SCP1 encodes an actin-bundling protein in yeast

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The association of F-actin (filamentous actin) with a large number of binding proteins is essential for cellular function. Actin-binding proteins control the dynamics of actin filaments, nucleate new filaments and facilitate formation of higher-order structures such as actin bundles. The yeast gene *SCP1* encodes a small protein with significant homology to mammalian SM22/transgelin. We have investigated the role of Scp1p in budding yeast to probe the fundamental role of this family of proteins. Here, we demonstrate that Scp1p binds to F-actin and induces the formation of tight F-actin bundles *in vitro*. Deletion of *SCP1* in yeast lacking

the actin-bundling protein, fimbrin (Sac6p), exacerbates the disrupted actin phenotype and enhances latrunculin-A sensitivity. Furthermore, Scp1p co-localizes with actin in cortical patches and its localization is lost in the presence of latrunculin-A. Our data support a role for Scp1p in bundling actin filaments and, in concert with Sac6p, acting as a second actin-bundling activity crucial to the stability of the yeast actin cytoskeleton.

Key words: actin-binding protein, calponin, F-actin, Scp1p, SM22, transgelin.

INTRODUCTION

Actin-binding proteins are required for the organization of the actin cytoskeleton by regulating F-actin (filamentous actin) dynamics and through the formation of higher-order structures such as actin bundles and networks [1]. These actin-binding proteins are, in turn, under the control of a plethora of intracellular and extracellular signals which control the spatio-temporal organization of the F-actin cytoskeleton. Scp1p was identified through a database search of the yeast genome and shows significant homology to the vertebrate smooth-muscle protein calponin, and the calponin-related protein Sm22 [2], also known as transgelin [3]. Sequence analysis shows that Scp1p contains a single CH (calponin homology) domain and a single CLR (calponin-like repeat) domain and so in this regard is more similar to the Sm22/transgelin subfamily of these proteins (Figure 1) [4], with Scp1p sharing 28 % identity and 48 % similarity with SM22 at the amino acid level. The mammalian protein SM22/transgelin binds to F-actin [3,5], but the function of the SM22/transgelin protein in higher eukaryotes has remained inconclusive; indeed, mice deficient for the smooth muscle-specific form of SM22, SM22 α , showed no overt smooth muscle phenotype [6]. Interestingly, however, the expression pattern of SM22 in various primary and cultured cells shows it to be a transformation- and aging-sensitive protein, with levels falling in transformed cells and increasing towards senescence [7,8]. The identification of a *Saccharomyces cerevisiae* homologue of a protein from the calponin family provides the opportunity to study the protein in a well-characterized and manipulable system. The high level of conservation among actin-binding proteins and their effects on the actin cytoskeleton indicates that its fundamental role is likely to have been maintained across eukaryotic evolution.

We have used both *in vitro* biochemical analysis and *in vivo* studies in yeast to demonstrate that Scp1p binds to F-actin and induces the formation of tight F-actin bundles at physiological salt concentrations and pH. The *in vivo* data support a role for Scp1p in stabilizing the actin cytoskeleton in concert with the previously characterized yeast actin-bundling protein Sac6p/fimbrin.

EXPERIMENTAL

Expression and purification of Scp1p

SCP1 was cloned into the pET vector pSJW1 [9,9a] following PCR from genomic *S. cerevisiae* DNA, using *Nde*I and *Sal*I restriction sites included in the PCR primers to generate the plasmid pSJW1-Scp1p. The sequence was verified before transformation into *Escherichia coli* BL21 (DE3) cells. Following induction with isopropyl β -D-thiogalactoside (0.5 mM, 2 h), Scp1p was targeted to the inclusion bodies, which were isolated as described previously [10]. Inclusion-body proteins solubilized in 6 M urea were then subjected to two rounds of column chromatography. First was cation-exchange using CM-Sephacrose CL-6B: columns were developed with a linear gradient of NaCl (0–500 mM) in 6 M urea, 20 mM Tris/HCl (pH 8.0) and 1 mM EGTA (buffer A). Scp1p-containing fractions eluted at around 100 mM NaCl, were identified by SDS/PAGE, pooled and then applied directly to a hydroxyapatite column in buffer A. The hydroxyapatite column was developed with a linear gradient of sodium phosphate (0–100 mM) in buffer A. Scp1p eluted at around 20 mM phosphate and fractions containing purified Scp1p were identified as above and pooled. Scp1p was refolded from 6 M urea by dialysis in serial 2-fold dilutions of 10 mM Mes pH 6.0. Purified dialysed Scp1p was subjected to CD spectroscopy in a Jasco J600 spectropolarimeter to demonstrate that it had undergone appropriate refolding after denaturation in urea.

Scp1p fragments and fusion proteins

A full-length GST (glutathione S-transferase)–Scp1p construct was generated by PCR of the Scp1p coding sequence from pSJW1-Scp1p and cloned into pGEX-4T (Amersham Biosciences) using *Eco*RI and *Xho*I restriction sites incorporated into the PCR primers. This plasmid was then used to generate a GST–CH domain construct (residues 1–142) by the insertion of a stop codon after codon 142 of Scp1p by QuikChange mutagenesis (Stratagene). cDNA corresponding to residues 142–200 of Scp1p,

Abbreviations used: F-actin, filamentous actin; G-actin, globular actin; CH, calponin homology; CLR, calponin-like repeat; GST, glutathione S-transferase; GFP, green fluorescent protein.

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Figure 1 Comparison of Scp1p and related proteins

(A) Representative single CH-domain-containing proteins. Proteins shown are calponin, SM22/transgelin and Scp1p. Both SM22 and Scp1p have a single CH and single CLR domain. (B) Sequence comparison of Scp1p-related proteins in different phyla using Clustal W [39]. Included in this comparison are sequences from *Echinococcus granulosa* (myophilin antigen; accession no. CAA82316), *D. melanogaster* (mp20; NP_476643), *Caenorhabditis elegans* (Cpn1; AAF01687); chicken (*Gallus gallus*, TGLN1; P19966); *Homo sapiens* (SM22/transgelin; NP_003177) and *S. cerevisiae* (SCP1; NP_015012), the relative positions of the CH and CLR are marked by horizontal bars above the sequences and the positions of predicted actin-binding sites (ABS) are shown below.

the calponin-repeat region, was generated by PCR from pSJW1-Scp1p and cloned into pET14b (Novagen) using *Nde*I and *Xho*I restriction sites incorporated into the PCR primers to generate a six-histidine tag at the N-terminus. Fusion proteins were expressed in *E. coli* BL21(DE3) as described above. Following lysis [9], but see [9a]), soluble-phase proteins were purified: the GST-CH domain on glutathione-Sepharose (Amersham Biosciences) and the His-tagged calponin-repeat region on Ni-NTA-agarose (Qiagen), according to the manufacturers' instructions.

Purified full-length Scp1p was allowed to degrade for at least 1 week at 4 °C through the action of a co-purifying bacterial protease until a relatively stable band of approx. 6 kDa was obtained. The mass of the 6 kDa band was determined by matrix-assisted laser-desorption/ionization-time-of-flight MS using a PE Biosystems Voyager 6138 mass spectrometer.

Analysis of actin interactions

Rabbit muscle F-actin was prepared according to a modification of the method of Spudich and Watt [11] as described previously [10].

For actin sedimentation assays, F-actin (5 μ M) was incubated in actin-binding buffer [12] in the absence or presence of increasing concentrations of Scp1p and centrifuged at 50000 *g* (high speed) or 13000 *g* (low speed). Equivalent volumes of supernatant and pellet fractions were subjected to SDS/PAGE. Falling-ball viscometry was performed as described previously [10] using 5 μ M G-actin (globular actin) and increasing concentrations of Scp1 from 0.1 to 5 μ M. Pyrenyl-actin polymerization, and depolymerization assays were performed as described previously [10] using 5 μ M G-actin and 0.5 μ M Scp1p. All data were baseline corrected for light scattering.

Electron microscopy analysis

Actin (5 μ M) was polymerized as above and incubated with or without 1 μ M Scp1 for 3 h. Actin filaments or Scp1p-actin filament complexes were then placed on to carbon formvar-coated discs, drained and negatively stained with Nanovan (2 s; Universal Biologicals), dried and visualized under a Zeiss 902 electron microscope.

Figure 2 Biochemical analysis of Scp1p interaction with actin

(A) Increasing concentrations of Scp1p, from 0.1 to 5 μ M, were incubated with 5 μ M F-actin and then subjected to high-speed centrifugation. Scp1p is seen to co-sediment with actin in the pellet (P), whereas Scp1p alone remained in the supernatant (S). (B) F-Actin was subjected to low-speed sedimentation after polymerizing in the presence of increasing concentrations of Scp1p. In the absence of Scp1p most F-actin was found in the supernatant fraction. However, after addition of at least 0.1 μ M Scp1p, more actin co-sedimented in the pellet fraction. (C) Falling-ball assays were performed to investigate the effect of Scp1p on the viscosity of an F-actin solution. Increasing concentrations of Scp1p were seen to decrease the viscosity of the solution, indicating either a severing or a very tight bundling activity.

In vivo analysis

Yeast cells were grown with shaking at 29 °C in liquid YPD (1% yeast extract, 2% Bacto-peptone and 2% glucose, supplemented with 40 μ g/ml adenine) or synthetic medium (lacking uracil; Sigma-Aldrich).

A plasmid was constructed to allow expression of Scp1p tagged at its N-terminus with GFP (green fluorescent protein), under the control of the galactose promoter. *SCP1* was cloned from genomic DNA by PCR using oligonucleotides oKA49-5' CTGGAATTCCAATGAGTTACGATAAGAAGGCAGATGTC and oKA50-3' TGAGAATTCTCTAACTATATCTCTCCGTTGTCCTAAC. KAY 302 cells (MAT α , *his3*- Δ 200, *leu2*-3,112, *ura3*-52, *trp1*-1) were transformed with the GFP-Scp1p plasmid (KAY 337; pGAL-GFPSCP1, *URA3*, *CEN*) and grown in selective media containing glucose. Logarithmic-phase cells were then transferred to media containing 2% raffinose overnight and then expression of GFP-Scp1 was induced for 3 h by addition of 1% galactose. Live cells, and cells fixed for co-localization with actin, were processed and visualized as described previously [13]. Briefly, for images of GFP-Scp1p expression in live cells, 3 μ l of logarithmic-phase cells were put on a slide, covered with a coverslip and sealed with nail polish. Cells were viewed and images recorded as

Figure 3 Effect of Scp1p on kinetics of pyrenyl-actin polymerization and depolymerization

(A) Compared with an actin-only control (black line, circles in inset) 0.5 μ M Scp1p had little effect on polymerization kinetics (grey line, squares in inset) other than to reduce the lag time of polymerization. An expanded view of the initial 40 s of polymerization is shown in the inset. (B) Inset: steady-state (after 24 h) polymerization levels of pyrenyl-actin in the presence (grey line) or absence (black line) of 0.5 μ M Scp1p. Main panel: depolymerization of pyrenyl-actin was slightly retarded in the presence of 0.5 μ M Scp1p (grey line) compared with actin only (black line).

described below. For double labelling with rhodamine-phalloidin (Molecular Probes), cells were fixed using ethanol as follows. Logarithmic-phase cells were pelleted then gently resuspended in ice-cold 70% ethanol and left on ice for 10 min. Cells were pelleted for 20 s and resuspended in rhodamine-phalloidin (5 mg/ml) in PBS containing 1 mg/ml BSA. After cells were left on ice for 5 min they were washed three times with PBS. Cells were resuspended in PBS and placed on poly-lysine treated slides.

Rhodamine-phalloidin staining for visualizing F-actin was performed as described previously [14]. Briefly, 1 ml of logarithmic-phase cells were fixed with 3.7% formaldehyde for 1 h and washed with PBS containing 1 mg/ml BSA and 0.1% Triton X-100. Cells were resuspended in 50 μ l of this buffer and 5 μ l of rhodamine-phalloidin was added and cells incubated in the dark for 30 min. Cells were then washed twice with PBS containing 1 mg/ml BSA and mounted on poly-L-lysine-coated slides. In

Figure 4 Electron microscopy reveals bundling of actin filaments induced by Scp1

Actin filaments were polymerized as described in the Experimental section and incubated with 1 μ M Scp1 for 3 h. Filaments were negatively stained with Nanovan and visualized under a Zeiss 902 electron microscope. Scale bars, 200 nm. Arrows show Scp1p-induced actin bundles.

all cases cells were viewed with a Olympus BX-60 fluorescence microscope with a 100-W mercury lamp and an Olympus 100 $\times$ Plan-NeoFluar oil-immersion objective. Images were captured using a Roper Scientific MicroMax 1401E cooled CCD camera using Scanalytics IP lab software on an Apple Macintosh 7300 computer.

Cells in which *SCP1* was deleted (KAY 448; MAT α , Δ *scp1::KanMx*, *his3* Δ , *leu2* Δ , *met15* Δ , *ura3* Δ) were obtained from Research Genetics (Invitrogen). Cells containing a *SAC6* disruption (AAY1201; MAT α *sac6::URA3*, *trp1-1*, *his3*- Δ 200, *leu2-3,112*, *ura3-52*, *lys2-801*) were a gift from Alison Adams, University of Arizona, Tucson, AZ, U.S.A. The strains were crossed, and resulting diploids sporulated. Tetrads were dissected using a Singer Instruments MSM micromanipulator. Each segregant from tetrads was assessed for actin organization using rhodamine-phalloidin as described above, for growth under a range of growth conditions, and for sensitivity to latrunculin-A [15]. Halo assays were performed as follows. An overnight culture of yeast was grown in YPD medium. Cells (10 μ l) were added to 2 ml of 2 $\times$ YPD, then 2 ml of molten 1% agar (cooled to 55 $^{\circ}$ C) was added and the cell suspension was poured on to the surface of a YPD plate. Dilutions of latrunculin-A were made (1, 2 and 5 mM) and 10 μ l of each dilution of the drug was pipetted on to the centre of a sterile 6-mm filter disc (Fisher). The disc was then placed on top of the agar. The plate was inverted and left at 25 $^{\circ}$ C for 24–48 h before halos were clearly visible. The diameter of halos formed were measured for the three concentrations of drugs applied to the discs. Relative apparent sensitivities were calculated from three independent experiments as described in [16].

SCP1 was cloned from genomic DNA using PCR and oligonucleotides as described above. The sequence was then cloned into a vector carrying the MET promoter (p416MET25 and p426MET25 [17]). The parent plasmid and *SCP1*-containing plasmid were transformed into wild-type yeast. Transformations were performed using lithium acetate as described previously [18]. Over-expression of *SCP1* was induced by switching growth

on to media lacking methionine either on agar plates or in liquid media.

RESULTS

In order to investigate the possible interaction of Scp1p with actin, Scp1 protein was purified from bacterial inclusion bodies and refolded from urea. To verify that the recombinant Scp1 protein was folded and monodispersed, the refolded protein was analysed by CD spectroscopy and analytical gel filtration. CD analysis revealed that Scp1p had a significant α -helical content, as expected for a single CH-domain-containing protein related to calponin [19]. As determined by gel filtration, Scp1p appeared to behave as a monomer in solution, typical of proteins from this family [2,20]. In order to examine whether Scp1p could bind directly to F-actin, a high-speed sedimentation assay was performed. As shown in Figure 2(A), Scp1p interacted directly with F-actin, with Scp1 being recovered almost exclusively in the pellet fraction along with the F-actin, whereas Scp1 alone was recovered exclusively in the supernatant fraction. Duplicate samples from the assay were also subjected to a low-speed sedimentation. F-actin does not pellet significantly under these conditions unless it has been induced to form higher-order structures by the action of a bundling protein. Some F-actin was recovered from the walls of the centrifuge tube in the pellet fraction as seen in the actin-only control, but as shown in Figure 2(B), with increasing concentrations of Scp1p there was a marked shift of actin from the supernatant fraction to the pellet fraction with all of the Scp1p being recovered in the pellet fraction with the F-actin. These data strongly suggest an actin-bundling or cross-linking activity for Scp1p. Experiments where a constant amount of Scp1p was incubated with various concentrations of F-actin produced qualitatively similar results, as did experiments where G-actin was polymerized in the presence of Scp1p.

To assess the actin-bundling activity of Scp1p further, a falling-ball viscometry assay was used to study the effects on the viscosity

of an F-actin solution polymerized in the presence of Scp1p. Somewhat surprisingly, Scp1p caused a dramatic decrease in viscosity even at the lowest concentrations tested, $0.1 \mu\text{M}$ Scp1p with $5 \mu\text{M}$ actin (Figure 2C), suggestive of a severing action rather than a bundling action. However, the actin-sedimentation data would suggest that Scp1p is simply an actin-bundling protein as there was no increase of actin in the supernatant fraction in Figure 2(A) that would indicate severing or actin monomer binding. However, to determine if Scp1p had any effect on severing or capping of actin filaments, the kinetics of actin polymerization and depolymerization were measured using pyrenyl-actin fluorescence in the absence and presence of Scp1p. At concentrations of $7.5 \mu\text{M}$ actin and up to $5 \mu\text{M}$ Scp1p, the presence of Scp1p did not have any significant effect on the rate of actin polymerization, apart from a slight reduction in the lag phase of polymerization (Figure 3A). The latter effect could be due to the stabilization of very short filaments by an F-actin side-binding or bundling activity. There was no effect on steady-state levels of actin polymerization, and depolymerization of pyrenyl-F-actin was slightly inhibited, another property of proteins with F-actin side-binding or bundling activity (Figure 3B).

To visualize any actin-bundling effect of Scp1p directly, actin filaments were incubated with $1 \mu\text{M}$ Scp1p for 3 h. Filaments were negatively stained and viewed by electron microscopy. The electron micrographs show that in the absence of Scp1p there are single filaments of F-actin, whereas in the presence of Scp1p the F-actin is found in tight parallel bundles (Figure 4, arrows) with very few single actin filaments. In summary, the *in vitro* biochemical data are consistent with Scp1p binding to F-actin and inducing the formation of tight bundles of F-actin. Scp1p does not appear to have any role in actin-polymerization kinetics, binding actin monomers or severing or capping actin filaments.

To define regions within Scp1p that might be responsible for actin binding and bundling we subjected Scp1p to limited proteolysis and examined the ability of the resultant fragments to interact with F-actin. As shown in Figure 5, a 6-kDa fragment of Scp1p interacted with F-actin under high-speed sedimentation conditions but remained soluble in the absence of F-actin (Figure 5A). MS of the Scp1p fragment revealed three predominant species of mass 6297, 6780 and 7345 Da with relative abundances of 15:1:2. From the mass of the fragments and known sequence of Scp1p it was possible to predict that the fragments corresponded to residues 139–195, 139–199 and 130–195 respectively. Internal sequencing of the most abundant fragment yielded the sequence YGYMKGASQATEGVVLGQ, corresponding to residues 177–194 of Scp1p and confirming the identity of the 6-kDa fragment as the C-terminal 60 residues of Scp1p, including the CLR and preceding residues, but excluding the CH domain. To substantiate further a role for the C-terminus of Scp1p in binding to actin, fusion proteins were generated to the CH domain and C-terminal regions and subjected to F-actin sedimentation. As can be seen in Figure 5(B), a GST–CH domain fusion did not interact with F-actin, whereas a significant proportion of His-tagged calponin-repeat fusion protein was found in the pellet fraction along with F-actin (Figure 5B, lane 3P) compared with the His-tagged calponin repeat alone, which was completely soluble (Figure 5B, lane 4P). Thus the actin-binding sequences in Scp1p appear to be predominantly in the C-terminal region including the calponin repeat.

To investigate the role of Scp1p further, antibodies were raised to the purified protein. Following affinity purification the antibodies were used to detect a single protein of an appropriate size (19.5 kDa; predicted 22.7 kDa) on a blot of whole-cell extracts separated by SDS/PAGE. Despite using a range of fixation protocols and processing methods, we have been unable to localize Scp1p in yeast cells using the antibodies. As the biochemical

Figure 5 Identification of an actin-binding region in Scp1

(A) Proteolytic fragments of Scp1p (approx. $5 \mu\text{M}$) were subjected to high-speed centrifugation in the absence (lanes 1) or presence (lanes 2) of $5 \mu\text{M}$ F-actin. On SDS/PAGE (7.5–20% gradient gels) the major 6-kDa band, identified as the C-terminus of Scp1p by MS, remained in the supernatant fraction (S); however, in the presence of F-actin it was recovered bound to F-actin in the pellet (P) fraction. (B) GST–CH domain ($10 \mu\text{M}$, lanes 1 and 2) or 6×His-tagged Scp1 C-terminus (6xH-CLR; $25 \mu\text{M}$, lanes 3 and 4) were incubated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of $5 \mu\text{M}$ F-actin. Following ultracentrifugation, GST–CH remained in the supernatant fraction even in the presence of F-actin (lane 1P), whereas some His-tagged CLR was found along with F-actin in the pellet fraction (lane 3P; SDS/PAGE, 15% gels). The bands below GST–CH in lanes 1S and 2S are degradation products of GST–CH; the bands above 6xH-CLR in lanes 3S and 4S are a co-purifying bacterial protein.

data support a role for Scp1 as a tight actin-bundling protein which would place Scp1p in close association with adjacent actin filaments, it may be that Scp1p is in a location where epitopes may be difficult to probe with antibodies in a fixed cell. We were however able to GFP tag Scp1 at its N-terminus and localize this tagged protein in cells. As shown in Figure 6(A), Scp1p localizes to small punctate patches at the cell cortex in a similar pattern to actin patches. To determine whether these patches were indeed actin patches we added the actin-monomer-sequestering drug latrunculin-A. In yeast this causes the rapid disassembly of the actin cytoskeleton such that within 15 min of addition no cortical patches remain [15]. Following latrunculin-A addition for 15 min no Scp1p staining was observed to localize in patches, strongly supporting an association with actin *in vivo*. We also fixed cells expressing GFP–Scp1p and co-stained for actin using

Figure 6 GFP-Scp1p localizes to cortical actin patches

(A) GFP-Scp1p was visualized in live cells and was seen to localize as small punctate patches at the cell cortex. The localization of Scp1p is dependent on the presence of F-actin, as addition of the F-actin-disrupting drug latrunculin-A (200 μ M for 15 min) disrupted Scp1p organization. (B) cells expressing GFP-Scp1 were fixed and stained using rhodamine-phalloidin to visualize F-actin. As shown, there was clear co-localization of Scp1p and actin in cells. Scale bar, 5 μ m.

rhodamine-phalloidin. As shown in Figure 6(B) there is a clear co-localization of actin patches and Scp1p.

To determine whether the interactions of Scp1p with actin are consistent with its *in vivo* role, we first obtained cells in which *SCP1* was deleted. In yeast, the actin cytoskeleton comprises two distinct structures visible by fluorescence light microscopy: actin patches which are punctate and localize to the cell cortex, and actin cables which are elongated structures that extend throughout the cell (reviewed in [21]). Both structures are highly polarized during active cell growth. *SCP1*-deletion strains were viable over the same range of temperatures as wild-type cells. Furthermore, when the actin cytoskeleton was observed in cells stained using rhodamine-phalloidin, the organization of cortical actin patches and actin cables also appeared as in the wild type (Figure 7A). This result suggested that the cell may express other proteins with partially redundant functions, making analysis of the *in vivo* function of Scp1p difficult. We reasoned that the most likely protein with an overlapping function was Sac6p, which is the yeast homologue of fimbrin and to date the only other yeast protein with a major role in actin bundling and filament stabilization [22,23].

Cells in which *sac6* is disrupted are temperature-sensitive and have an aberrant actin phenotype with poorly polarized cortical actin patches and fewer actin cables. We crossed yeast cells carrying a *sac6* disruption with a strain lacking *scp1* to determine

whether Sac6p and Scp1p perform an overlapping function. Cells carrying both deletions were obtained, demonstrating that the combination of deletions is not lethal. However, the $\Delta sac6 \Delta scp1$ cells were considerably more impaired in growth than either single mutant (Figure 7B). Furthermore, the actin phenotype was severely aberrant, with only 2% of $\Delta sac6 \Delta scp1$ cells containing polarized cortical actin compared with 18% of $\Delta sac6$ cells, 88% of $\Delta scp1$ cells and 90% of wild-type cells (Figure 7A). This result suggests that Scp1p acts in concert with Sac6p to organize the yeast F-actin cytoskeleton.

We used a halo assay test to assess the latrunculin-A sensitivity of the $\Delta sac6 \Delta scp1$ double-mutant strain and compared it with the wild-type and each single-deletion strain. The results are shown graphically in Figure 7(C) and importantly show a clear actin phenotype for the $\Delta scp1$ strain alone which was not so apparent from rhodamine-phalloidin staining (Figure 7A). The effect of latrunculin-A on the $\Delta scp1$ strain was not simply a general sickness phenotype, as this strain was no more sensitive than the wild-type strain to a range of compounds, including 5 mM caffeine, 10–50 μ g/ml Benomyl, 3% formamide and 0.5–5% H_2O_2 . Furthermore the latrunculin-A sensitivity of the two deletion strains appears to be additive, with the double-deletion strain showing 7-fold increased sensitivity to latrunculin-A when compared with the wild-type strain (Figure 7C).

Figure 7 *In vivo* analysis supports a role for Scp1p in stabilization of the actin cytoskeleton

(A) Rhodamine-phalloidin staining was performed to assess the F-actin organization in wild-type cells, single-deletion strains $\Delta scp1$ and $\Delta sac6$, and the double-deletion strain $\Delta sac6 \Delta scp1$. In the absence of both bundling proteins the actin cytoskeleton was severely depolarized. Scale bar, 5 μ m. (B) Strains carrying either an *scp1* deletion or a *sac6* disruption were crossed and the resulting diploid strains sporulated and the tetrads dissected. Shown are segregants from two tetrads. In each case there is a 2:2 segregation of markers for each mutation (G418 resistance for *scp1* and URA⁺ for *sac6*). Growth at 37 °C indicated slow growth for *sac6* mutants and no growth for the double mutants. d.o URA, uracil drop out. (C) Latrunculin-A sensitivity of strains was tested using a halo assay in which a nascent lawn of cells was exposed to a range of concentrations of latrunculin-A from 0.5 to 5 mM. Sensitivity is relative to the wild-type (WT) strain; data are means $\pm$ S.D. ($n=3$).

Finally, we over-expressed Scp1p to investigate whether this would affect actin structures. Previously, it has been noted that deletion of the gene encoding the actin-binding protein Abp1p has no clear phenotype while its over-expression causes an aberrant actin phenotype [24]. *SCP1* was cloned behind an inducible methionine promoter (see the Experimental section) and its expression induced both on plates and in liquid media. As shown in Figure 8(A), over-expression of *SCP1* on agar plates causes lethality to cells. Lethality is observed whether Scp1p is overproduced from a CEN plasmid or is on a high-copy-number 2- μ m plasmid. When induced for shorter periods in liquid media a clear disruption of normal actin structures was seen, which became increasingly severe over the time course (Figure 8B). After 4 h many cells showed aberrant clumps of actin at the site of bud formation. Other actin patches in cells appeared mostly depolarized with few normal actin cables observed by rhodamine-phalloidin staining. At later time points (20 h) the cells appeared smaller and mostly unbudded. The actin was still observed to be mostly in clumps or thickened cables.

DISCUSSION

The biochemical, electron-microscopic and cellular analyses provide a compelling case for Scp1p acting as a major actin-bundling protein in *S. cerevisiae*. While we observe biochemically that Scp1p is able to bundle F-actin, the mechanism for the bundling remains to be elucidated. In order for a protein to bundle actin, it requires two or more sites of interaction with actin, or if it has only one actin-binding site, it must oligomerize to give more than one actin-binding site per complex. Like the related proteins SM22 and calponin [2,20], purified Scp1p was determined to be monomeric by analytical gel filtration and must therefore possess multiple sites for interaction with actin. It is known that single CH domains are not sufficient to bind F-actin [19,25], and as shown here (Figure 5B), the CH domain of Scp1p also does not appear to be able to interact with F-actin, but CH domains may serve to locate the actin-binding protein on an actin filament [19,26]. One possibility therefore, is that the sequences C-terminal to the Scp1p CH domain contain distinct sites capable of interacting with actin;

Figure 8 *SCP1* over-expression induces aberrant actin structures

(A) Wild-type yeast cells (KAY 302) were transformed with either an empty vector or one carrying *SCP1* under the control of an inducible MET promoter (see the Experimental section). Transformants were struck either on control plates (left) or on plates lacking methionine to induce expression of *SCP1* (right). d.o. URA, uracil drop out. (B) The top panel shows yeast cells stained with rhodamine-phalloidin to visualize F-actin. Actin patches and cables typical of wild-type cells were observed in all cases. *SCP1* over-expression induces aberrant actin structures to form (lower panel), which are visible as large aggregates of F-actin and very thick prominent cables in cells. Scale bars, 5 μ m.

identification of a 6-kDa fragment of Scp1p corresponding to the C-terminus and including the CLR would tend to support this. Deletion analysis of SM22 [5] and calponin (for a discussion see [27,28]) has revealed the existence of two distinct actin-interaction sites located within the first CLR and in the region between the CH domain and first CLR of both SM22 and calponin. Many of the key residues in these two regions are conserved within Scp1p (see Figure 1B), and these two sites could provide the basis for the actin-bundling activity of Scp1p. An alternative possibility is that the basic nature of Scp1p (pI 9.5) may allow it to exert a bundling effect by acting as a poly-cation that in turn interacts with acidic

actin filaments. The CH domain may then act to optimize this Scp1p-actin interaction. Such a mechanism has been proposed previously for actin bundling by other positively charged proteins and polypeptides including calponin [29]. In general the F-actin-bundling activity of calponin *in vitro* (like transgelin [3]) is only seen at lower salt concentrations and may simply be due to non-specific electrostatic interactions. Actin-binding assays performed herein were all carried out at physiological salt concentrations which would discount an electrostatic component to actin binding. As discussed above, the CH domain is not required for actin binding itself; indeed the nematode protein UNC-87, comprised almost exclusively of CLRs, 7 in total, is an extremely efficient and tight actin-bundling protein [30]. It is likely therefore that sequences C-terminal of the CH domain and within the CLR of Scp1p provide the necessary multiple actin-binding sites to enable Scp1p to bundle actin. This was confirmed by identification of a C-terminal fragment capable of interacting with F-actin. The precise residues in Scp1p involved in interacting with F-actin awaits further analysis.

Falling-ball viscometry of Scp1p revealed a decrease in the viscosity of actin filament networks. It would be expected that if Scp1p, like transgelin, was inducing a meshwork or gel of F-actin it would impede the passage of the falling ball, indicative of an increase in viscosity. A decrease in viscosity is typical of proteins that sever filaments, resulting in an increase in rate of movement of the ball. However, some proteins that produce very tight parallel bundles of F-actin, e.g. scruin [31–33], produce a similar kinetic profile to severing. Tight F-actin bundles result in the formation of free channels in the actin gel through which the falling ball is able to move more freely, giving the appearance of a decrease in viscosity. Such a mechanism for the interaction of Scp1p with F-actin is substantiated both by the low-speed sedimentation data indicating an increase in Scp1p and F-actin in the pellet fraction, and the tightly bundled actin filaments induced by Scp1p as observed by electron microscopy.

Cellular analyses in *S. cerevisiae* revealed both a genetic interaction between *SAC6* and *SCP1* and also a common, additive and specific sensitivity to latrunculin-A. Latrunculin-A is an actin-disrupting drug which has been used to indicate the level of dynamics within the actin cytoskeleton [15,34]. Latrunculin-A acts to sequester monomeric actin, and the binding of the drug to G-actin renders the actin incapable of being incorporated into new filaments [35]. Thus cells with stable actin structures show little sensitivity to the effects of latrunculin-A, while cells with highly dynamic or unstable F-actin are very susceptible to its effects. In keeping with this premise the deletion of either *Sac6p* or *Scp1p* increased the sensitivity of yeast to latrunculin-A, and when both proteins were deleted together there was an additive latrunculin-A sensitivity, indicating that the deletion of both actin-bundling proteins was even more deleterious to the yeast than either alone. Conversely, over-expression of Scp1p also induced aberrant actin structures presumably as a consequence of over-stabilization of actin dynamics and a reduction in normal actin filament treadmilling. F-actin-bundling proteins like *Sac6p* and Scp1p reduce actin filament disassembly, leading to filament stabilization. One other actin-bundling protein described in yeast, coronin, does not share this property [36]. A systematic MS screen of protein complexes in *S. cerevisiae* revealed a complex containing Scp1p and another actin-binding protein Abp1p [37]. The localization of Scp1p to actin patches (herein) and the identification of a complex containing Scp1p and Abp1p, another major actin-binding and actin patch protein, further substantiates a role for Scp1p in actin organization in *S. cerevisiae*.

In summary we report the presence of a yeast actin-binding protein Scp1p which behaves *in vitro* as an actin-bundling protein.

In vivo Scp1p co-localizes with actin patches and its role appears to overlap with that of yeast fimbrin (Sac6p). This supports the idea that Scp1p functions in concert with Sac6p to increase the stability of F-actin. Over-expression of Scp1p was seen to be extremely detrimental to the organization of the actin cytoskeleton and to cell viability, suggesting that levels of Scp1p are likely to be tightly regulated within cells.

A paper by Goodman and colleagues [38], published while this paper was under revision, has demonstrated substantially the same findings, with respect to a role for Scp1p in the regulation of the yeast actin cytoskeleton, as presented in this paper.

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