Published in final edited form as:

Cytoskeleton (Hoboken). 2014 June ; 71(6): 351–360. doi:10.1002/cm.21170.

Autonomous and *in trans* functions for the two halves of Srv2/CAP in promoting actin turnover

Faisal Chaudhry^{#1}, Silvia Jansen^{#1}, Kristin Little¹, Cristian Suarez², Rajaa Boujemaa-Paterski², Laurent Blanchoin², and Bruce L. Goode^{1,#}

¹Department of Biology, Rosenstiel Basic Medical Science Research Center, Brandeis University, Waltham, MA, 02454, U.S.A.

²Laboratoire de Physiologie Cellulaire & Végétale, Institut de Recherches en Technologies et Sciences pour le Vivant, Centre National de la Recherche Scientifique/Commissariat à l'énergie atomique et aux énergies alternatives/Institut National de la Recherche Agronomique/Université Joseph Fourier, Grenoble 38054, FRANCE

[#] These authors contributed equally to this work.

Abstract

Recent evidence has suggested that Srv2/CAP (cyclase-associated protein) has two distinct functional roles in regulating actin turnover, with its N-terminus enhancing cofilin-mediated severing of actin filaments and its C-terminus catalyzing actin monomer recycling. However, it has remained unclear to what degree these two activities are coordinated by being linked in one molecule, or whether they can function autonomously. To address this, we physically divided the protein into two separate halves, N-Srv2 and C-Srv2, and asked whether they are able to function *in trans* both in living cells and in reconstituted assays for F-actin turnover and actin-based motility. Remarkably, in F-actin turnover assays the stimulatory effects of N-Srv2 and C-Srv2 functioning *in trans* were quantitatively similar to those of intact full-length Srv2. Further, in bead motility assays and *in vivo*, the fragments again functioned *in trans*, although not with the full effectiveness of intact Srv2. From these data, we conclude that the functions of the two halves of Srv2/CAP are largely autonomous, although their linkage improves coordination of the two functions in specific settings, possibly explaining why the linkage is conserved across distant plant, animal, and fungal species.

Keywords

actin; cofilin; cyclase-associated protein; yeast; cytoskeleton

INTRODUCTION

Approximately 70% of proteins expressed in eukaryotic cells have a multi-domain layout, in which each domain can be considered as an evolutionary conserved module with a distinct structure and an independent function (Vogel et al., 2004; Han et al., 2007; Yang and

[#]Corresponding author: goode@brandeis.edu.

Bourne, 2009). Throughout evolution, combining and recombining domains has greatly increased the functional variability of proteins, creating a wide diversity of integrated functions, e.g., ‘scaffolding’ of two or more other proteins to form a complex, holding substrates near enzymes, or linking protein activities to membranes (Forslund and Sonnhammer, 2012). In some cases, the function of a protein with two or more domains depends on the direct connection between those domains. In other cases, the two domains continue to support largely independent activities, but their linkage can enhance function and/or provide regulatory control over that process. It is almost impossible to predict the nature of the relationships between domains; this can only be determined empirically through direct biochemical and genetic tests.

Srv2/CAP (cyclase-associated protein) is a highly conserved actin-binding protein that consists of multiple domains and has two separate activities in promoting actin turnover (reviewed in Ono et al., 2013). The C-terminal half of Srv2 (C-Srv2) consists of two actin-binding domains, a WH2 domain and an adjacent ‘ β -sheet’ domain (Dodatko et al., 2004). Together, these elements give C-Srv2 its high affinity interaction with ADP-G-actin ($K_d = 18$ nM) and 100-fold lower affinity interaction with ATP-G-actin ($K_d = 2$ μ M). C-Srv2 also competitively displaces cofilin from ADP-actin monomers and catalyzes nucleotide exchange (ATP for ADP) (Balcer et al., 2003; Mattila et al., 2004). Through these catalytic effects, C-Srv2 ‘recycles’ actin monomers, which is an important step in rapid actin network turnover in vivo. Further, these functions of C-Srv2 can be disrupted by specific point mutations in both the WH2 domain (e.g. *srv2-98*) and the β -sheet domain (e.g. *srv2-108* and *srv2-109*), and the same mutations cause defects in actin organization in vivo (Matilla et al., 2004; Chaudhry et al., 2010).

In contrast, the N-terminal half of Srv2 (N-Srv2) makes no apparent contributions to actin monomer recycling, and rather enhances cofilin-mediated severing (Chaudhry et al., 2013). N-Srv2 contains a short oligomerization domain (OD) at its N-terminus that mediates its hexamerization (Quintero et al., 2009; Chaudhry et al., 2013), followed by a domain with a six-helix fold, referred to as the helical folded domain (HFD) (Ksiasek et al., 2003; Yusof et al., 2005). The HFD alone forms anti-parallel dimers (Mavoungou et al., 2004), but within the context of intact N-Srv2 hexamers, the individual HFDs are organized as six symmetrical protrusions (or blades), giving N-Srv2 the appearance of a ninja throwing star (Chaudhry et al., 2013). Using dual-color total internal reflection fluorescence (TIRF) microscopy, we previously showed that N-Srv2, as well as full-length (FL) Srv2, enhances cofilin-mediated severing of actin filaments. Similar effects were also reported for CAP1, the mammalian homologue of Srv2/CAP (Normoyle et al., 2013), suggesting that this function is conserved. This view is further supported by the observation that point mutations at conserved surfaces on the HFD domain (*srv2-90* and *srv2-91*) disrupt N-Srv2 enhancement of cofilin-mediated severing, and the same mutations impair actin cytoskeleton organization in vivo (Chaudhry et al., 2013).

From the observations described above, it has been proposed that Srv2/CAP is a bifunctional protein, with distinct effects on actin filaments (mediated by its N-terminus) and actin monomers (mediated by its C-terminus). However, physical linkage between these two halves of the protein has been maintained across evolutionarily distant plant, animal, and

fungal species (Moriyama and Yahara, 2002; Balcer et al., 2003; Mattila et al., 2004; Chaudhry et al., 2007; Chaudhry et al., 2013). This prompted us to ask whether the two halves of the protein, after being physically separated, could function *in trans* both in living cells and in vitro.

RESULTS

N-Srv2 and C-Srv2 function *in trans* to promote steady state F-actin turnover in vitro

To test whether the two separate halves of Srv2/CAP are capable of functioning *in trans*, we used a biochemical assay for steady state F-actin turnover. This assay measures the rate of F-actin turnover by monitoring the kinetics of inorganic phosphate (P_i) release. When steady state is reached in the assays (our conditions), the rate of P_i release is directly proportional to rate of actin subunit turnover in filaments, assuming that subunits are not blocked from undergoing P_i release before their dissociation from filament ends.

Previously, we showed that yeast cofilin (Cof1) increases actin turnover in this assay by approximately 4-fold, and that addition of FL-Srv2 further increases the rate of turnover by another 4-fold (Quintero et al., 2009). Moreover, FL-Srv2 carrying point mutations in the N-terminal HFD (e.g., *srv2-91*) or the C-terminal WH2 domain (e.g., *srv2-98*) abolished the enhanced turnover effects (Quintero et al., 2009; Chaudhry et al., 2013). Thus, the ability of FLSrv2 to accelerate cofilin-mediated actin turnover requires activities present in both its N- and C-terminal halves.

Here, as expected, we observed that FL-Srv2 increased the rate of Cof1-mediated F-actin turnover in a concentration-dependent manner, and that neither N-Srv2 nor C-Srv2 alone was sufficient to stimulate turnover (Fig 1B). However, the combined action of physically separated N-Srv2 and C-Srv2 led to a strong stimulation of Cof1-mediated F-actin turnover, equivalent to FL-Srv2 at a range of concentrations. These observations demonstrate that the uncoupled halves of Srv2 are capable of functioning *in trans*, and remarkably, that they do as efficiently as FL-Srv2. Importantly, we could detect no physical interactions between purified N-Srv2 and C-Srv2 either in the presence or absence of actin monomers (ADP- or ATP-G-actin) (Fig 1C and 1D). These results suggest that N-Srv2 and C-Srv2 work independently, and support the view that they make independent contributions to actin turnover.

It is also interesting that we do not see an increase in steady state actin turnover in the presence of either half of Srv2 alone, but only when both halves, or intact Srv2, are present. This suggests that even if N-Srv2 enhances severing and disassembly by ~4-fold, then nucleotide exchange on cofilin-bound ADP-actin monomers becomes rate limiting, and the activities of C-Srv2 are required to 'see' the 4-fold increase in turnover from N-Srv2. Consistent with this view, C-Srv2 stimulates nucleotide exchange rate on cofilin-bound ADP-actin by far greater than 4-fold (Chaudhry et al., 2010), suggesting that filament disassembly is once again rate limiting in the presence of intact Srv2 or both halves.

N-Srv2 and C-Srv2 can function *in trans* in a reconstituted motility assay

We next investigated the ability of N-Srv2 and C-Srv2 to function *in trans* in a reconstituted actin-based bead motility assay. In this assay, beads coated with the VCA portion of WASp are mixed with G-actin, profilin, Arp2/3 complex, capping protein, and Cof1. Branched actin nucleation is initiated at the bead surface by VCA activation of Arp2/3 complex, leading to formation of actin comet tails that propel beads through the solution (Achard et al., 2010). When actin assembly reaches steady state, after ~40 min, comet tail length and bead velocity stabilize, allowing simultaneous evaluation of disassembly (indicated by steady state tail length) and monomer recycling (indicated by steady state bead velocity).

Consistent with previous reports (Reymann et al., 2011), we found that Cof1 was required for sustained bead motility (Figure 2A), and led to fragmentation of the actin tails, indicated by macroscopic release of large portions of the aged networks (Movie S1). Addition of FL-Srv2 elevated the rate of motility only in the presence, but not absence, of Cof1 (Figure 2B and 2C, reactions *b* and *g*; Movie S2). Simultaneous addition of N-Srv2 and C-Srv2 also increased the rate of motility specifically in the presence of Cof1 (Figure 2C, reactions *f*; Movie S4), though not to the same extent as intact FL-Srv2. The tail length also decreased in reactions containing Cof1 + FL-Srv2, or Cof1 + N-Srv2 + C-Srv2, consistent with enhanced F-actin disassembly (Fig 2B).

In the presence of Cof1, neither N-Srv2 nor C-Srv2 alone further increased the rate of bead motility, demonstrating that both halves of Srv2 are required for enhanced motility. In fact, in each case, motility was modestly decreased (Figure 2B and 2C, reactions *d* and *e*; Movies S3 and S4), although we still do not understand the basis for these negative effects. Importantly, addition of N-Srv2 alone to reactions containing Cof1 did not change the average tail length (not shown), but altered the qualitative manner in which the tails disassembled. With Cof1 (but no Srv2), comet tails were trimmed by the release of large portions of the aged actin network into the medium. With Cof1 + N-Srv2, tails dissolved rapidly at their trailing ends without releasing macroscopic portions of the network (Movie S3). These effects are consistent with more rapid and efficient filament severing by Cof1 + N-Srv2, producing smaller severing products.

Taken together, these data show that FL-Srv2 increases the rate of actin-based bead motility specifically in the presence of Cof1, and that this enhancement requires activities contained in both the N- and C-terminal halves of Srv2. Further, the two halves are capable of enhancing motility *in trans*, albeit not as efficiently as FL-Srv2.

N-Srv2 and C-Srv2 can function *in trans* in vivo

We next asked whether N-Srv2 and C-Srv2 can function *in trans* in cells. We previously showed that normal actin organization and dynamics *in vivo* depends on activities in both the N- and C-termini of Srv2 (Matilla et al., 2004; Quintero et al., 2009; Chaudhry et al., 2010; Chaudhry et al., 2013). However, the importance of the linkage between these two halves has not been tested. To address this issue, we generated strains expressing each half of Srv2 (integrated), *N-srv2* and *C-srv2*, individually and in combination, under the control of the *SRV2* promoter. We compared cell growth and actin organization in these strains to

control *SRV2* and *srv2* strains (Fig 3). *N-srv2* was sufficient to partially rescue the growth defects of *srv2* at 37°C (Fig 3A), whereas *C-srv2* minimally improved growth at this temperature. In contrast, co-expression of *N-srv2* and *C-srv2* almost fully rescued the growth defects of *srv2* at both 25°C and 37°C. We also compared the actin organization in these strains (Fig 3B-C). Consistent with previous reports, *srv2* caused a striking reduction in actin cable levels, depolarized actin patches, and enlarged cell morphologies (Fig 3B). These *srv2* defects were fully rescued by an integrated *SRV2* gene, whereas *C-srv2* failed to rescue the defects and *N-srv2* provided only a partial rescue of actin defects. However, co-expression of *N-srv2* and *C-srv2* provided a strong rescue of actin defects, suggesting that the two halves are capable of functioning *in trans* in vivo (Fig 3B-C). Nonetheless, co-expression of *N-srv2* and *C-srv2* did not rescue actin organization as well as full-length *SRV2*, consistent with our observations above in the bead motility assays, indicating that optimal function is achieved only with direct physical linkage of the two halves.

C-Srv2 function requires a direct link between its two actin monomer-binding domains

We next dissected C-Srv2, asking whether its two actin-binding domains, WH2 and β -sheet, must be physically linked in order for it to carry out its functions in actin monomer recycling. Our previous studies showed that both the WH2 and β -sheet domains make critical contributions to C-Srv2's ability to catalyze nucleotide exchange on cofilin-bound ADP-actin monomers (Matilla et al., 2004; Chaudhry et al., 2010). To test the importance of this linkage, we divided full-length Srv2 at a new boundary, between the WH2 and β -sheet domains, giving rise to NW and B constructs (Fig 4A). We then compared the activities of NW and B to those of FL-Srv2, C-Srv2, and N-Srv2 in both actin filament disassembly and actin monomer recycling assays.

In filament disassembly assays (Chaudhry et al., 2013), NW (which encompasses NSrv2) had similar effects to FL-Srv2 and N-Srv2, increasing the rate of Cof1-mediated disassembly (Fig 4B). The β -sheet (B) alone had no effects in this assay, consistent with C-Srv2 lacking this activity (Chaudhry et al., 2013). In monomer recycling assays, measuring the rate of nucleotide exchange on ADP-actin monomers, Cof1 inhibited recycling, and FL-Srv2 (100 nM) stimulated exchange both in the presence and absence of Cof1 (Fig 4C). This agrees well with earlier studies showing that FL-Srv2 and C-Srv2 each enhance nucleotide exchange (Chaudhry et al., 2013). In contrast, N-Srv2, NW, and B (100 nM) each failed to stimulate nucleotide exchange, as did NW + B. These results suggest that the two actin-binding domains of C-Srv2 do not function *in trans* to promote nucleotide exchange, and must be directly linked. At much higher concentrations (4 μ M), construct B showed some stimulatory effects on nucleotide exchange on G-actin, but only in the absence of Cof1 (Fig 4C). Thus, the WH2 domain is critical for promoting nucleotide exchange in the presence of cofilin. These results may be explained by previous observations showing that the WH2 increases β -sheet's affinity for ADP-G-actin by 20-fold and is required for displacing cofilin from ADP-actin monomers (Matilla et al., 2004). This is also consistent with WH2 domains and cofilin having overlapping binding sites on G-actin (Hertzog et al., 2004; Chereau et al., 2005; Paavilainen et al., 2008).

Finally, to test the relevance of these results *in vivo*, we generated integrated strains expressing NW and B, alone or in combination under control of the *SRV2* promoter, and compared them to cells co-expressing *N-srv2* and *C-srv2* (N+C) for cell growth and actin organization. This showed that the temperature sensitive growth defects and actin organization defects of *srv2* were almost fully rescued by co-overexpression of N+C, as mentioned above, but not by NW + B (Fig 5A and 5B). These *in vivo* observations are highly consistent with our biochemical observations, and emphasize that while N-Srv2 and C-Srv2 have distinct functions in actin turnover and can be uncoupled to function *in trans*, splitting the WH2 and β -sheet domains markedly disrupts C-Srv2 function.

DISCUSSION

Srv2/CAP is one of the signature components of the eukaryotic actin cytoskeleton, being ubiquitously expressed in all cell and tissue types, and evolutionarily conserved in sequence, activities, and *in vivo* functions across distant species of plants, animals, and fungi (Goode et al., 2006; Ono, 2013). Genetic studies have shown that loss of Srv2/CAP in a variety of model organisms leads to decreased actin filament network dynamics, accumulation of excessive F-actin, and defects in cell morphogenesis, endocytosis, cell motility, and cytokinesis (Noegel et al., 1999; Bertling 2004; Baum et al., 2000; Benlali et al., 2000). Thus, the *in vivo* importance of Srv2/CAP as a regulator of actin cytoskeleton dynamics has been firmly established. However, only recently have the underlying mechanisms by which Srv2/CAP governs actin dynamics been unraveled. These discoveries came more slowly, in part because Srv2/CAP has two separate roles in stimulating actin turnover, and in part because these effects are only revealed in the presence of cofilin.

The two roles of Srv2/CAP in stimulating actin dynamics

Initial biochemical studies on Srv2/CAP concluded that it is an ‘actin monomer sequestering protein’ based on the observation that C-terminal fragments inhibited spontaneous actin polymerization *in vitro* (Mattila et al., 2004; Noegel et al., 1999). However, this activity is observed for most proteins that bind actin monomers, due to their interference with actin-actin self-interactions required for nucleation. Indeed, similar inhibitory effects on nucleation *in vitro* have been reported for other actin monomer binding proteins such as profilin and WASP whose *in vivo* roles are in stimulating rather than inhibiting actin dynamics (Pollard et al., 1984; Higgs et al., 2000; Yang et al., 2000). For Srv2/CAP, its role in promoting rather than inhibiting actin dynamics did not become apparent until its activities were tested in the presence of cofilin (Moriyama and Yahara, 2002; Balcer et al., 2003). In these two studies, it was shown that the C-terminal half of Srv2/CAP catalyzes conversion of cofilin-bound ADP-G-actin to ATP-G-actin, recycling actin monomers and cofilin. The mechanism for these effects was later clarified when it was shown that C-Srv2 has 100-fold higher affinity for ADP- versus ATP-actin monomers (K_d 18 nM), competitively displaces cofilin from ADP-actin, and uses conserved surfaces on its WH2 and β -sheet domains for these functions (Mattila et al., 2004; Chaudhry et al., 2010). Collectively, these studies suggested that the monomer recycling function of Srv2/CAP is conserved, and indeed similar activities have been demonstrated for *Arabidopsis thaliana* CAP1 (Chaudhry 2007).

More recently, a second function for yeast and mammalian Srv2/CAP was discovered, in stimulating cofilin-mediated severing and disassembly of filaments (Chaudhry et al., 2013; Normoyle et al., 2013). Importantly, this new activity maps to distinct surfaces in the N-terminus rather than C-terminus of Srv2/CAP, and has been genetically uncoupled from the monomer recycling function (Chaudhry 2013). With this knowledge, it has become clear that Srv2/CAP is a bifunctional regulator of actin dynamics, serving to both enhance filament disassembly and catalyze monomer recycling. In this study, we found that both activities of Srv2/CAP are required for it to increase the rate of actin-based bead motility and to increase the rate of steady state F-actin turnover in P_i release assays. Further, we showed that both halves of Srv2 are required for function *in vivo*. The co-requirement for N-Srv2 and C-Srv2 activities can be explained by the interdependent relationship between filament disassembly and monomer recycling. At steady state, an increase in the rate of filament disassembly leads to a more rapid accumulation of ADP-actin monomers, and then the rate of monomer recycling becomes limiting for new assembly. Reciprocally, an increase in the rate of monomer recycling promotes filament growth, and then disassembly becomes rate limiting for sustained turnover. Thus, increasing the rates of disassembly and monomer recycling in parallel is required to achieve accelerated steady state turnover.

How are the two activities of Srv2/CAP coordinated during actin turnover?

We compared the activities of intact Srv2 and its two halves, alone and together (*in trans*) in three distinct settings, testing their ability to: (1) enhance actin-based bead motility, (2) enhance steady state F-actin turnover, and (3) complement *SRV2* function *in vivo*. Remarkably, in each of these cases, N-Srv2 + C-Srv2 complemented the functions of intact Srv2 *in trans*. One of the most important implications of these observations is that the distinct steps in actin turnover catalyzed by N-Srv2 and C-Srv2 do not need to occur in physical proximity. This is especially evident in the P_i release assays, where N-Srv2 + C-Srv2 functioned *in trans* as efficiently as intact Srv2 over a wide range of concentrations. Further, we could detect no direct interactions between the two halves, in the presence or absence of actin. In addition, if interactions between the two halves were important for their functions, the efficiency of these two halves functioning *in trans* (compared to intact Srv2) would be expected to diminish at lower concentrations, but this was not what we observed. From these observations we conclude that the functions of the two halves of Srv2/CAP are largely independent, and require minimal coordination or contact.

Nonetheless, in the bead motility assays and *in vivo*, the two halves functioning *in trans* did not reach the full effectiveness of intact Srv2, suggesting that under certain conditions the activities of the two halves are more effectively coordinated when linked. What key difference in these assays explains these observations? The major distinction is that in the P_i release assays, where the two halves are as effective as intact Srv2, filaments and other proteins are uniformly dispersed throughout the reaction, while in the bead motility assays (and *in vivo*) filaments are locally concentrated. Since N-Srv2 interacts with cofilin-decorated filaments, it is possible that in the intact protein, N-terminal interactions with filaments help localize C-terminally-mediated actin monomer recycling activities to the disassembling filaments to improve turnover efficiency. This might also explain why the two halves functioning *in trans* were not as effective as intact Srv2 specifically in bead

motility assays and in vivo. Alternatively, our data do not rule out the possibility of an intramolecular effect and/or direct feedback effect between the two halves of Srv2/CAP that contributes to the efficiency of their functions in vivo and in the bead motility assays. Perhaps the demands in the motility assays and in vivo are higher than in the P_i release assays, and under these conditions the interplay between the two halves begins to make a difference. Although we were not able to detect any physical interactions between N-Srv2 and C-Srv2, either in the presence or absence of G-actin, this does not rule out the possibility that when they are connected in the same molecule they physically interact and/or conformationally affect each others' activity states.

Conclusions

There are two broad conclusions we draw from our data. The first is that Srv2/CAP is a bifunctional protein comprised of two modules that operate to a large extent independently of one another. N-Srv2 and C-Srv2 have distinct structures, oligomerization states, biochemical activities, and genetic interactions. The two halves of the protein support genetically and biochemically separable mechanistic steps in promoting actin turnover. Within C-Srv2, we found that its two actin-binding domains, WH2 and β -sheet, must be connected for efficient activity in recycling actin monomers. In a previous study, we showed that N-Srv2 activity in enhanced filament severing requires both its oligomerization domain and HFD domain (Chaudhry et al., 2013). Thus, from all indications, N-Srv2 and C-Srv2 define two autonomous functional modules that have each been conserved across evolution in this protein family. The second major conclusion we reach is that despite their predominantly independent functions, a physical connection between the two modules can improve the efficiency of their concerted functions in vivo and in bead motility assays, pointing to a possible regulatory interplay between the two halves of the protein. These observations may explain why the linkage between these two distinct modules has been maintained across evolutionarily distant species of plants, animals, and fungi.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmid Construction

DNA coding regions of C-Srv2 (a.a. 253-526) and B (β -sheet domain) (a.a. 369-526) were PCR-amplified and subcloned into the *EagI* and *XhoI* sites of the yeast integration vector, pRS306 (*URA2*). The coding regions of N-Srv2 (a.a. 1-259) and NW (a.a. 1-368) were subcloned into the *BamHI* and *XhoI* sites of pRS305 (*LEU2*). These constructs were integrated BGY330 (*srv2 ::HIS3*) by linearizing the plasmids with *XcmI*, transforming, and growing cells on selective media lacking uracil and/or leucine. This produced strains KLY23 (*srv2 ::HIS3*, *C-srv2::URA3*), KLY6 (*srv2 ::HIS3*, *srv2-B::URA3*), KLY2 (*srv2 ::HIS3*, *N-srv2::LEU2*), KLY5 (*srv2 ::HIS3*, *NW-srv2::LEU2*), and KLY4 (*srv2 ::HIS3*, *N-srv2::LEU2*, *C-Srv2::URA3*). To generate plasmids for expressing and purifying 6His-tagged full-length (FL) Srv2 and Srv2 fragments, coding regions were PCR-amplified from an *SRV2* plasmid (pBG334) and subcloned into the *NcoI* and *NotI* sites of pHAT2. All plasmids were verified by sequencing.

Protein Purification

Rabbit skeletal muscle actin (RMA) was purified and gel filtered as described (Graziano et al., 2013). ATP-G-actin was converted to ADP-G-actin as described (Pollard, 1986). RMA was labeled with Oregon-green for TIRF microscopy, and pyrenyl-iodoacetamide for F-actin disassembly assays. *S. cerevisiae* profilin were expressed and purified from BL21(DE3) *E. coli* as described (Moseley et al., 2004). Cof1 was purified as described (Chaudhry, et al., 2013). 6His-tagged FL-Srv2, C-Srv2, N-Srv2, NW and B polypeptides were expressed in *E. coli* BL21-RP cells, and purified by nickel affinity chromatography and gel filtration using a Superose 6 column. All proteins besides actin were stored in Buffer A (20 mM Tris, pH 8.0, 50 mM KCl and 1 mM DTT).

Phosphate Release Assays for F-actin Turnover

The kinetics of steady state F-actin turnover were measured by phosphate (P_i) release using EnzChek kit (Life Technologies, Grand Island, NY). Variable concentrations of Srv2 polypeptides and Cof1 as indicated (Figure 1A) were mixed with polymerization buffer (2 mM $MgCl_2$, 0.5 mM ATP, 50 mM KCl), 0.2 mM 2-amino-mercapto-7-methylpurine ribonucleoside, and 0.1 units of purine nucleoside phosphorylase (PNP). Actin polymerization was initiated by addition of G-actin (8 μ M). Absorbance at 360 nm was monitored at 25°C in a Tecan fluorescence multi-well plate reader (Tecan Group Ltd, Männedorf, Switzerland). After actin assembly reached steady state, a constant rate of P_i production was observed, and data were collected for 15 min. The data were corrected for path length, and slopes (rates of turnover) were determined from a linear curve fit.

Supernatant Depletion Assays

Binding reactions contained 1 μ M soluble C-Srv2 and 10 μ M GST or GST-N-Srv2 immobilized on glutathione beads, either in the presence or absence of 2 μ M ATP- or ADP-G-actin as indicated (Figure 1C). Reactions were incubated for 20 min at 4°C, then the beads were pelleted, and equal volumes of supernatants were analyzed on Coomassie stained gels.

Reconstituted Bead Motility Assays

Carboxylate polystyrene microspheres (2 μ m diameter, 2.6% solids-latex suspension, Polysciences, Inc) were mixed with 2 μ M GST-pWA in buffer B (10 mM HEPES [pH 7.5], 0.1 M KCl, 1 mM $MgCl_2$, 1 mM ATP, and 0.1 mM $CaCl_2$) for 15 min at 20°C on thermoshaker. The beads coated with pWA were washed in buffer B containing 1% BSA and stored on ice for 48 hours in buffer B containing 0.1% BSA. GST-pWA coated beads were mixed with a motility medium containing 6 μ M G-actin, 18 μ M profilin, 150 nM Arp2/3 complex, 35 nM capping protein (α/β heterodimer), and 0.5 mM Cof1, FL Srv2, N-Srv2 and C-Srv2, in buffer B containing 1% BSA, 0.2% methylcellulose, 3 mM DTT, 0.13 mM DABCO, 1.8 mM ATP. Images were captured on an upright BX61 Olympus microscope equipped with a 40x dry objective (UPLFLN, NA=0.75), a XY motorized stage (Marzhauser, Germany) and a CoolSnap HQ2 camera (Roper Scientific, GmbH, Germany), driven by MetaMorph software (Molecular Devices, Downingtown, PA).

Cell Imaging

To visualize cellular actin organization, yeast strains were grown to early/mid log phase (OD_{600} 0.3-0.5) in YEPD (yeast-extract, peptone, 2% glucose) supplemented with adenine at 25°C, then fixed with 2% formaldehyde for 30 min, washed, and stained with Alexa488-phalloidin (Molecular Probes; Eugene, OR). Cells were imaged in mounting media (10 mM $NaPO_4$ (pH 7.4), 75 mM NaCl, 4.3 mM p-phenylenediamine, 0.01 mg/ml DAPI, 45% glycerol (v/v)) at room temperature (25°C) using an Axioskop-2 mot plus microscope (Carl Zeiss; Thornwood, NJ) equipped with a 100x plan apochromat objective (N.A. 1.40) (Carl Zeiss; Thornwood, NJ), and an ORCA-ER digital CCD camera (Hamamatsu Photonics; Bridgewater, NJ). Images were acquired using OpenLab software (Improvision; Lexington, MA) and analyzed using ImageJ (<http://rsbweb.nih.gov/ij/>).

F-actin Disassembly Assays

2 μ M F-actin (10% pyrene labeled) and 100 nM CapZ was added to different concentrations of Srv2 polypeptides and/or Cof1, then rapidly mixed with 3 μ M vitamin-D-binding protein (VDBP)/human plasma Gc-globulin (Sigma-Aldrich) to induce net disassembly. Decrease in fluorescence was monitored for 900 s at 25°C at 365 nm excitation and 407 nm emission in a fluorescence spectrophotometer (PTI).

Nucleotide Exchange Assays

ADP-G-actin (2 μ M) was mixed with different concentrations of Srv2 polypeptides and/or Cof1 in CDT buffer (0.2 mM $CaCl_2$, 0.2 mM DTT, 10 mM Tris pH 8.0) or buffer alone, and then added to 50 μ M ϵ -ATP. The reaction was monitored for 200 s at 25°C at 350 nm excitation and 410 nm emission in a fluorescence spectrophotometer (PTI, Photon Technology International, Lawrenceville, NJ). Exchange rates were calculated from linear fitting of the first 50 s of each reaction curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

REFERENCES

- Balcer HI, Goodman AL, Rodal AA, Smith E, Kugler J, Heuser JE, Goode BL. Coordinated regulation of actin filament turnover by a high-molecular-weight Srv2/CAP complex, cofilin, profilin, and Aip1. *Curr Biol*. 2003; 13:2159–69. [PubMed: 14680631]
- Baum B, Li W, Perrimon N. A cyclase-associated protein regulates actin and cell polarity during *Drosophila* oogenesis and in yeast. *Curr Biol*. 2000; 10:964–73. [PubMed: 10985383]
- Benlali A, Draskovic I, Hazelett DJ, Treisman JE. act up controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the *Drosophila* eye disc. *Cell*. 2000; 101:271–81. [PubMed: 10847682]
- Bertling E, Hotulainen P, Mattila PK, Matilainen T, Salminen M, Lappalainen P. Cyclase-associated protein 1 (CAP1) promotes cofilin-induced actin dynamics in mammalian nonmuscle cells. *Mol Biol Cell*. 2004; 15:2324–34. [PubMed: 15004221]
- Chaudhry F, Breitsprecher D, Little K, Sharov G, Sokolova O, Goode BL. Srv2/cyclase associated protein forms hexameric shurikens that directly catalyze actin filament severing by cofilin. *Mol Biol Cell*. 24:31–41. [PubMed: 23135996]

- Chaudhry F, Guerin C, von Witsch M, Blanchoin L, Staiger CJ. Identification of Arabidopsis cyclase-associated protein 1 as the first nucleotide exchange factor for plant actin. *Mol Biol Cell*. 2007; 18:3002–14. [PubMed: 17538023]
- Chaudhry F, Little K, Talarico L, Quintero-Monzon O, Goode BL. A central role for the WH2 domain of Srv2/CAP in recharging actin monomers to drive actin turnover in vitro and in vivo. *Cytoskeleton (Hoboken)*. 67:120–33. [PubMed: 20169536]
- Chereau D, Kerff F, Graceffa P, Grabarek Z, Langsetmo K, Dominguez R. Actin bound structures of Wiskott-Aldrich syndrome protein (WASP)-homology domain 2 and the implications for filament assembly. *Proc Natl Acad Sci U S A*. 2005; 102:16644–9. [PubMed: 16275905]
- Dodatk T, Fedorov AA, Grynberg M, Patskovsky Y, Rozwarski DA, Jaroszewski L, Aronoff Spencer E, Kondraskina E, Irving T, Godzik A, Almo SC. Crystal structure of the actin binding domain of the cyclase-associated protein. *Biochemistry*. 2004; 43:10628–41. [PubMed: 15311924]
- Forslund K, Sonnhammer EL. Evolution of protein domain architectures. *Methods Mol Biol*. 2012; 856:187–216. [PubMed: 22399460]
- Graziano BR, Jonasson EM, Pullen JG, Gould CJ, Goode BL. Ligand-induced activation of a formin-NPF pair leads to collaborative actin nucleation. *J Cell Biol*. 201;595–611. [PubMed: 23671312]
- Han JH, Batey S, Nickson AA, Teichmann SA, Clarke J. The folding and evolution of multidomain proteins. *Nat Rev Mol Cell Biol*. 2007; 8:319–30. [PubMed: 17356578]
- Hertzog M, van Heijenoort C, Didry D, Gaudier M, Coutant J, Gigant B, Didelot G, Preat T, Knossow M, Guittet E, Carlier MF. The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. *Cell*. 2004; 117:611–23. [PubMed: 15163409]
- Higgs HN, Pollard TD. Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J Cell Biol*. 2000; 150:1311–20. [PubMed: 10995437]
- Ksiazek D, Brandstetter H, Israel L, Bourenkov GP, Katchalova G, Janssen KP, Bartunik HD, Noegel AA, Schleicher M, Holak TA. Structure of the N-terminal domain of the adenylyl cyclase-associated protein (CAP) from *Dictyostelium discoideum*. *Structure*. 2003; 11:1171–8. [PubMed: 12962635]
- Mattila PK, Quintero-Monzon O, Kugler J, Moseley JB, Almo SC, Lappalainen P, Goode BL. A high-affinity interaction with ADP-actin monomers underlies the mechanism and in vivo function of Srv2/cyclase-associated protein. *Mol Biol Cell*. 2004; 15:5158–71. [PubMed: 15356265]
- Mavoungou C, Israel L, Rehm T, Ksiazek D, Krajewski M, Popowicz G, Noegel AA, Schleicher M, Holak TA. NMR structural characterization of the N-terminal domain of the adenylyl cyclase-associated protein (CAP) from *Dictyostelium discoideum*. *J Biomol NMR*. 2004; 29:73–84. [PubMed: 15017141]
- Moriyama K, Yahara I. Human CAP1 is a key factor in the recycling of cofilin and actin for rapid actin turnover. *J Cell Sci*. 2002; 115:1591–601. [PubMed: 11950878]
- Moseley JB, Goode BL. The yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiol Mol Biol Rev*. 2006; 70:605–45. [PubMed: 16959963]
- Moseley JB, Sagot I, Manning AL, Xu Y, Eck MJ, Pellman D, Goode BL. A conserved mechanism for Bni1- and mDia1-induced actin assembly and dual regulation of Bni1 by Bud6 and profilin. *Mol Biol Cell*. 2004; 15:896–907. [PubMed: 14657240]
- Noegel AA, Rivero F, Albrecht R, Janssen KP, Kohler J, Parent CA, Schleicher M. Assessing the role of the ASP56/CAP homologue of *Dictyostelium discoideum* and the requirements for subcellular localization. *J Cell Sci*. 1999; 112(Pt 19):3195–203. [PubMed: 10504325]
- Normoyle KP, Brieher WM. Cyclase-associated protein (CAP) acts directly on F-actin to accelerate cofilin-mediated actin severing across the range of physiological pH. *J Biol Chem*. 287:35722–32. [PubMed: 22904322]
- Ono S. The role of cyclase-associated protein in regulating actin filament dynamics - more than a monomer-sequestration factor. *J Cell Sci*. 126:3249–58. [PubMed: 23908377]
- Paavilainen VO, Oksanen E, Goldman A, Lappalainen P. Structure of the actin depolymerizing factor homology domain in complex with actin. *J Cell Biol*. 2008; 182:51–9. [PubMed: 18625842]
- Pollard TD. Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J Cell Biol*. 1986; 103:2747–54. [PubMed: 3793756]

- Pollard TD, Cooper JA. Quantitative analysis of the effect of Acanthamoeba profilin on actin filament nucleation and elongation. *Biochemistry*. 1984; 23:6631–41. [PubMed: 6543322]
- Quintero-Monzon O, Jonasson EM, Bertling E, Talarico L, Chaudhry F, Sihvo M, Lappalainen P, Goode BL. Reconstitution and dissection of the 600-kDa Srv2/CAP complex: roles for oligomerization and cofilin-actin binding in driving actin turnover. *J Biol Chem*. 2009; 284:10923–34. [PubMed: 19201756]
- Reymann AC, Suarez C, Guerin C, Martiel JL, Staiger CJ, Blanchoin L, Boujemaa Paterski R. Turnover of branched actin filament networks by stochastic fragmentation with ADF/cofilin. *Mol Biol Cell*. 22:2541–50. [PubMed: 21613547]
- Vogel C, Bashton M, Kerrison ND, Chothia C, Teichmann SA. Structure, function and evolution of multidomain proteins. *Curr Opin Struct Biol*. 2004; 14:208–16. [PubMed: 15093836]
- Yang C, Huang M, DeBiasio J, Pring M, Joyce M, Miki H, Takenawa T, Zigmond SH. Profilin enhances Cdc42-induced nucleation of actin polymerization. *J Cell Biol*. 2000; 150:1001–12. [PubMed: 10973991]
- Yang S, Bourne PE. The evolutionary history of protein domains viewed by species phylogeny. *PLoS One*. 2009; 4:e8378. [PubMed: 20041107]
- Yusof AM, Hu NJ, Wlodawer A, Hofmann A. Structural evidence for variable oligomerization of the N-terminal domain of cyclase-associated protein (CAP). *Proteins*. 2005; 58:255–62. [PubMed: 15558566]

Figure 1. N-Srv2 and C-Srv2 function *in trans* to promote F-actin turnover at steady state
A. Schematic of Srv2 domains and constructs. OD, oligomerization domain. HFD, helical folded domain. P1 and P2, polypeptide motifs. WH2, WASP-homology 2 domain. **B.** Concentration-dependent effects of Srv2 polypeptides on Cof1-mediated steady state F-actin turnover, measured by rate of phosphate (P_i) release from actin subunits. **C.** Supernatant depletion assays comparing binding of 1 μ M C-Srv2 to different concentrations of GST (control) or GST-N-Srv2 immobilized on beads. Beads were pelleted, and levels of C-Srv2 in the supernatants were quantified on Coomassie-stained gels by densitometry using ImageJ software. **D.** Supernatant depletion assays comparing binding of 1 μ M C-Srv2 to 10 μ M immobilized GST (control) or GST-N-Srv2, both in the presence and absence of 2 μ M ATP- or ADP-G-actin.

Figure 2. Effects of Srv2 polypeptides on actin-based bead motility

A. Images of steady state actin comet tails assembled for 60 min. At time zero, GST-pVCA-coated beads were mixed with 6 μ M Alexa568-actin monomers, 18 μ M profilin, 150 nM Arp2/3 complex, 35 nM capping protein, with and without 0.5 μ M Cof1 and/or 0.5 μ M Srv2 polypeptide (FL-Srv2, N-Srv2, C-Srv2, or N-Srv2 and C-Srv2). Tail formation was monitored by time-lapse fluorescence microscopy. Additional time points are shown in Figure S1. **B.** Changes in average comet tail length over time. **C.** Changes in average bead motility rate over time. In B and C, error bars represent SEM (n= 20-30 propulsive actin comets).

Figure 3. In vivo analysis of *N-srv2* and *C-srv2* in trans functions

A. Strains were grown to log phase, serially diluted, plated on YEPD plates, and grown for 2 days at 25 and 37°C. **B.** Representative images of cells grown at 25°C, fixed and stained with Alexa488-phalloidin. **C.** Scored actin phenotypes (n>100 cells).

Figure 4. Effects of Srv2 polypeptides on rates of F-actin disassembly and G-actin nucleotide exchange

A. Schematic of Srv2 constructs. Abbreviations as in Figure 1A. **B.** Effects of Srv2 polypeptides on kinetics of Cof1-mediated F-actin disassembly. At time zero, 3 μ M F-actin (5% pyrene labeled), 100 nM Cof1, and/or 100 nM Srv2 polypeptides, was mixed with an actin monomer-sequestering agent (3 μ M vitamin D-binding protein). Control reactions contained each Srv2 polypeptide in the absence of Cof1. **C.** Effects of Srv2 polypeptides (100nM) on kinetics of etheno-ATP nucleotide exchange on 2 μ M ADP-G-actin in the presence and absence of 5 μ M Cof1. Rates were averaged from two experiments. Error bars are SD.

Figure 5. In vivo analysis of *srv2* NW and B functions

A. Strains were grown to log phase, serially diluted, plated on YEPD plates, and grown for 2 days at 25 and 37°C. **B.** Representative images of cells grown at 25 and 37°C, fixed and stained with Alexa488-phalloidin.