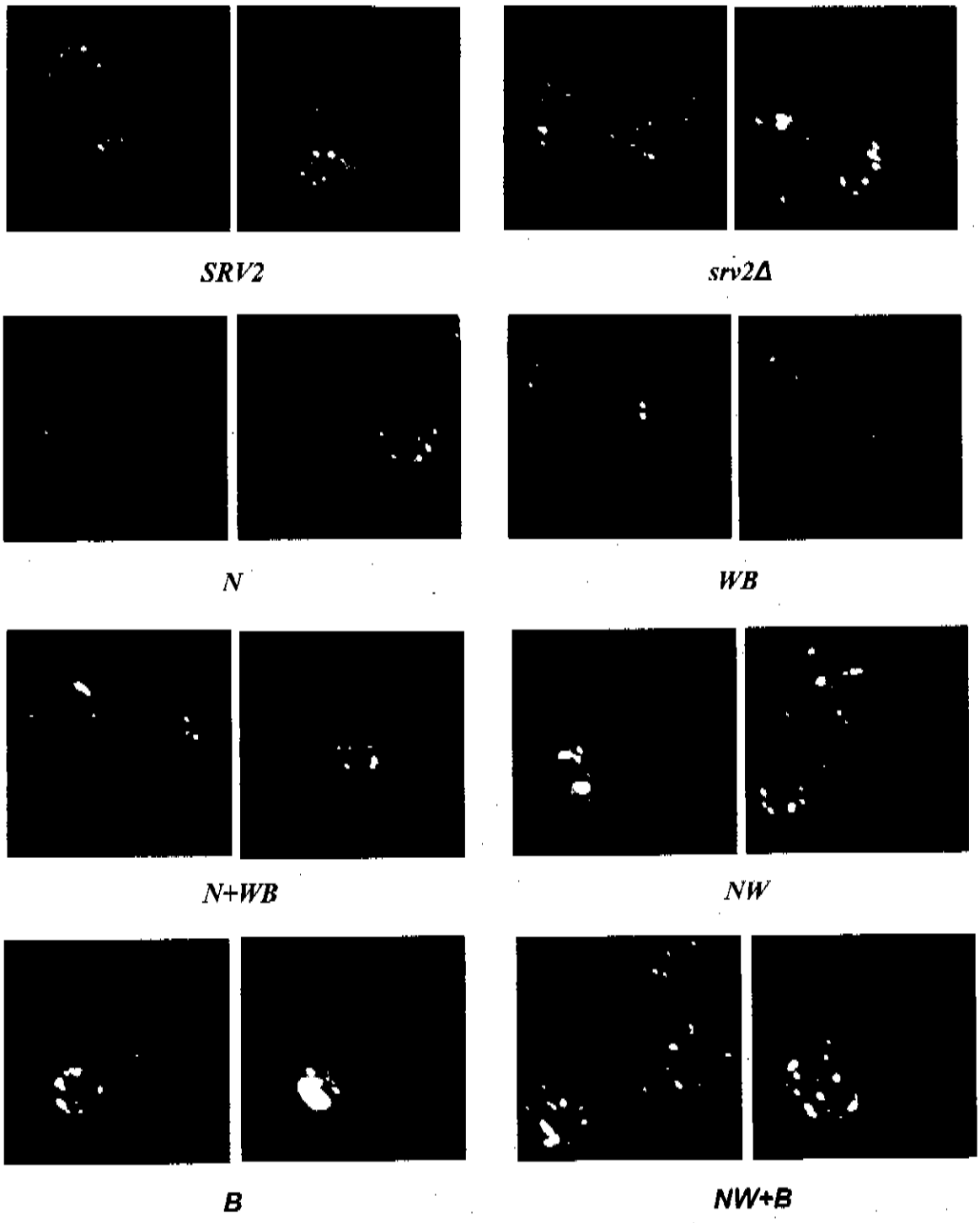


Figure 2.4--- Actin staining of Srv2/CAP mutants
SRV2, srv2Δ, N, WB, N+WB, NW, B, NW + B cells were grown to log phase in YPD medium and then stained with Alexa Phalloidin and visualized in the FITC channel. Wild type cells show visible longitudinal actin cables and some actin patches polarized to the bud.

Consistent with the biochemical data, the strain carrying both the separate N and C-termini showed a nearly wild-type phenotype (Figure 2.4). Overall, the cells have more prominent actin cables and fewer depolarized patches than either the *N* and *WB* cells, though they have some defects compared to wild-type cells. Since the two halves may not perfectly co-localize *in vivo* and thus always be able to function together, some level of defect is to be expected. The cells have visible longitudinal cables, but fewer in number than WT cells, and modest defect in actin patch polymerization. The strain expressing the separate *NW+B* fragments had more severe defects. These cells show prominent, disorganized actin cables, moreso than either the *NW* or *B cells*. These results further suggest that the N and C-termini can cooperate *in trans*, but the NW and B fragments cannot.

Along with measuring temperature sensitivity and actin morphology, analysis of the cell morphology and comparison with healthy wild-type cells provides more evidence of the functionality of Srv2/CAP. To analyze the cell morphology of my mutants, I analyzed Calcofluor-stained cells using CalMorph freeware, which can quantify the mother axis ratios and mother cell size among other phenotypes. The mother axis ratio compares the width and length of the mother cells, measuring a cell's roundness. WT cells are somewhat elliptical, whereas *srv2Δ* cells are rounder (Figure 2.5A) and larger (Figure 2.5B). The *N+WB* strain was the least round and most similar to the WT cells. The *B* strain showed a greatly enlarged cell shape, suprisingly even larger than the *srv2Δ* cells. The *N* and *N+ WB* cells showed sizes similar to the *SRV2* cells, while *WB*, *NW*, and *NW+ B cells* were similar in size to *srv2Δ* cells (Figure 2.6B). Interestingly, the N cells showed less defect in cell morphology than the NW cells. Overall, this suggests all of the cells have morphological defects, with the most severe and dominant defects

in *B* cells, the least severe defects in the *N+WB* cells. A round phenotype combined with delocalized patches suggests polarization and endocytic defects (Chant, 1999), (Shaw et al., 2001). This result could suggest that while a loss of Srv2/CAP affects a cell's polarity and endocytosis, the presence of *B* further interferes with these processes.

Interestingly, *all* of my mutant cells were measured rounder than even the *srv2Δ* cells (2.5A). Since the program seems to not be able to recognize which axis is longitudinal and latitudinal to the bud, there is a possibility that *srv2Δ* are elliptical but wider at the incorrect axis. This can be observed in Figure 2.4. This signifies a serious defect in polarity but would be measured by the program as a normal, elliptical cell. The other possibility is that my mutant cells are indeed rounder and show a greater morphological defect than *srv2Δ* cells. While a loss of Srv2/CAP affects a cell's polarity and endocytosis, the presence of isolated domains may further interfere with these processes. This result would indicate that the regions regulate the activity of each other as part of their role in Srv2/CAP function.

a. b.

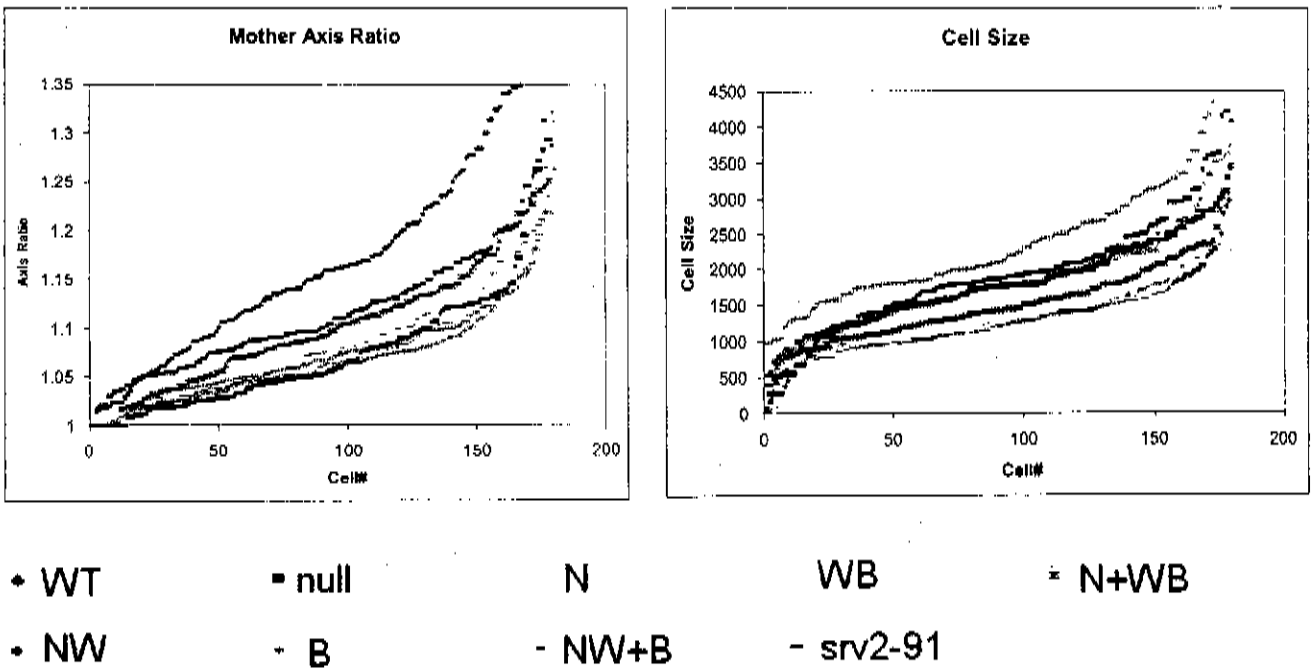
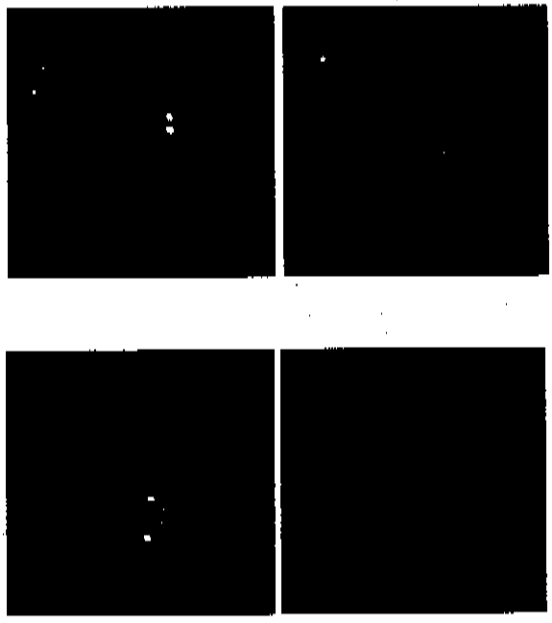


Figure 2.5--- Cell morphology
a. Graph of the mother axis ratio, which quantifies the "roundness" of the cells. A ratio of 1.0 signifies a perfectly round cell, while a ratio >1.0 signifies a more elliptical cell. This data was obtained through CalMorph freeware.
b. Graph of the mother cell size, obtained through CalMorph freeware.

Recently in our lab, it was shown that the N-terminus of Srv2/CAP plays an important role in actin regulation. A cofilin-actin binding site was identified in this region through a mutation analysis. The *srv2-91* allele disrupts this interaction (Quintero-Monzon et al., 2009). To determine if other regions beside this site are important in Srv2/Cap's actin regulation function, I also compared the phenotypes of my *WB* cells with cells containing the *srv2-91* mutation. I found that overall, the *WB* strain was sicker than the *srv2-91* strain. The *WB* and *srv2-91* cells had similar actin defects (Figure 2.6A) and both showed an exaggerated round phenotype (Figure 2.5A). While *WB* cells were enlarged slightly, however, the *srv2-91* cells were not enlarged (Figure 2.5B). Furthermore, the *WB* cells showed a more severe TS phenotype than the *srv2-91*

cells (Figure 2.6B). These results suggest that other sites in the N-terminus other than the known site for cofilin-actin binding are important for overall Srv2/CAP function. This fact has already been known, and at this point I cannot predict whether these other sites are important for actin regulation. It was previously discovered that loss of the N-terminal coiled coil domain introduces minor defects in Futhermore, the N domain region interacts with Ras and Cyr1 (Fedor-Chaiken et al., 1990), (Gerst et al., 1991), (Hubberstey and Mottillo, 2002); loss of this function could be contributing to the severity of the cell size and TS phenotypes, but more investigating will have to be done to rule out other regions in the N domain important for actin turnover. Future investigation should utilize cells with constitutively active Ras protein to lessen the possibility that phenotypes acquired through loss of the N domain are related to loss of Ras regulation (Fedor-Chaiken et al., 1990).

a.



b.

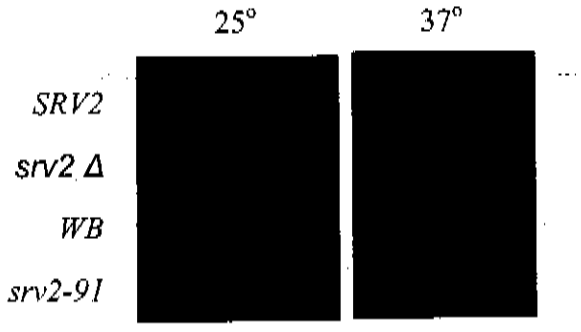


Figure 2.6--- Actin morphology and growth phenotypes of *WB* and *srv2-91*
a. *srv2-91* and *WB* cells were grown to log phase in YPD medium and then stained with Alexa Phalloidin.
b. Cells were grown to an OD600 = 0.3 in YPD medium and a 1:10 serial dilution of each culture was plated on YPD and grown for two days at 25° or 37°C

Discussion

Insight into the functional relationships of Srv2/CAP domains

Srv2/CAP is a large, multi-domain protein with two known cellular functions. Its interactions with Ras and Cyr1 are isolated to the N-terminal 36 amino acids, and the remainder of the protein facilitates actin filament turnover (Nishida et al., 1998). It was previously thought that the B fragment of Srv2/CAP was sufficient for its actin interactions (Freeman et al., 1995); however, recent studies confirm that the W also binds actin (Chaudhry et al., 2010). Furthermore, we now know the multiple domains are important for actin turnover function *in vitro* and *in vivo* (Quintero-Monzon et al., 2009). My results using fragments of Srv2/CAP support this observation; the loss of any domain results in actin and cell defects *in vivo* and no fragment tested is sufficient for actin recycling activities *in vitro*. To gain more insight into the mechanism of the protein, it is important to learn more about how the domains of this protein work together. Studying possible *in trans* mechanisms not only explores a possible new mechanism for Srv2/CAP function, but reveals more information about how the different domains function with each other.

The current model of Srv2/CAP function describes a complex mechanism relying on the unique properties of the N, W, and B regions (Quintero-Monzon et al., 2009). The first step of this mechanism seems clear cut; it is known that the N has affinity for ADF/cofilin-bound actin, and not actin or ADF/cofilin alone, and that disruption of ADF/cofilin-actin binding has serious effects on Srv2/CAP function (Quintero-Monzon et al., 2009). This likely means that Srv2/CAP functions by the N region binding cofilin-bound actin before the W and B regions come to play. The steps following this, specifically how the N, W, and B regions may interact to trigger

dissociation of ADF/cofilin and promote nucleotide exchange on the actin monomer, remain less defined.

The evidence that the N and WB can synergize, but not the NW and B regions, suggests that the central W region and the B region should be physically linked for efficient function. Previous data demonstrate the W and B regions can work *in trans* at very high concentrations support this model (Chaudhry et al., 2010). As previously mentioned, the W and B fragments bind ADP-actin monomers (Freeman et al., 1995), (Mattila et al., 2004), (Chaudhry et al., 2010). At the high concentrations required for *in trans* activity (4 μM), nearly all of the actin should be bound to both the WH2 domain of W (K_d = 1.5 μM) (Chaudhry et al., 2010) and B fragments (K_d = 20 nM) (Mattila et al., 2004). This suggests that the ability of the WB to facilitate nucleotide exchange depends on binding of both the WH2 and B regions simultaneously. The fact that the WB of Srv2 has greater affinity for ADP-actin than either WH2 or B region is another reason to believe that physical linkage of the WH2 and B regions is important. WH2 alone has a K_d around 1.5 μM and binds to ADP and ATP actin without preference (Chaudhry et al., 2010). While the β-sheet binds ADP-actin exclusively, its affinity for ADP-actin is twenty-fold higher when also physically linked to WH2. Binding by both of these regions could stabilize actin monomers in a conformation that opens up the nucleotide pocket and facilitates exchange of ATP for ADP. My data indicate that linkage of W and B facilitate the most efficient ADP-actin binding, leading to nucleotide exchange.

Previous data suggests that the ability of the N to bind ADF/cofilin is critical to Srv2/CAP's actin turnover function (Quintero-Monzon et al., 2009) and that Srv2/CAP and ADF/cofilin synergize to promote nucleotide exchange (Chaudhry et al., 2010). Furthermore, it is known that both the W and B regions are required for nucleotide exchange activity (Chaudhry

et al., 2010) and I have shown that this function requires linkage of these fragments *in vitro* and *in vivo*. My data supports two potential models for the Srv2/CAP mechanism: one where the N and WB fragments function simultaneously and one where they function sequentially. If the domains act sequentially, then it follows that the interaction of the N with the ADF/cofilin-actin complex must alter the properties of the ADF/cofilin-bound monomer. In a subsequent step, the WB region would bind the ADP-actin monomer and facilitate nucleotide exchange. Alternatively, the fragments could function simultaneously with ADF/cofilin to facilitate nucleotide exchange on the actin monomer; once the monomer is converted to an ATP-bound state, ADF/cofilin would then dissociate. More analysis is needed to distinguish which one of these two models might be at work.

Dominant effects of Srv2/CAP fragments

Through analysis of the Srv2/CAP fragments *in vivo*, I observed interesting phenotypes that suggested that separation of W and B domains may lead to dominant negative effects. Expression of NW causes stronger actin (Figure 2.4), cell growth (Figure 2.3), and cell morphology defects (Figure 2.5) than expression of the smaller N fragment. This suggests that the W region causes additional damage to the actin network when fused only to N. The WH2 domain of this region is known to bind both ATP and ADP-bound actin monomers, and the solitary peptide inhibits nucleotide exchange on actin (Chaudhry et al., 2010). Perhaps the NW region is thus able to promiscuously bind to actin monomers and inhibit nucleotide exchange. This binding may reduce the concentration of the cellular pool of ATP-actin monomers and thus actively disrupt actin assembly. Furthermore, the NW fragment may inhibit recycling of ADP-actin to ATP-actin. The biochemical data, which demonstrates that the NW and B fragments

together do not promote nucleotide exchange, supports this hypothesis. These results stress the importance of the W and B regions being physically linked for the C-terminus to perform its proper function, and show that the B and W alone can cause aberrant effects. A different explanation is that the NW peptide was not being expressed at the same level as the N peptide, which led to the worse phenotype for NW. Since all of the fragments have a 6-His tag, I will be able to use an anti-6-His antibody to evaluate expression levels.

Previously in our lab, an *srv2-98* mutant was created, which was a full-length protein with a non-functional WH2 domain. These cells showed an enlarged, round cell morphology, along with severe growth and actin defects. These data demonstrate that WH2 domain plays a critical role in Srv2/CAP function (Chaudhry et al., 2010). Interestingly, the *N* cells, which lack both a functional WH2 domain and B region, were far healthier than these mutants. These data suggest that the B region, when not linked to a functional W region, may cause worse defects than loss of both the W and B region.

I also observed that *B* cells have worse actin defects (Figure 2.4) and cell morphology defects (Figure 2.5) than the *srv2Δ* cells. This result fascinatingly indicates that the presence of B is more detrimental to the cell than complete loss of Srv2/CAP. The B region is known to bind ADP-actin monomers. Interestingly, it was also discovered that binding of the WB region of Srv2/CAP inhibits actin monomer addition at the generally fast-growing barbed end of the filament. Profilin, which binds ATP-actin monomers and directs addition of actin monomers to the barbed end, partially relieves this inhibition (Mattila et al., 2004). Profilin binds the W region. It could be that the B fragment, when not linked with W, actually interferes with actin polymerization by directing polymerization to the *pointed* end instead of the *barbed* end of the filament. Prominent short, disorganized and tangled cables were observed in some *B* cells and

many *NW+B* cells; this supports the model that *B* directly interferes with proper actin filament treadmilling and directed growth. This can be tested further using *in vivo* assays for actin turnover.

Another puzzling observation was that the *N* cells, while showing actin defects, were relatively healthy and showed normal cell morphology. Since it is known that the signaling functions of Srv2/CAP are contained in the N-terminus (Nishida et al., 1998), it could be that maintaining this function enables healthy cell morphology. To test this, I plan to create a strain expressing *N-91*, the N fragment carrying the 91 mutation that would interfere with ADF/cofilin-actin binding. If this strain shows greater defects than the *N* cells, we would understand that the interaction of the N domain with Ras explains the phenotype of *N* cells.

My thesis work has unveiled two important clues about the mechanism of Srv2/CAP. First, we now know that the N region of Srv2/CAP can be physically separated from the C-terminus without seriously affecting Srv2/CAP function. Second, we know that the two actin binding sites in the C-terminal W and B regions cannot be physically separated. This suggests that proper function of Srv2/CAP requires simultaneous action of both actin binding sites. Since efficient coordination of the functions of the N-terminal and C-terminal halves does not require their linkage or stable association, we now know that Srv2/CAP likely involves a sequential or transient but simultaneous interaction between the N and C terminal halves. This characterization of the functional relationships of the N, W, and B regions provides a clearer picture of overall Srv2/CAP mechanism in promoting actin turnover. Further structural information on these complexes will lend more insight into Srv2/CAP's mechanism.

In the future, it would be exciting to not only better understand Srv2/CAP's actin regulatory mechanism, but to explore how Srv2/CAP links cell signaling through Ras and

Adenylyl Cyclase and actin regulation (Fedor-Chaiken et al., 1990), (Hubberstey and Mottillo, 2002). Because of this linkage, Srv2/CAP plays a role in many critical cellular events which one may not expect from an actin turnover promoting protein. Loss of Srv2/CAP has also been implicated in the accumulation of ROS (reactive oxygen species) and the onset of yeast apoptosis (Gourlay and Ayscough, 2006). Another study has observed over-expression of the mammalian homolog of Srv2 in pancreatic cancer and demonstrated that mammalian CAP contributes to cancer cell motility (Yamazaki et al., 2009). Obviously no single scientist can tackle all of these issues, but these exciting observations and future avenues for study clearly demonstrate the importance of Srv2/CAP research to a broad range of biological questions.

Experimental Procedures

Yeast Strains and Plasmid Construction – Standard methods were used for all DNA manipulations and for growth and transformation of yeast strains. The promoter and coding regions of the WB and β-sheet polypeptide were each PCR-amplified and subcloned into the EagI and XhoI sites of pRS306. The promoter and coding regions of the HFD and NW polypeptides were likewise subcloned into the BamHI and XhoI sites of pRS305. Plasmids were linearized with XcmI and transformed into BGY330 (*srv2Δ::HIS3*). Transformants were selected for growth on –Ura for the WB and β-sheet and –Leu for HFD and NW. For purification of wild type and truncations of 6His-tagged Srv2 proteins, the polypeptides were PCR-amplified from a wild-type Srv2 template (pBG334) and subcloned into the NcoI and NotI sites of pHAT2. All plasmids were sequenced. To GFP-tag Srv2/CAP, a kanMX6 marked GFP tag was PCR-amplified according to the Longtine method (Longtine et al., 1998)and transformed into BGY1061 (*Abp1-RFP::His3).*

Protein Purification – Rabbit skeletal muscle actin was purified as previously described (Spudich and Watt, 1971), and converted to ADP-actin (Pollard, 1986). NW fragments were expressed in *Escherichia coli* BL21-RP cells. Cultures were grown to log phase at 37° and then induced with 0.4 mM IPTG for 16 h at 20°. Each 1 liter of cells was pelleted and purified as previously described (Chaudhry, et al in revision). Full length, WB, N, and β-sheet polypeptides and Cof1 were purified similarly as described (Quintero-Monzon et al., 2009), (Mattila et al., 2004)

Nucleotide Exchange Assays – Nucleotide exchange rates on ADP-actin monomers was determined by measuring change of fluorescence upon incorporation of ε -ATP (Sigma Aldrich). Briefly, 2 μM rabbit muscle actin in G-buffer (10 mM Tris, pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM DTT, no ATP) was monitored alone or mixed with proteins in Tris/NaCl buffer and added to 50 μM ε-ATP. The reaction was monitored at 350 nm excitation and 410 nm emission at 25° C in a fluorescence spectrophotometer for at least 250s (Photon Technology International, Lawrenceville, NJ). Exchange rates were determined from the initial, linear slopes of the curves.

Inhibition of Spontaneous Actin Assembly Assays – To test the ability of purified full length Srv2 or Srv2 fragments to inhibit spontaneous nucleation of actin monomers, 3μM 5% pyrene-labeled rabbit muscle actin monomers were combined with 4μM FL-Srv2, Srv2 fragments, or Pfy1, incubated for 2 minutes, and added to 0.05 volume of 20X initiation mix. Actin polymerization was monitored at 365 nm excitation and 407 nm emission in a Tecan fluorescence multi-well plate reader (Tecan Group Ltd, Mannedorf, Switzerland) at 25°C. Rates of actin polymerization were calculated from the slopes of assembly curves.

Phosphate Release Assays – Kinetics of P_i release during steady state turnover of F-actin was measured by P_i release using EnzChek kit (Molecular Probes). Variable concentrations of Cof1 and/or Srv2 fragments were mixed with polymerization buffer (2 mM $MgCl_2$, 0.5 mM ATP, 50 mM KCl), 0.2 mM 2-amino-mercapto-7-methylpurine ribunucleoside, and 0.1 units of purine nucleoside phosphorylase (PNP). Actin polymerization was initiated by addition of rabbit muscle actin monomers (8 μM final concentration). Absorbance at 360 nm was monitored at 25°C in a Tecan fluorescence multi-well plate reader (Tecan Group Ltd, Mannedorf, Switzerland). After actin assembly reached steady state, a constant P_i production was observed, and at that point data were collected for 15 minutes. After the data were corrected for path length, the slopes for steady state were determined from a linear curve fit.

Fluorescent Microscopy – To visualize actin organization, yeast cells were grown to log phase in YPD (yeast peptone dextrose) medium, fixed in 2% formaldehyde for 30 minutes at room temperature, and stained with Alexa488-phalloidin (Invitrogen). Images were acquired on a Zeiss E600 microscope (Thornwood, NY) equipped with a Hammamatsu Orca ER CCD camera (Bridgewater, NJ) running Openlab software (Improvision Inc, Waltham, MA).

Miscellaneous – The stability of wild type and truncated Srv2 proteins was determined by measuring fluorescence-monitored urea denaturation as previously described (Vinson et al., 1998)with the following modification: the urea stocks were prepared with 10mM potassium phosphate pH 7.0 buffer.

Supplementary Materials

I. Table of yeast strains used and generated

Name	Genotype
BGY330	*Matα, his3Δ200, ura3-52, leu2-3,112, trp1-1(am), lys2-801(oc), SRV2::HIS3*
BGY311	*Matα, his3Δ200, ura3-52, leu2-3,112, trp1-1(am), lys2-801(oc)*
BGY1061	*Matα, his3Δ200, ura3-52, leu2-3,112, ABP1-RFP::HIS3*
KLY1	*Matα, his3Δ200, ura3-52,leu2-3,112, trp1-1(am), lys2-801(oc), N::LEU2*
KLY2	*Matα, his3Δ200, leu2-3,112, trp1-1(am), lys2-801(oc), WB::URA3*
KLY3	*Matα, his3Δ200, ura3-52,leu2-3,112, trp1-1(am), lys2-801(oc), N::LEU2, WB::URA3*
KLY4	*Matα, his3Δ200, ura3-52, leu2-3, trp1-1(am), lys2-801(oc), B-srv2--srv2::URA3*
KLY5	*Matα, his3Δ200, ura3-52, leu2-3,112, trp1-1(am), lys2-801(oc), NW::LEU2*
KLY6	*Matα, his3Δ200, ura3-52,leu2-3,112, trp1-1(am), lys2-801(oc), N::LEU2, B-srv2::URA3*
KLY7	*Matα, his3Δ200, ura3-52, leu2-3,112, ABP1-RFP::HIS3, SRV2-GFP::KANmx6*

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