Mechanism and biological role of profilin-Srv2/CAP interaction

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Summary

Profilin and cyclase-associated protein (CAP, known in yeast as Srv2) are ubiquitous and abundant actin monomer-binding proteins. Profilin catalyses the nucleotide exchange on actin monomers and promotes their addition to filament barbed ends. Srv2/CAP recycles newly depolymerized actin monomers from ADF/cofilin for subsequent rounds of polymerization. Srv2/CAP also harbors two proline-rich motifs and has been suggested to interact with profilin. However, the mechanism and biological role of the possible profilin-Srv2/CAP interaction has not been investigated. Here, we show that *Saccharomyces cerevisiae* Srv2 and profilin interact directly (K_D ~1.3 μM) and demonstrate that a specific proline-rich motif in Srv2 mediates this interaction in vitro and in vivo. ADP-actin monomers and profilin do not interfere with each other's binding to Srv2, suggesting that these three proteins can form a ternary complex. Genetic and cell biological analyses on an Srv2 allele (*srv2-201*) defective in binding profilin reveals that a direct interaction with profilin is not essential for Srv2 cellular function. However, *srv2-201* causes a moderate increase in cell size and partially suppresses the cell growth and actin organization defects of an actin binding mutant profilin (*pfy1-4*). Together these data suggest that Srv2 is an important physiological interaction partner of profilin.

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Introduction

The actin cytoskeleton is highly dynamic and must be remodelled rapidly to perform its essential roles in cell morphogenesis and motility, and other membrane-associated events such as endocytosis, phagocytosis and cytokinesis. Organization and dynamics of the actin cytoskeleton are regulated by a large set of proteins that interact with monomeric and/or filamentous actin (reviewed by Pollard and Borisy, 2002; Paavilainen et al., 2004; Nicholson-Dykstra et al., 2005). A key player in the rapid turnover of actin networks is ADF/cofilin, which severs and/or depolymerises actin filaments (Carlier et al., 1997). This leads to the rapid accumulation of ADF/cofilin-bound ADP-actin monomers, which are inhibited from undergoing nucleotide exchange. Two conserved cellular factors implicated in recycling ADP-actin monomers to an ATP-bound and assembly-competent state are profilin and Srv2.

Profilins are small proteins (12-15 kDa) that bind ATP-actin monomers with high affinity and catalyze nucleotide exchange (ATP for ADP) on actin (Witke, 2004). When bound to an actin monomer, profilin inhibits spontaneous filament nucleation and blocks addition of monomers to the pointed ends of filaments. On the other hand, profilin promotes addition of actin subunits to the free barbed ends of filaments (Pantaloni and Carlier, 1993) and targets actin growth to barbed ends capped by regulators such as Ena/VASP and formins (reviewed by Krause et al., 2003; Goode and Eck, 2007). Genetic studies have demonstrated that profilin plays an essential role in regulating actin dynamics in flies, yeasts, mammals and *Dictyostelium* (Witke, 2004). Together with ADF/cofilin, Arp2/3 complex and capping protein, profilin is one of the core components sufficient to promote actin-based motility of *Listeria* in vitro (Loisel et al., 1999). In addition to binding G-actin, profilin interacts with phosphoinositides and proline-rich sequence motifs. The PtdIns(4,5)P_2- and actin-binding surfaces on profilin partially overlap and thus actin binding is inhibited by phosphoinositides (Skare and Karlsson, 2002; Lambrechts et al., 2002). However, the polyproline-binding site on profilin is separate and completely non-overlapping with its actin-binding site; thus, profilin can simultaneously interact with poly-L-proline and G-actin (Mahoney et al., 1997). Poly-L-proline binding is thought to target profilin–actin-monomer complexes to sites of rapid actin assembly in cells by delivering actin monomers to polymerizing filament barbed ends (Witke, 2004). Indeed, profilin binds to many proline-rich proteins that are known to promote filament barbed end elongation, including VASP, N-WASP and formins (Reinhard et al., 1995; Suetsugu et al., 1998; Vavylonis et al., 2006). Consistent with these roles, mutant profilins defective in polyproline binding fail to complement profilin-null cells in yeast (Wolven et al., 2000; Lu and Pollard, 2001).

Cyclase-associated protein (CAP) is a highly conserved actin monomer binding protein found in all eukaryotic organisms examined (Hubberstey and Mottillo, 2002). It was first identified in budding yeast as both a factor associated with adenylyl cyclase (Field et al., 1990) and a suppressor of the activated *ras* allele (Fedor-Chaiken et al., 1990). The yeast CAP homolog is named Srv2 and binds to adenylyl cyclase

complex through a short segment of its N-terminus, whereas the C-terminal half of the protein binds to actin monomers and plays a central role in regulation of actin dynamics in cells (Gerst et al., 1991; Kawamukai et al., 1992; Freeman et al., 1995; Yu et al., 1999; Mattila et al., 2004). Recent study also revealed that Srv2 links actin dynamics and Ras signaling during apoptosis (Gourlay and Ayscough, 2006). However, the adenylyl cyclase binding activity does not appear to be conserved in animals and plants, whereas its role in regulating the actin cytoskeleton is conserved in all Srv2 or CAP proteins tested so far (Hubberstey and Mottillo, 2002).

Srv2/CAP has been demonstrated to be a part of a multimeric complex that recycles actin monomers from ADF/cofilins, making them available for new rounds of filament assembly (Moriyama and Yahara, 2002; Balcer et al., 2003; Bertling et al., 2004). In budding yeast, the native Srv2 complex oligomerizes apparently as a hexamer with each Srv2 molecule capable of binding to one actin monomer (Balcer et al., 2003). Deletion of the *SRV2* gene in yeast results in abnormally large cell size, random budding pattern, and defects in actin distribution (Gerst et al., 1991; Votjek et al., 1991). The role of Srv2 or CAP in regulating the actin cytoskeleton is conserved in a wide range of eukaryotes: Srv2 or CAP defective cells have severe abnormalities in actin organization in *Dictyostelium discoideum*, *Drosophila,* plants and mammals (Gottwald et al., 1996; Noegel et al., 1999; Baum et al., 2000; Benlali et al., 2000; Barrero et al., 2002; Bertling et al., 2004). In addition, mutations that specifically disrupt actin monomer binding by yeast Srv2 cause defects in cell morphology and actin organization, demonstrating that G-actin binding plays a crucial role in Srv2 function in vivo (Mattila et al., 2004).

Srv2 harbors two conserved proline-rich regions and based on high-throughput yeast two-hybrid screens and biochemical studies using synthetic peptides it has been proposed that Srv2 may interact with profilin (Drees et al., 2001; Lambrechts et al., 1997). However, the mechanism of this potential interaction and its role in actin dynamics has not been investigated. Here, we demonstrate that yeast Srv2 directly interacts with profilin and we have mapped the binding interfaces of these proteins. Furthermore, co-immunoprecipitation of Srv2 and profilin from cell extracts and genetic analyses with an *srv2* mutant that specifically disrupts profilin-binding demonstrate that the Srv2-profilin interaction occurs in vivo and contributes to the regulation of cellular actin organization.

Results

Yeast Srv2 interacts directly with profilin

To examine whether yeast Srv2 interacts with profilin, we expressed and purified its C-terminal half ($Srv2_{253-526}$). This Srv2 fragment contains the complete actin-binding site of the protein (Mattila et al., 2004) and two proline-rich regions (P1 and P2) suggested to be involved in profilin interactions. Interaction of this Srv2 fragment with profilin was examined by a supernatant depletion pull-down assay in which the ability of bead-immobilized GST-$Srv2_{253-526}$ to deplete profilin from the supernatant was measured. The assay was carried out at physiological salt concentration and pH 7.5, and the average values of five independent experiments are shown in Fig. 1B. Wild-type $Srv2_{253-526}$ efficiently decreased the amount of profilin remaining in the supernatant, suggesting that these two proteins directly interact in vitro (Fig. 1A,B).

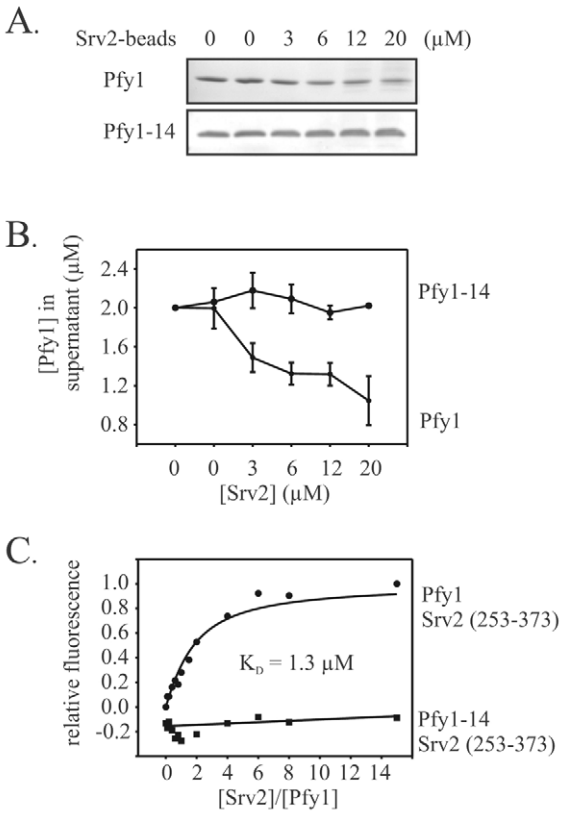

Fig. 1. Srv2 binds directly to profilin (K_D=1.3 μM). (A) Profilin binding to GST-$Srv2_{253-526}$ on glutathione agarose beads measured by supernatant depletion pull-down assays. Reactions contained 2 μM wild-type profilin (Pfy1) or mutant profilin with impaired binding to polyproline (Pfy1-14) and variable concentrations of Srv2 (0-20 μM). The amount of Pfy1 in the supernatant was examined by SDS gel electrophoresis. First lane, no beads; second lane, control-beads; lanes 3-6, 3, 6, 12 and 20 μM Srv2 immobilized on beads. (B) Quantification of five independent pull-down assays comparing Pfy1 with Pfy1-14. Srv2 efficiently depleted Pfy1 from the supernatant, but failed to deplete Pfy1-14. Standard deviations are indicated with error bars. (C) Tryptophan fluorescence assay for determining the dissociation constant of the Srv2-profilin complex. Reactions contained 1 μM profilin. Pfy1 binds to $Srv2_{253-373}$ with a K_D of 1.3 μM, whereas Pfy1-14 shows no detectable affinity for $Srv2_{253-373}$.

Profilin interacts with proline-rich sequence motifs and its binding to these motifs can be disrupted by mutating specific aromatic residues that are highly conserved on the surface of profilin (Wolven et al., 2000; Lu and Pollard, 2001). We next examined the interaction of $Srv2_{253-526}$ with a mutant yeast profilin (Pfy1-14) that harbours tyrosine-to-alanine substitutions at residues 119 and 125 and consequently has markedly reduced affinity for proline-rich sequences compared with wild-type profilin (Wolven et al., 2000). Supernatant depletion pull-down assays revealed that Pfy1-14 does not bind $Srv2_{253-526}$ with a detectable affinity, suggesting that the proline-rich region(s) of Srv2 play an essential role in this interaction (Fig. 1A,B).

A tryptophan fluorescence assay was applied for determining the dissociation constant of the Srv2-profilin interaction. In order to diminish the background fluorescence

from Srv2, we purified an Srv2 fragment corresponding to residues 253-373. This segment includes both proline-rich regions but does not contain any tryptophan or tyrosine residues. Titration of $Srv2_{253-373}$ (0-15 μM) binding to profilin (1 μM) yielded a K_D of 1.3 μM for the interaction (Fig. 1C). Consistent with results from the supernatant depletion pull-down assay described above, Pfy1-14 did not bind to Srv2 with detectable affinity in the tryptophan fluorescent assay (Fig. 1C).

Profilin binds to the first proline-rich motif (P1) of Srv2

Profilin binds to proline-rich target motifs (Tanaka and Shibata, 1985; Witke, 2004), two of which are found in Srv2/CAP (P1 and P2, Fig. 2A). A mutant profilin with defects in binding polyproline (Pfy1-14) failed to interact with Srv2 (Fig. 1), suggesting that polyproline regions in Srv2 are required for binding profilin. Therefore, we introduced mutations designed to disrupt P1 and P2 separately in Srv2. Three and two proline-to-alanine substitutions were introduced into P1 to generate *srv2-201* and *srv2-202* alleles, respectively. Three proline-to-alanine substitutions were introduced into P2 to generate *srv2-203* (Fig. 2A). Supernatant depletion assays carried out with Srv2-201, Srv2-202, and Srv2-203 GST-fusion proteins demonstrated that Srv2-203, which contains mutations in P2, bound to profilin with a similar affinity to wild-type Srv2. Interestingly, Srv2-201 and Srv-202, in which P1 was mutated, showed no detectable binding to profilin in this assay (Fig. 2B). Binding of Srv2-201 to profilin was also examined by tryptophan fluorescence assay using a shorter fragment of Srv2-201 corresponding to residues 253-373. High concentrations of this fragment induced only a small increase in tryptophan fluorescence, suggesting non-saturable, low-affinity binding (estimated K_D greater than 20 μM). This provides further evidence that the P1 region of Srv2 is essential for profilin binding.

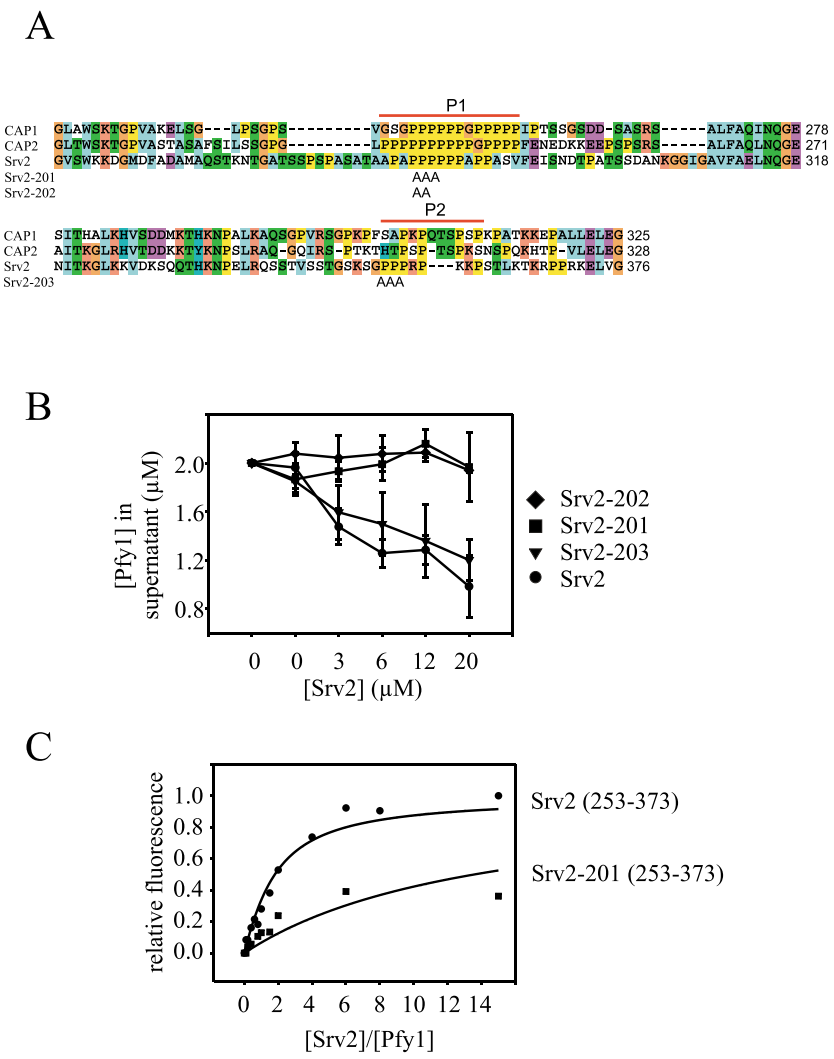

Fig. 2. The P1 polyproline sequence is required for Srv2 binding to profilin. (A) Sequence alignment of the two mouse Srv2/CAP isoforms (CAP1 and CAP2) and yeast Srv2. Positions of the proline-to-alanine substitutions in Srv2-201, Srv2-202 and Srv2-203 mutants are indicated below the sequences. (B) Quantification of four independent supernatant depletion pull-down assays. Wild-type Srv2 and Srv2-203 bind profilin efficiently, whereas Srv2-201 and Srv2-202 fail to bind profilin in this assay. Standard deviations are indicated by error bars. (C) Tryptophan fluorescence assay of wild-type $Srv2_{253-373}$ and mutant $Srv2\text{-}201_{253-373}$. Wild-type $Srv2_{253-373}$ but not $Srv2\text{-}201_{253-373}$ induces a saturable increase in tryptophan fluorescence of 1 μM profilin.

The first proline-rich region of Srv2 (P1) is not involved in actin binding

The C-terminal half of Srv2 binds ADP-G-actin with much higher affinity (K_D ~0.02 μM) than ATP-G-actin (K_D=1.9 μM) (Mattila et al., 2004). Mutational analysis showed that the actin-binding activity of yeast Srv2 is derived largely from the C-terminal β-strand domain (residues 396-526) with important additional contributions made by adjacent sequences (residues 253-368) that contain P1 and P2 (Mattila et al., 2004). To examine the role of Srv2/CAP's polyproline regions in actin binding, we carried out NBD-actin monomer binding assays to compare the ADP-G-actin binding affinities of wild-type, Srv2-201, Srv2-202, and Srv2-203 proteins under physiological ionic conditions. In these assays, we used Srv2 fragments corresponding to residues 253-526 as described previously (Mattila et al., 2004). NBD-actin assays showed that Srv2-203, containing mutations in P2, binds to ADP-G-actin with a similar affinity to wild-type Srv2. Interestingly, the affinity of Srv2-202 for ADP-G-actin was only 10% of that of wild-type Srv2, whereas Srv2-201 actin-binding was not affected (Fig. 3). These data suggest that neither one of the proline-rich regions is directly involved in actin binding, but that introducing two proline-to-alanine substitutions in P1 may induce a conformational change in the Srv2 polypeptide that affects interactions of the nearby β-strand domain (residues 396-526) with ADP-G-actin. Most importantly, these assays

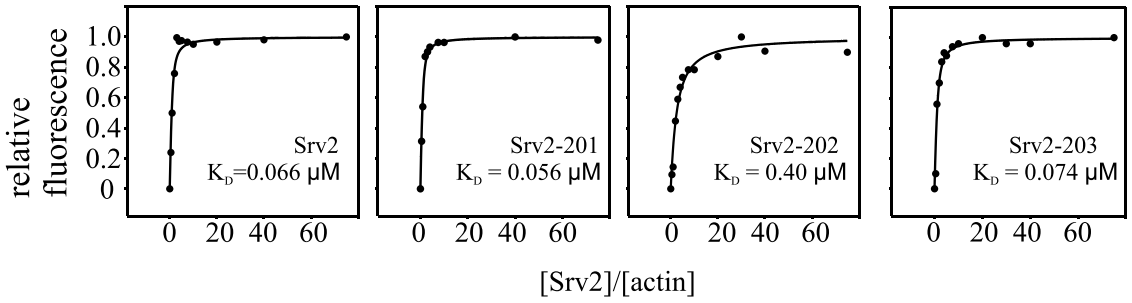

Fig. 3. Interactions of wild-type Srv2 and mutants Srv2-201, Srv2-202 and Srv2-203 with ADP-G-actin. An increase in the fluorescence of 0.2 μM NDB-labeled G-actin was measured under physiological salt conditions at pH 8.0. Dissociation constants were calculated from the binding curves. Srv2, Srv2-201 and Srv2-203 bound to actin monomers with high affinity (K_D=0.066 μM, 0.056 μM and 0.074 μM, respectively), whereas Srv2-202 bound actin monomers with considerably lower affinity (K_D=0.4 μM).

show that Srv2-201 (three proline-to-alanine substitutions in P1) displays severe defects in binding profilin but binds to G-actin with wild-type affinity.

Srv2 can simultaneously interact with actin and profilin

If the binding sites of two proteins overlap on a mutual ligand, they typically interfere with each other's binding interactions with the ligand. Sometimes binding of one protein also alters the conformation of the ligand and thus changes the affinity of the ligand for other proteins. We thus telabested whether the C-terminal half of Srv2 (253-526) can bind simultaneously to both profilin and G-actin, or whether these two proteins interfere with each other's binding to Srv2. To examine the effects of profilin on Srv2 binding to actin, we monitored the fluorescence change of Srv2-bound 0.2 μM NBD-labeled actin. Addition of 1 μM $Srv2_{253-526}$ induced an ~30% increase in the NBD fluorescence owing to the interaction between ADP-G-actin and $Srv2_{253-526}$. Titration of the reaction with 0.2-40 μM wild-type profilin did not significantly decrease the fluorescence, suggesting that even in the presence of 80-fold molar excess of profilin, the majority of $Srv2_{253-526}$ was still bound to actin. Importantly, previous studies showed that, in contrast to Srv2, profilin does not bind ADP-actin with high affinity (reviewed by Pollard et al., 2000). In addition, it was reported that NBD labelling of actin decreases its affinity to profilin (Malm, 1984). Thus, profilin does not significantly compete with Srv2 for ADP-G-actin binding in these assays. The same assay was also carried out with Srv2-201, which binds to actin with normal affinity but is defective in profilin binding. Titration of the Srv2-201–ADP-G-actin complex yielded very similar results to those obtained with wild-type Srv2, suggesting further that the small decrease in fluorescence of Srv2–ADP-G-actin complexes after addition of high concentrations of profilin does not result from competitive interactions between profilin and actin for binding Srv2 (Fig. 4A).

We next carried out supernatant depletion pull-down assays to examine the ability of Srv2 to bind profilin in the presence of actin. GST-$Srv2_{253-526}$ bound to glutathione agarose beads efficiently decreased the concentration of profilin in the supernatant. Titration of the reaction with increasing amounts of purified ADP-G-actin did not cause a significant reduction in the amount of profilin depleted by Srv2, suggesting that ADP-G-actin does not interfere with profilin binding to Srv2. It is important to note that Srv2 has a very high affinity for ADP-G-actin (K_D ~0.02 μM) and thus 10 μM ADP-G-actin is expected to saturate approximately half of the 20 μM Srv2 molecules. Therefore, if actin interferes with profilin binding to Srv2, one should observe a ~50% decrease in the amount of depleted profilin in the presence of 10 μM ADP-G-actin compared with reactions without actin. We also examined the pellet fractions of these reactions and found that both profilin and actin were present in the pellet (data not shown). To confirm that profilin did not bind to Srv2 indirectly, through associations with ADP-actin, the same assay was carried out with Srv2-201, which could not deplete profilin from the supernatant. In this case, addition of actin did not decrease the amount of profilin in the supernatant, and only actin was detected in the pellet fractions as analyzed by SDS gels (Fig. 4B, and data not shown). Together, these experiments suggest that Srv2, profilin and ADP-G-actin form a ternary complex in which profilin and ADP-G-actin bind to Srv2 through separate interactions. However, further work is required to confirm the presence of this complex and to reveal the stoichiometry of these proteins in the complex.

Profilin interacts with Srv2 in vivo

To test whether Srv2 and profilin also associate in vivo, we carried out a co-immunoprecipitation assay from wild-type (*SRV2*), *srv2-201* and *srv2Δ* yeast cell lysates. Wild-type or mutant Srv2 proteins were immunoprecipitated with an antibody against Srv2 and the amount of profilin in the lysates was detected by using an anti-profilin antibody. This assay revealed that profilin co-immunoprecipitated with wild-type Srv2. By contrast, only very small amounts of profilin were detected in immunoprecipitates from the *srv2-201* strain and from the *srv2Δ* strain (Fig. 5A).

Srv2 localizes to patch-like structures that partially co-localize with cortical actin patches in wild-type yeast cells (Gourlay and Ayscough, 2006). Mutations in the Srv2 proline-rich region P2 were previously shown to affect the subcellular localization of this protein, as a result of disrupting molecular interactions with the actin-patch resident protein Abp1 (Lila and Drubin, 1997). We thus compared the localization of Srv2 protein in *SRV2* and *srv2-201* yeast strains (which harbors mutations in the Srv2 proline-rich region P1) by immunofluorescence microscopy. In both strains, Srv2 localized to patch-like structures that partially overlapped with the cortical actin cytoskeleton (Fig. 5B).

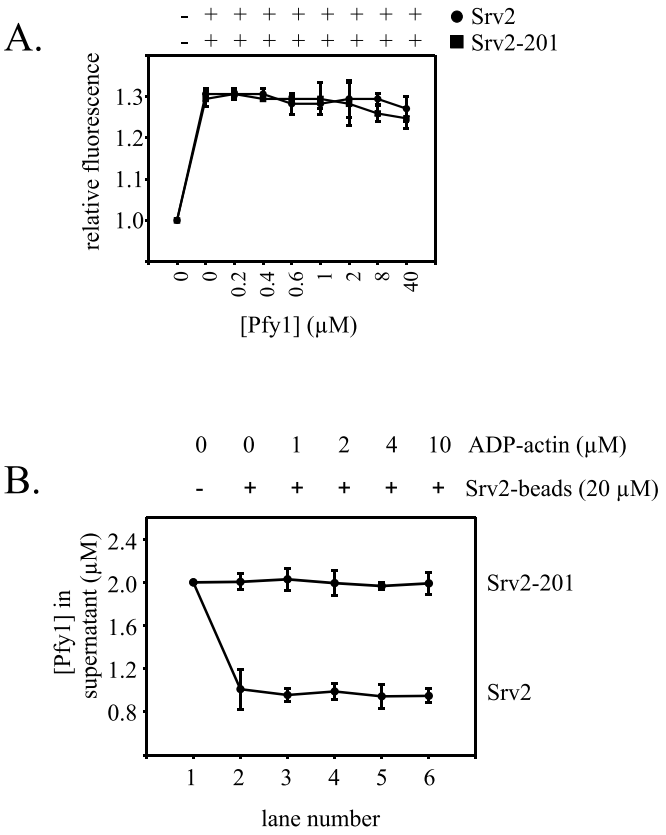

Fig. 4. Actin and profilin do not interfere with each other's binding to Srv2. (A) The presence of profilin does not alter the affinity of Srv2 for G-actin. Addition of 1 μM Srv2 or Srv2-201 to 0.2 μM NBD-labeled ADP-G-actin resulted in a ~30% increase in the fluorescence. Addition of profilin (0-40 μM) did not significantly reduce the NBD fluorescence signal, suggesting that profilin does not affect the binding of Srv2 or Srv2-201 to ADP-G-actin. Standard deviations are indicated by error bars. (B) Actin-binding does not change Srv2 affinity for profilin. Supernatant depletion pull-down assays were carried out with reactions containing 2 μM profilin; the average of five independent assays is shown. Lane 1, profilin alone; lanes 2-6, 20 μM GST-Srv2 on beads; lanes 3-6, variable concentrations of ADP-G-actin (1, 2, 4, 10 μM). Addition of ADP-G-actin does not change the amount of profilin in the supernatant, demonstrating that actin monomers do not interfere with Srv2-profilin interaction. Results using Srv2-201 show that profilin does not bind indirectly to Srv2 through interaction with G-actin in this assay. Standard deviations are indicated by error bars.

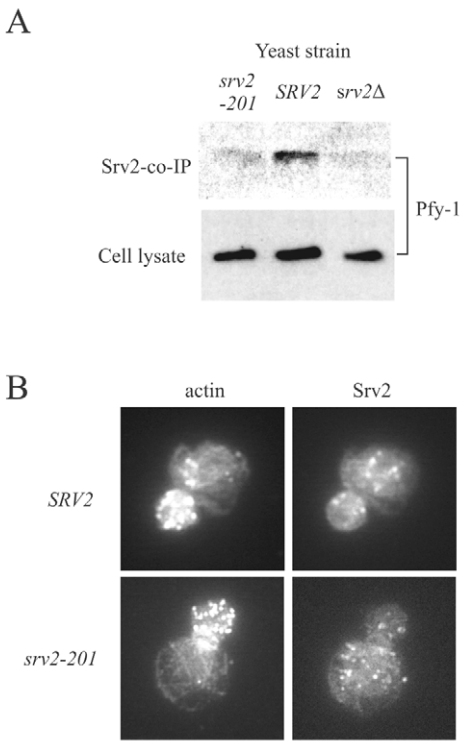

Fig. 5. Srv2 interacts with profilin in vivo. (A) Immunoprecipitation of Srv2 with anti-Srv2 antibody was carried out from wild-type *SRV2*, *srv2-201* and *srv2Δ* yeast strains. The blot was probed with anti-profilin antibody and demonstrates specific co-immunoprecipitation of profilin with wild-type Srv2. By contrast, only very small amounts of profilin were detected in immunoprecipitates from *srv2-201* and *srv2Δ* cells. The amounts of profilin in cell lysates before co-immunoprecipitation are shown as a control. (B) Srv2 localizes in patch-like manner in *SRV2* and *srv2-201* cells. The Srv2 dots occasionally co-localize with cortical actin patches.

Together, these data show that full-length Srv2 and profilin interact with each other in vivo and that the P1 polyproline motif is necessary for this interaction. However, unlike the P2 motif that is crucial for Srv2 localization, through an interaction with the SH3 domain of Abp1 (Lila and Drubin, 1997; Balcer et al., 2003), the P1 motif does not contribute to the subcellular localization of Srv2.

Genetic evidence for an in vivo functional interaction between Srv2 and profilin

Identification of an *srv2* mutant allele (Srv2-201) defective in binding profilin but with normal actin-binding interactions enabled us to examine the functional role of the Srv2-profilin interaction in yeast cells. To facilitate this analysis, we integrated wild-type (*SRV2*) and mutant (*srv2-201*) alleles at the *SRV2* locus of an *srv2Δ* yeast strain. These isogenic strains (*SRV2*, *srv2-201* and *srv2Δ*) were grown in parallel at different temperatures (25, 30 and 37°C) and compared for defects in cell growth, morphology, and actin cytoskeleton organization. At all temperatures, the *srv2Δ* strain showed reduced rates of cell growth compared with wild-type cells, as well as enlarged cell size and depolarized actin patches and cables (not shown), consistent with previous studies (Gerst et al., 1991; Vojtek et al., 1991). By contrast, *SRV2* and *srv2-201* strains displayed indistinguishable rates of cell growth and actin cytoskeleton organization (not shown). However, despite the absence of obvious defects in cell growth, the *srv2-201* mutation caused a statistically significant increase in average cell size compared with the isogenic wild-type *SRV2* strain, albeit not to the same extent as the *srv2Δ* mutation (supplementary material Fig. S1). These data show that the srv2-profilin interaction is not crucial for cell growth, but makes a supportive contribution to Srv2 regulation of cell morphology.

In addition, we tested the *srv2-201* mutation for possible

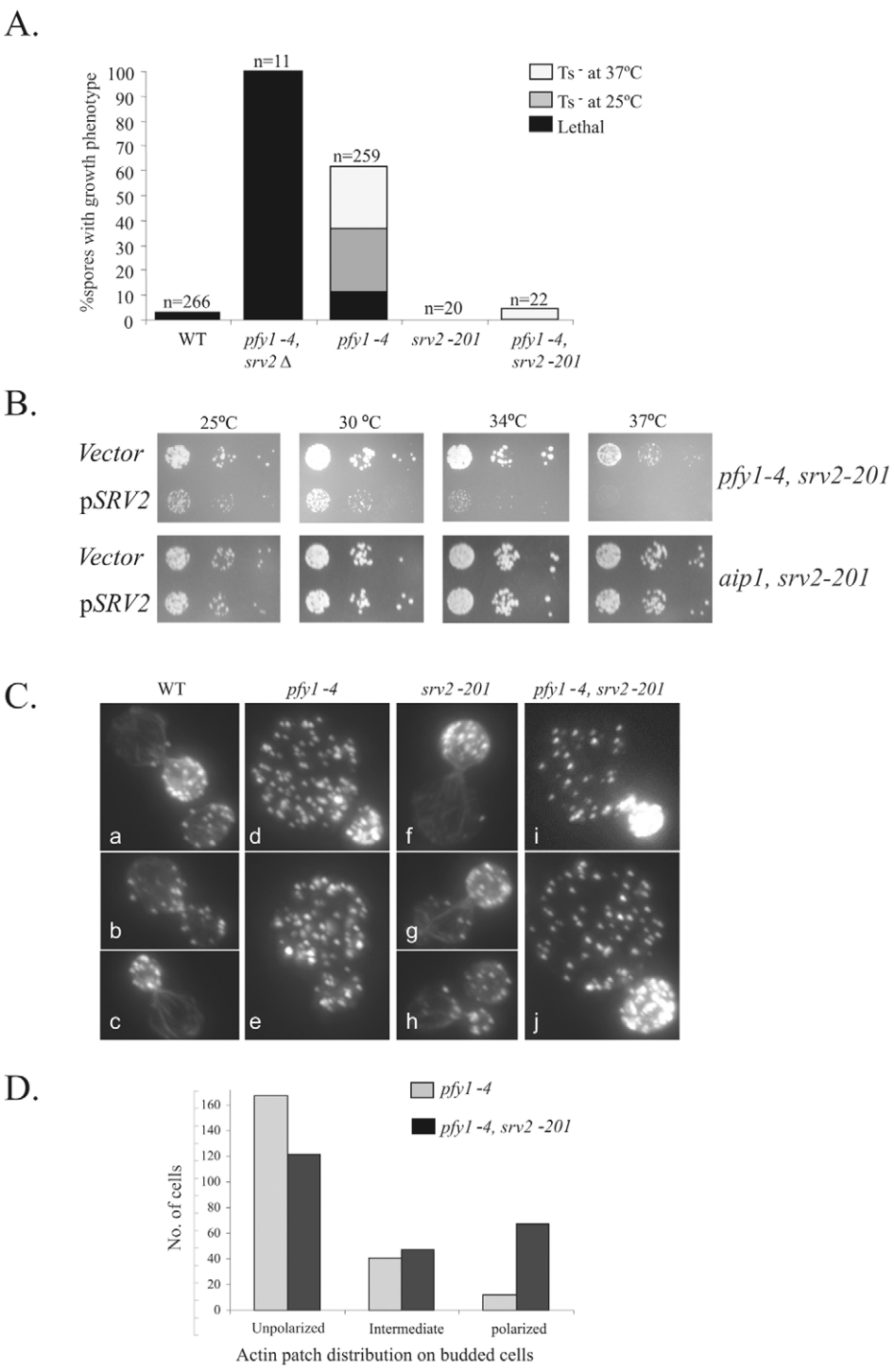

Fig. 6. The *srv2-201* allele suppresses defects in cell growth and actin organization caused by *pfy1-4*. (A) Comparison of cell growth defects for progeny from a cross between *pfy1-4* and *srv2-201* strains. Diploids from this cross were sporulated, tetrads were dissected. Lethality of spores was scored on the tetrad plates. Viable haploid strains from these plates were re-plated on YPD and scored for cell growth at 25 and 37°C. n, number of haploid progeny with the indicated genotype analyzed. (B) Transformation assay showing that *srv2-201* suppresses the growth defects of *pfy1-4*. Double mutant *pfy1-4, srv2-201* cells were transformed with a low copy *URA*-marked empty vector (pRS316) or p*SRV2* (pBG334). Serial dilutions of transformed cells were plated on Ura^- selective medium and grown at 25, 30, 34 and 37°C. As a control, double mutant *aip1, srv2-201* cells were transformed with same vectors. (C) Cells from A above were grown to log phase, chemically fixed and stained with Alexa Fluor 488-phalloidin to label F-actin structures. (D) Quantitative comparison of actin patch polarization defects in *pfy-4* and *pfy1-4 srv2-201* cells. Medium-budded cells (*n*>200, each strain) were scored for actin patch distribution in the bud. Actin patches were classified as being polarized, unpolarized or intermediate polarized.

genetic interactions with *pfy1-4*, *cof1-19*, and *aip1Δ* mutations, each of which is synthetic lethal with *srv2Δ* (Wolven et al., 2000; Balcer et al., 2003). Interestingly, *srv2-201* partially suppressed the growth defects caused by *pfy1-4*, but showed no genetic interactions with *cof1-19* or *aip1Δ* mutations, as determined by tetrad analysis and by carefully comparing the resulting single and double haploid mutant strains for cell growth at a range of temperatures (Fig. 6A and data not shown). Thus, suppression of *pfy1-4* growth defects by *srv2-201* is a highly specific genetic interaction. As an independent test of this suppression, we transformed the double mutant strains *pfy1-4, srv2-201* and *aip1Δ, srv2-201* with either a low copy wild-type *SRV2* plasmid or empty vector. Transformed cells were serially diluted and compared for growth at a range of temperatures (Fig. 6B). Consistent with the genetic results above, this assay showed that the defects caused by *pfy1-4* are suppressed by *srv2-201*, since the cells carrying the *SRV2* plasmid were significantly more impaired for growth than cells carrying empty vector. Yeasts carrying mutations *aip1Δ, srv2-201* did not show similar effects. Further, this suggests that

physical interactions between Pfy1-4 and Srv2 lead to dominant negative effects in vivo (see Discussion).

A close examination of actin organization in these mutant strains revealed that *srv2-201* partially rescues the actin patch polarization defects caused by the *pfy1-4* mutation. Single mutant *pfy1-4* cells exhibited greatly diminished actin cable staining and a highly depolarized distribution of actin patches, consistent with previous reports (Wolven et al., 2000); notably, there was no enrichment of actin patches in the bud (Fig. 6C; panels d and e). By contrast, *srv2-201 pfy1-4* double mutant cells exhibited an enrichment of actin patches in the bud compared with *pfy1-4* cells (Fig. 6C; panels i and j). These effects were scored, comparing the number of medium-budded *pfy1-4* cells and *pfy1-4 srv2-201* cells showing polarized, partially polarized, or completely unpolarized actin patch distribution (Fig. 6D). Thus, the partial suppression of *pfy1-4* growth defects by *srv2-201* correlates with partial restoration of actin polarization. Interestingly, restoration of visible actin cables was not observed in *pfy1-4 srv2-201* cells. This suggests that the profilin-Srv2 functional interaction may specifically influence actin patches.

Discussion

Srv2/CAP is a highly abundant protein that binds actin monomers and ADF/cofilin (Mattila et al., 2004; Moriyama and Yahara, 2002) to promote the rapid actin filament turnover in cells (Balcer et al., 2003; Bertling et al., 2004). In addition to actin and ADF/cofilin, Srv2/CAP has been proposed to interact physically with profilin (Lambrechts et al., 1997; Drees et al., 2001; Balcer et al., 2003). Further, an Srv2-profilin functional interaction was suggested by earlier studies showing that defects arising from truncation of the yeast Srv2 C-terminus are partially suppressed by overexpression of profilin (Votjek et al., 1991). However, the mechanism and biological role of this possible Srv2-profilin interaction has not been demonstrated so far. In this study, we used *Saccharomyces cerevisiae* as a model organism to examine this interaction and revealed that: (1) Srv2 binds directly to profilin (K_D=1.3 μM) in vitro; (2) The profilin-binding site is located in the first proline-rich region (P1) of Srv2; (3) Profilin and ADP-G-actin can bind simultaneously to the C-terminus of Srv2; (4) The Srv2-profilin interaction also occurs in vivo, but is not essential for Srv2 function in yeast cells. Nevertheless, Srv2 interaction with profilin participates in regulating actin organization and cell morphology, as revealed by the ability of *srv2-201* to partially suppress the defects of *pfy1-4*.

Previously, Lambrechts et al. (Lambrechts et al., 1997) applied fluorescence spectroscopy to examine the interaction between mammalian profilin I and a 15-residue-long synthetic peptide corresponding to P1 of mammalian CAP1. These studies defined an interaction with a K_D of approximately 5 μM. This affinity is approximately four times lower than measured here using a 120-residue yeast Srv2 fragment, suggesting either that yeast Srv2 binds to profilin with higher affinity than the mammalian protein or that sequences adjacent to the polyproline motif affect the binding interaction with profilin. It remains to be shown, whether this affinity is the same or even higher with full-length protein. It was previously shown that in vitro ten proline residues is the optimal length of a polyproline peptide for binding profilin (Perelroizen et al., 1994). The P1 motif in yeast Srv2 spans eleven residues, of which nine are prolines. By contrast, P2 spans eight residues, of which five are prolines (see Fig. 2A). Our experiments showed that profilin binding to Srv2 requires P1, whereas P2 does not contribute to profilin binding. This is in good agreement with previous studies demonstrating the importance of P2 for interactions with the SH3 domain of Abp1 (Freeman et al., 1996; Lila and Drubin, 1997; Balcer et al., 2003). Together, these data suggest that P1 defines the profilin-binding site on Srv2 (and is thus not required for localization of Srv2 in cells), while P2 binds to Abp1 and other SH3 domain proteins that control the localization of Srv2 in cells.

One unexpected result from our study was that some, but not all mutations in P1 impair binding of Srv2 to actin. Proline-rich regions are known to form a helix-like structure containing approximately three prolines per turn. Srv2-201, which has three proline-to-alanine substitutions (amino acids 278-280), showed a severe defect in profilin-binding but interacted with actin with wild-type affinity. This suggests that in Srv2-201 replacing a 'full turn' of prolines in the helix retains a functional conformation similar to wild-type Srv2. However, in Srv2-202 the two proline-to-alanine substitutions might have disrupted the register of the helix and thereby resulted in decreased affinity for ADP-G-actin. These data suggest that in the three-dimensional structure of Srv2, the profilin- and actin-binding sites may be in close proximity, making it possible that the Srv2-bound profilin and actin monomer can also interact with each other. This possibility is further supported by our previous mutational analysis demonstrating that the C-terminal β-sheet structure (residues 369-526) is sufficient to bind actin but its affinity for actin is greatly enhanced by adjacent sequences N-terminal to this domain (Mattila et al., 2004). However, it is important to note that although these binding sites may be located close to each other in the three-dimensional structure of Srv2, our studies suggest that profilin and ADP-G-actin can bind simultaneously to Srv2.

Deletion of the *SRV2* gene in budding yeast results in impaired cell growth, enlargement of cell size, and severe depolarization of cortical actin patches (Gerst et al., 1991; Votjek et al., 1991). Similar phenotypes were also detected from yeast cells expressing mutant Srv2 proteins with defects in ADP-G-actin binding, demonstrating that the Srv2 interaction with actin monomers is essential for Srv2 function in vivo (Mattila et al., 2004). Our co-immunoprecipitation analysis demonstrated that Srv2 interacts with profilin in vivo. Interestingly, studies with a mutant Srv2 impaired specifically in binding profilin (*srv2-201*) revealed first that this interaction is not essential for Srv2 cellular functions. The *srv2-201* cells grew at wild-type rates at a range of temperatures and did not display detectable defects in actin cytoskeleton organization at the light microscopy level. On the other hand, *srv2-201* cells were enlarged compared with wild-type cells, indicating that direct interactions between Srv2 and profilin must contribute to Srv2 function in maintaining polarized cell growth. Further, we showed that a profilin mutant (*pfy1-4*) with defective actin binding interactions but normal affinity for polyproline is compromised in vivo by its direct interactions with Srv2. This was indicated by the ability of *srv2-201* to partially suppress the cell growth defects of *pfy1-4*. Together, these genetic data indicate that Srv2 is an important physiological target of profilin. One explanation for the ability of *srv2-201* to suppress *pfy1-4* defects is that in cells expressing wild-type Srv2, Pfy1-

4 molecules (with reduced actin-binding affinity) may become sequestered on Srv2 in a dominant negative fashion, and thereby diminish the pool of Pfy1-4 molecules available to deliver actin monomers to the growing barbed ends of filaments. In this case, disrupting the profilin binding site on Srv2 (*srv2-201*) would liberate Pfy1-4 molecules to perform their role in actin filament growth. Importantly, this does not rule out other possible explanations. However, regardless of the exact mechanism underlying these effects, this genetic interaction demonstrates that the Srv2-profilin interaction occurs in vivo (supported further by Srv2 and profilin co-immunoprecipitation) and can strongly affect the polarization of actin patches and cell growth. Given that the P1 motif is highly conserved in Srv2/CAP homologues, these profilin-Srv2/CAP interactions are likely to make similar contributions to actin dynamics in other organisms.

Together with previously reported observations, our new data on the Srv2-profilin interaction supports the following model for how Srv2/CAP regulates actin dynamics. ADF/cofilin severs actin filaments and promotes dissociation of ADP-G-actin monomers from filament pointed ends (Bamburg, 1999) and the N-terminus of Srv2/CAP binds to ADF/cofilin–G-actin complexes (Moriyama and Yahara, 2002). Srv2/CAP forms a high molecular weight multimeric complex with G-actin (Balcer et al., 2003), which in yeast localizes to the cortical actin cytoskeleton through interactions with the actin filament binding protein Abp1 (Freeman et al., 1995; Lila and Drubin, 1997). We propose that following the interaction of the N-terminus of Srv2/CAP with ADF/cofilin–G-actin, ADP-actin dissociates from cofilin and is transferred to the C-terminal actin-binding domain of Srv2/CAP (Mattila et al., 2004). This transfer may occur either in *cis* (within the same Srv2 molecule) or in *trans* (between two different Srv2/CAP molecules in the same complex). Because the profilin- and actin-binding sites are predicted to be adjacent in the three-dimensional structure of Srv2/CAP (see above), we propose that the Srv2/CAP-bound ADP-actin monomer next interacts with profilin. Some combination of these interactions (profilin and Srv2/CAP with ADP-actin) stimulates nucleotide exchange to produce ATP-G-actin. It was previously shown that Srv2 and profilin have additive biochemical effects in promoting nucleotide exchange in vitro (Balcer et al., 2003). Owing to the low affinity of Srv2/CAP for ATP-G-actin (K_D=1.9 μM), the profilin-actin monomer complex rapidly dissociates from Srv2/CAP and becomes available for filament barbed end assembly. However, it is important to note that our genetic studies revealed that the interaction with profilin is not essential for yeast Srv2 function in vivo. Thus, we speculate that the actin monomer can also be recycled from Srv2/CAP to profilin if profilin is not directly bound to Srv2/CAP, but that the direct interaction between Srv2/CAP and profilin increases the efficiency of this process.

Previous studies have shown that many proteins contain proline-rich motifs that interact with profilin, used to recruit ATP-actin-profilin complexes to specific sites and promote filament barbed end growth (e.g. Reinhard et al., 1995; Suetsugu et al., 1998; Evangelista et al., 2003). Our study reveals that proline-rich sequence motifs can also be used for a different function, loading profilin molecules onto actin monomers and thus replenishing the assembly-competent actin monomer pool. In the future it will be important to elucidate whether this function is also conserved in Srv2/CAP proteins of other organisms as well as to gain structural information of the Srv2/CAP–profilin–actin-monomer complex.

Materials and Methods

Plasmid constructs, mutant integration, and genetic analyses

For expression as glutathione-S-transferase (GST) fusion proteins, *SRV2* DNA fragments encoding amino acid residues 253-526 (pPL143) or 253-373 (pPL241) were cloned to pGAT2 vector (Peränen et al., 1996) using *Nco*I and *Hin*dIII sites at the 5′ and 3′ ends as described (Mattila et al., 2004). Specific mutations were introduced into these fragments to generate plasmids pPL267 (*srv2-201*), pPL339 (*srv2-201* residues 253-373), pPL268 (*srv2-202*) and pPL266 (*srv2-203*). A mutant allele of yeast profilin (*pfy1-14*) defective in polyproline binding (Wolven et al., 2000) was cloned into the pBAT4 vector (pPL338) for expression in *E. coli* (Peränen et al., 1996).

To generate yeast strains BGY1190 and BGY1191, an integration plasmid was constructed carrying the *SRV2* coding region plus 492 bp upstream and 496 bp downstream sequences. The *TRP1* gene was inserted 54 bp downstream of the *SRV2* stop codon to generate pBG652. From this template, the *srv2-201* mutant allele was generated by QuikChange site-directed mutagenesis (Stratagene; Cedar Creek, TX). All plasmids were sequenced. Wild-type and mutant *SRV2::TRP1* alleles were integrated at the *SRV2* locus in the haploid yeast strain BGY330 (*Mat α, his3Δ200, leu2-3,112, ura3-52, trp1-1, lys2-801, srv2Δ::HIS3*), replacing *srv2Δ::HIS3*. Integration at the correct locus was confirmed by PCR analysis of isolated genomic DNA. Genetic interactions with *pfy1-4, aip1Δ and cof1-19* mutations were tested by crossing haploid *srv2-201* and *SRV2* strains to each of the following strains: (1) Mat a, *his3Δ200, leu2-3,112, ura3-52, trp1-1, lys2-801, pfy1-4::LEU2*; (2) Mat a, *his3Δ200, leu2-3,112, ura3-52, trp1-1, lys2-801, aip1Δ::URA3*; (3) Mat a, *his3Δ200, leu2-3,112, ura3-52, trp1-1, lys2-801, cof1-19::LEU2*. Diploids were selected and then sporulated, tetrads were dissected and resulting haploids (wild type, single mutants and double mutants) were serially diluted and compared for growth on YPD plates at 25, 30, 34 and 37°C.

Protein expression and purification

All Srv2 fragments and profilins used in this study were expressed and purified using the *E. coli* BL-21 (DE3) strain. Wild-type and mutant fragments of the Srv2 C-terminal region (253-526 and 253-373) were purified as GST fusions as previously described (Mattila et al., 2004). In all cases except for supernatant depletion pull-down assays, the GST-tag was removed by thrombin cleavage. Wild-type Pfy1 (pBG073) and mutant Pfy1-14 (pPL338) were purified as described (Wolven et al., 2000). Rabbit muscle actin was prepared from acetone powder (Pardee and Spudich, 1982).

Actin monomer binding assay

Rabbit muscle actin was labeled with NBD (7-chloro-4-nitrobenz-2-oxa-1,3-diazole-Cl) as described (Detmers et al., 1981). ATP-actin was converted to ADP-actin by incubation with hexokinase-agarose beads for 2 hours at +4°C (Pollard, 1986). Hexokinase-agarose beads were prepared by coupling pure hexokinase (Sigma) to CNBr-activated Sepharose 4B beads (Amersham Biosciences AB, Sweden) according to the manufacturer's instructions. Binding of wild-type and mutant C-terminal Srv2 proteins Srv2-201, Srv2-202 and Srv2-203 to actin was measured by change in fluorescence of NBD-actin (excitation and emission wavelengths of 482 nm and 535 nm, respectively). Binding reactions [total volume 100 μl, reaction buffer 2 mM Tris-HCl pH 8.0, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 0.2 mM ADP, 1 mg/ml Bovine serum albumin (Sigma)] contained a constant concentration of actin (0.2 μM) and variable concentrations of other proteins and were carried out at room temperature. Data were collected with BioLogic MOS250 fluorometer (Claix, France) and fitted and analyzed using SigmaPlot 9.0 software (Systat Software, USA) and equations as described (Mattila et al., 2004).

Tryptophan fluorescence assay

Interactions between yeast profilin (1 μM) and $Srv2_{253-373}$ or $Srv2\text{-}201_{253-373}$ (0 to 15 μM) were measured by change in profilin tryptophan fluorescence at excitation and emission wavelengths of 295 nm and 350 nm, respectively. These shorter fragments of Srv2 were prepared to diminish Srv2 background tryptophan fluorescence. The background Srv2 fluorescence values (without profilin) were subtracted from the fluorescence values of Srv2-profilin samples to obtain the fluorescence changes resulting from Srv2-profilin interactions. All reactions were carried out in a sample volume of 100 μl in the following buffer: 30 mM Tris-HCl pH 7.5, 100 mM NaCl. The fluorescence was recorded after a 20-minute incubation at room temperature. Binding constants were measured from the curves as described above for actin monomer binding assays.

Supernatant depletion pull-down assays

For these assays, GST-fusions of wild-type and mutant Srv2 fragments were produced in *E.coli*. Cells were lysed and GST-fusion proteins were bound to

glutathione-Sepharose 4B beads (Amersham Biosciences AB, Sweden) as described (Mattila et al., 2004). Beads containing GST or beads only washed with reaction buffer were used as a control. Total amount of protein bound to the beads was estimated from Coomassie stained SDS-PAGE gels. Reactions of 250 μl were prepared in 30 mM Tris-HCl pH 7.5, 100-150 mM NaCl with a constant final concentration of profilin (2 μM) and variable (0 to 20 μM) concentrations of wild-type and mutant Srv2 immobilized on beads. After 20 min incubation at room temperature, the samples were centrifuged at 13,000 ***g*** for 5 minutes to pellet Srv2-beads and bound profilin. Amount of unbound profilin remaining in the supernatant was examined on Coomassie stained 15% SDS-PAGE gels and quantified using TINA software.

Binding interference assays

The ability of profilin to interfere with Srv2 binding to G-actin was measured in an applied NBD-actin fluorescence assay. All samples were prepared as described above for except that the concentrations of actin (0.2 μM) and Srv2 (1 μM) were constant and profilin concentration was varied (0 to 40 μM). Data was collected and analyzed as above. The stability of the Srv2-profilin association in the presence of G-actin was measured and analyzed by a modified version of that described above using 2 μM profilin in the supernatant, 20 μM Srv2 on beads and a variable concentration of ADP-actin (0 to 10 μM). The assay was carried out in 2 mM Tris-HCl pH 8.0, 0.1 mM $CaCl_2$, 0.1 mM DTT, 0.2 mM ADP, 50 mM NaCl.

Co-immunoprecipitation

The co-immunoprecipitation assay from *SRV2*, *srv2-201* and *srv2Δ* strains was carried out as described (Falck et al., 2004). Proteins were examined by western blotting as described previously (Vartiainen et al., 2003). The antibodies against yeast Srv2, profilin and actin were generated in chicken, mouse and guinea-pig, respectively.

Microscopy

To visualize filamentous actin organization in cells, yeast were grown in liquid culture to early log phase (OD_{600}=0.1) then fixed with formaldehyde, washed and stained with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR). Images were captured on a Zeiss Axioskop2 mot plus (Carl Zeiss, Thornwood, NY) using a Hamamatsu IEEE1394 digital CCD camera (Hamamatsu Photonics, Bridgewater, NJ). Image analysis was performed using Openlab™ software (Improvision, Lexington, MA). For analysis of cell size, DIC images of non-fixed cells (OD_{600}=0.1) were captured using the instrumentation described above. With system software, unbudded cells and mother cell compartments of budded cells were traced and the area within each compartment was computed. For each strain, at least 250 cells were analyzed.

Analysis of cell growth

SRV2, *srv2-201* and *srv2Δ* strains were directly compared for rates of cell growth at 30°C and 37°C. Cell cultures grown in YPD liquid media were maintained in log phase (OD_{600}<0.5) for 12 hours, during which time aliquots of cells were removed and counted by cytometry and plated on YPD to determine cell viability as colony forming units. All assays were performed in duplicate.

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