Interaction in Vivo and in Vitro between the Yeast Fimbrin, SAC6P, and a Polymerization-defective Yeast Actin (V266G and L267G)*

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A mutant yeast actin (GG) has decreased hydrophobicity in a subdomain 3/4 hydrophobic plug believed to be involved in a hydrophobic cross-strand “plug-pocket” interaction necessary for actin filament stability. This actin will not polymerize in vitro but is compatible with cell viability. We have assessed the ability of Sac6p, the yeast homologue of the actin filament stabilizing and bundling protein fimbrin, to restore polymerization in vitro and to facilitate GG-actin function in vivo. Sac6p rescues GG-actin polymerization at 25 °C but not at 4 °C. The actin polymerizes into bundles at room temperature with a fimbrin:actin molar ratio of 1:4. At this ratio, every actin monomer contacts a Sac6p actin binding domain. Following cold-induced depolymerization, actin/Sac6p mixtures repolymerize beginning at 15 °C instead of the 25 °C required for de novo assembly, because of the presence of residual actin-Sac6p nuclei. Generation of haploid ΔSac6/GG-actin cells from either diploid or haploid cells was unsuccessful. The facile isolation of cells with either mutation alone indicates a synthetic lethal relationship between this actin allele and the SAC6 gene. Sac6p may allow GG-actin function in vitro by stabilizing the actin in bundles thereby helping maintain sufficient levels of an otherwise destabilized actin monomer within the cell.

Actin, required for a number of intracellular processes ranging from muscle contraction to cytokinesis, has a primary structure that is largely conserved from yeast to human (1). Actin is present in cells as two interchangeable forms, monomeric G-actin and filamentous F-actin. Actin filament stability is determined by a number of monomer/monomer contacts both along and perpendicular to the filament axis. A major source of cross-strand stabilization according to Holmes’ model of F-actin (2, 3) involves the interaction of hydrophobic residues in a subdomain 3/4 loop of an actin monomer with a hydrophobic pocket on the opposing strand of the filament. In yeast actin, this hydrophobic plug consists of four residues Val266–Leu267–Gly268–Leu269 (1). One prediction of this proposal was that interference with the plug-pocket interaction would prevent polymerization of actin resulting in cytotoxicity.

Our laboratory previously constructed an actin mutant (GG) in which the first two residues in the subdomain 3/4 loop were replaced by glycines (4, 5). The GG mutant cells are cold-sensitive and slow growing, and at all temperatures cells contain no visible actin cables and are characterized by delocalized cortical patches. Purified GG actin alone cannot polymerize in vitro at any temperature tested. However, polymerization can be restored by inclusion of phalloidin or beryllium fluoride (BeFx), although the effects of these agents on GG-actin polymerization differ in a temperature-dependent fashion. Phalloidin will rescue polymerization at temperatures all the way down to 4 °C, presumably because it forms an inter-strand interaction (3, 4, 6), which substitutes for the one disturbed by the GG mutation. BeFx on the other hand, restores polymerization at 25 °C but not at 4 °C (4). This agent substitutes for the γ-phosphate of ATP that is released from the interior of the monomer following polymerization and restores the filament to a more stable ADP-Pi state, like state, apparently by strengthening monomer-monomer contacts along the filament axis (7, 8). Presumably, at some point, this increased stabilization is not enough to overcome the additional destabilization caused by decreasing temperature in the already compromised plug-pocket interaction, and the filament disassembles.

The polymerization defect of the GG-mutant, coupled with its compatibility with yeast viability, contradicts the prediction of Holmes and colleagues (2, 3) cited above. This result also immediately raises the question of what allows GG actin to function in vivo, since F-actin is required for actin-based processes. In vivo, actin function is modulated by a number of actin monomer-binding proteins and filament capping, cross-linking, severing, and bundling proteins (9). One such protein is a Ca2+-sensitive filament bundling protein called fimbrin or plastin. Fimbrin was first identified as a component of the chicken intestinal brush border microvillus (10) and exists in humans as three highly homologous isoforms, T-, L-, and I-plastin (11, 12). Fimbrin consists of one calcium binding domain and two tandem actin binding domains, ABD1 and ABD2. The calcium binding domain includes the first N-terminal 100 amino acids and contains two EF-hand motifs. Each of the actin binding domains is composed of two calponin homology subdomains (13). Fimbrin binds to the actin filament between two adjacent monomers along the filament axis (14) and thus might be expected to stabilize GG-actin filaments in much the same way that BeFx does. In the presence of calcium, ABD1 is apparently occluded leading to an inhibition of the ability of the protein to bundle actin filaments.

Sac6p, also known as yeast fimbrin, has 36–43% identity to human and chicken fimbrins, respectively (15). The SAC6 gene was identified as a suppressor of a yeast actin mutation (16), and the protein encoded by this gene Sac6p was independently isolated by actin-filament affinity chromatography (17). Sac6p colocalizes with actin in vivo (17) and is capable of bundling yeast actin filaments in vitro (15) although its ability to induce actin polymerization has not been described. With a complete SAC6 gene deletion, the cell is viable but grows at a reduced

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1 The abbreviations used are: BeFx, beryllium fluoride; PCR, polymerase chain reaction; ABD, actin binding domain; WT, wild type.
rate at 23 °C and cannot grow at 37 °C. These null mutant cells do not have normal actin cables and exhibit an abnormal cortical actin distribution. In addition, they are defective in both morphogenesis and endocytosis (18). Human T- and L-plastin can complement the defect of SAC6 deletion in Saccharomyces cerevisiae (19). It has also been demonstrated that deletion of SAC6 leads to a substantial increase in the G/F actin ratio, presumably because of filament destabilization (20).

Because of the properties associated with Sac6p in vitro, and the hypothesized function for the protein as an F-actin stabilizer in vivo, we wished to determine if Sac6p played a major role in the ability of the polymerization-defective mutant GG-actin to support yeast life. Here, we report our studies of the interaction of Sac6p with this mutant actin in vitro and the effect of deleting the SAC6 gene in a cell producing GG-actin as its only actin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The BCA protein assay kit was obtained from Pierce. QiAquick Gel Extraction Kit was obtained from Qiagen. Zymolyase was purchased from ICN. Bacto-yeast extract, Bacto-peptone, and Bacto-tryptone were from Difco. DE52 DEAE-cellulose was purchased from Whatman. Affigel-10 gel and Bio-Gel HT hydroxypapatite were purchased from Bio-Rad.

**Purification of Wild-type and GG Mutant Yeast Actin**—Wild-type and GG mutant actins were purified using a DNase-I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23). Actins were type and GG mutant actins were purified using a DNase I-agarose chromatography procedure as described previously (22, 23).

**Actin Polymerization Assays—**Polymerization of Mg\(^2+)\-GG-actin at 25 °C was assayed by monitoring the increase in light scattering following the addition of MgCl\(_2\) and KCl to final concentrations of 2 and 50 mM, respectively, in the absence or in the presence of Sac6p. Experiments were performed using a mini-cuvette with capacity of 150 μl in a thermostatted cuvette chamber of a SPEX Fluorolog 3 fluorescence spectrometer with excitation and emission wavelengths set at 360 nm.

**Centrifugation Assays of the Actin-Sac6p Interaction**—To measure F-actin bundling induced by Sac6p, the material was centrifuged for 15 min at 12,000 rpm in a TLA-100 rotor in a Beckman TL-100 ultracentrifuge (26). Centrifugation was performed at 25 °C. When centrifugation was complete, the contents of the pellet were resolved by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. Following staining of the gel with Coomassie Brilliant Blue R-250, the bands were quantitated by scanning densitometry. To measure total F-actin formation, the experiment was performed as just described except that centrifugation was performed at 70,000 rpm for 30 min. In all cases, the Sac6p was centrifuged by centrifugation at 70,000 rpm in a TLA-100 rotor 30 min at 4 °C before use.

**Electron Microscopy—**Actin samples were deposited on carbon-coated Formvar grids, negatively stained with 1.5% uranyl acetate, and visualized with a Hitachi model 7000 electron microscope (University of Iowa Central Electron Microscope Facility).

**Construction of Diploid GG-Actin/sac6 Double Mutants—**Genotypes of yeast strains described below are shown in Table I. We first disrupted one of the chromosomal actin genes from strain Y266 with the HIS3 gene. A PatI site was introduced into the actin coding sequence in the codon for E\(_{520}\), and an NcoI site was introduced at the beginning of the actin coding sequence. Neither of these changes altered the amino sequence of the protein. This actin sequence, along with the ACTI promoter and 3′-untranslated region, was isolated as an EcoI-BamHI fragment from pRSWT and cloned into pRS313 to make pRS313ACT. The HIS3 gene was introduced between the NcoI and PatI sites of the actin coding sequence in pRS313ACT to make pHIS3ACT1:HIS3. The EcoI-BamHI fragment was removed from pHIS3ACT1:HIS3 and inserted into a chromosomal actin gene by homologous replacement to produce strain yPAR266-1. Correct insertion of the sequence was determined by PCR and Southern analysis.

We next integrated the coding sequence for GG-actin along with the yeast actin promoter at a ura3-32 site of yPAR266-1 by selecting for reconstitution of URA3 function to produce yPAR266-2 (27). The SAC6 gene was deleted from yPAR266-2 as follows. We obtained a plasmid from Allison Adams, pAAB123, containing the SAC6 gene whose coding sequence had been replaced by the LEU2 gene. An EcoRI-PstI fragment containing the SAC6 gene with the LEU2 replacement was released and used to disrupt one of the SAC6 genes of yPAR266-2 by homologous recombination to give yPAR266-3. Correct insertion of the gene was verified by PCR using three different sets of primers. Details of the proof are presented under “Results.”

**Attempted Construction of Haploid GG-Actin/sac6 Double Mutants—**Strain yPAR266-2 was sporulated, and tetrads were dissected. Twenty two haploid colonies with the phenotype his\(^+\), ura\(^+\) were identified which express only GG actin, and one, called yPAR266-4, was used for the following experiments. We then used the fragment from AAB123 to disrupt the SAC6 gene in this cell as above and selected for growth on leu-- medium. No transformants were observed. Control experiments showed the cells were competent and that the fragment could transform diploid cells as above.

**RESULTS**

Interaction of Sac6p with GG-actin at 25 °C—The focus of this paper was to establish whether or not Sac6p, the yeast homologue of fimmbrin, was an important factor in restoring the ability of the mutant GG actin to form functional filaments in vivo. We first assessed the ability of Sac6p to restore GG-actin

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**TABLE I**

Yeast strains used in this study

| Strain    | Genotype                        |
|-----------|---------------------------------|
| y266      | MATa/MATa ura3-32 leu2-2 his3-3 his3 trp1/trp1 |
| yPAR266-1 | MATa/MATa act1::HIS3/+ leu2-2 ura3-3 his3 his3 his3 trp1/trp1 |
| yPAR266-2 | MATa/MATa act1::HIS3/+ leu2-2 ura3-3 his3 his3 trp1/trp1 |
| yPAR266-3 | MATa/MATa act1::HIS3/+ sac6::LEU2 leu2-2 leu2 |
| Sac6p     | G-G-URA3 ura3-3 his3 his3 his3 trp1/trp1 |

* From Dr. Robert J. Deschenes (University of Iowa).
polymerizability in vitro at 25 °C. In the absence of added Sac6p, the Mg\textsuperscript{2+} form of GG-actin remains in an unpolymerized state even in the presence of F-buffer (Fig. 1). When Sac6p was added at an actin:fimbrin ratio of 10:1, we observed an immediate increase in light scattering suggesting the fimbrin-induced formation of actin filaments.

To ascertain the morphology of this scattering material, we examined the material at the end of the experiment by electron microscopy following negative staining of the sample. Fig. 2 shows Sac6p induces the formation of bundles of actin filaments instead of single filaments, in agreement with its known properties as a filament-bundling protein. The morphology of those bundles we observed was like those seen in the interaction of F-actin with fimbrin (28).

With WT actin, fimbrin will cause maximum bundling at fimbrin:actin ratios much lower than the one used above. However, for rescue of GG-actin polymerizability, it might have been necessary for all actin monomer-monomer associations along the longitudinal axis to be stabilized by Sac6p. We thus determined what amount of Sac6p was required for maximal actin filament formation. The results, described in Fig. 3, demonstrate that at the actin and fimbrin concentrations used, maximum actin assembly occurred at a fimbrin:actin ratio of about 1.3. This is in good agreement with a value of 1 fimbrin to 2.5 actin monomers at saturation as determined previously with the Dictyostelium discoideum fimbrin homologue (29).

To quantify the interaction between Sac6p and GG-actin better, we carried out a cosedimentation study. Sac6p and Mg\textsuperscript{2+}-GG actin were combined in the presence of F-buffer at 25 °C, and assembly of the complex was allowed to proceed to completion. Aliquots of the resulting mixture were centrifuged at low speed, sufficient to pellet only large bundles, and at high speed, sufficient to pellet both bundles and individual actin filaments. The results are shown in Fig. 4. At a Sac6p:actin ratio of 1:1, essentially all of the actin pelleted under the low speed conditions, indicating that under these conditions few if any individual filaments were formed. In the absence of Sac6p, no actin was observed in the low speed pellet (data not shown). Densitometry of the actin and Sac6p bands showed that, for this experiment, the Sac6p:actin molar ratio in either the high or low speed pellet was between 1:3 and 1:4 with about 50–60% of the actin pelleting in the low speed pellet. The result is consistent with the saturation curve presented in Fig. 3. This experiment was repeated four times with comparable results suggesting that the minimal amount of Sac6p needed for stabilization of a GG-actin filament is approximately one actin binding domain per two monomers along the filament axis. Furthermore, together with our EM data, these results suggest that at this initial ratio of Sac6p:
actin, all of the actin that pellets is in the form of bundles and not individual filaments.

Effect of Temperature on the Sac6p-GG Actin Interaction—
Our previous work showed that mutations in the subdomain 3/4 hydrophobic plug where the GG mutation is located produce a cold-sensitive polymerization defect. In the case of GG-actin which does not polymerize at all by itself at 25 °C, BeFx, which strengthens longitudinal contacts along the filament axis, re-stores polymerization at 25 but not 4 °C. Since Sac6p binds along the filament axis, its ability to stabilize GG-actin filaments should exhibit the same type of cold sensitivity. To test this hypothesis, we combined Sac6p and GG actin in F-buffer at 4 °C and followed the change in light scattering as a function of temperature. Fig. 5 demonstrates that no noticeable polymerization occurs until 25 °C. At this temperature polymerization ensues and rapidly reaches completion.

We next determined whether pre-formed GG-actin-Sac6p assemblies were cold-sensitive. We also wished to know whether cold-induced disassembly, if it occurred, was reversible. GG-actin and Sac6p were coassembled in F-buffer at 25 °C, and the temperature was lowered to 4 °C. Fig. 6 shows that decreasing temperature led to a decrease in light scattering until at 4 °C where the total light scattering had decreased by 85%. We then raised the temperature. No polymerization was observed at 10 °C. However, at 15 °C we observed an immediate increase in light scattering which reached an intermediate plateau. Additional partial light scattering increases occurred at 20 and 25 °C until the original plateau level was reached. Examination of the samples at these intermediate positions revealed that at 4 °C, residual spherical or linear aggregates were present (Fig. 7A). At 15 °C, although individual filaments were occasionally observed (Fig. 7B1), the beginnings of small bundles were observed in the vast majority of fields (Fig. 7B1). Finally, return of the temperature to 25 °C results in the formation of mature bundles with few if any noticeable free filaments resembling the behavior of material initially assembled at 25 °C (Fig. 7C).

Effect of Ca$^{2+}$ on the GG-Actin-Sac6p Interaction—We demonstrated above that in the presence of Sac6p, GG-actin assembled at lower temperatures if it had first been polymerized and then disassembled rather than polymerized de novo. This differential response suggested that the earlier onset of polymerization following disassembly might be due to the presence of residual Sac6p-actin complexes following the decrease in temperature to 4 °C. To test this hypothesis, we needed a method to dissociate completely the Sac6p from the actin.

Earlier work with fimbrin homologues from both Dictyostelium and humans demonstrated that the interaction of fimbrin with actin was inhibited in the presence of Ca$^{2+}$ (29, 30). We wished to determine if the Sac6p-GG actin interaction was also calcium-sensitive. The Mg$^{2+}$ form of monomeric GG-actin was combined with Sac6p in F-buffer in the presence of different calcium concentrations, and the change in light scattering was monitored as a function of time. The results are shown in Fig. 8. Two hundred μM Ca$^{2+}$ drastically retarded the rate of polymerization in comparison with the Ca$^{2+}$-free sample, whereas 400 μM Ca$^{2+}$ completely inhibited Sac6p-dependent actin assembly.

We then determined whether eliminating residual Sac6p-actin complexes by introducing Ca$^{2+}$ following cold-induced disassembly would alter the repolymerization characteristics of the system. The results are shown in Fig. 9. Mg$^{2+}$-GG actin was polymerized at 25 °C in the presence of Sac6p. Following completion of polymerization, the temperature was reduced to 4 °C, and the mixture was allowed to disassemble until a plateau had been reached 20 min after the solution had reached 4 °C. Four hundred μM Ca$^{2+}$ was then added and the solution allowed to incubate an additional 30 min at 4 °C. Finally, 400 μM EGTA was added, and the temperature was allowed to rise. We observed no increase in light scattering until the temperature of the solution had reached 25 °C, similar to the response for de novo polymerization of the sample.

Sac6p Is Required for GG-Actin Function in Vivo—Based on our results that Sac6p could restore GG-actin polymerization in vitro, we wished to determine whether the reaction was required for maintenance of GG-actin function in vivo. We thus constructed a diploid strain of yeast, yPAR26-2, in which one of the chromosomal actin genes was disrupted with HIS3, and the GG-actin coding sequence was integrated in the chromosome along with the actin promoter at the ara3-52 site. The cells were sporulated, and 27 tetrads were dissected. Statistically, one-fourth of the spores should not be viable since they would have no active actin gene and one-fourth of the cells should express only GG-actin. From the 27 tetrads, 81 surviving colonies were obtained with 22 of them expressing only
GG-actin. Thus, GG-actin appears completely compatible with germination and vegetative growth.

We next used insertion of the LEU2 gene to delete one of the Sac6p coding sequences from strain yPAR266-2 and selected for the desired disrupted diploid cells by the ability to grow on leu2 medium. Deletion of one of the genes was confirmed by PCR as shown in Fig. 10. Three sets of primers were used. Two sets included primers within the LEU2 coding sequence and sequences outside of both ends of the SAC6 coding region. Each gave the fragment expected if the appropriate deletion had occurred. PCR using two primers within the SAC6 coding region confirmed the presence of one active SAC6 gene in the diploid cells. Following sporulation, 100 tetrads were dissected, and the spores were allowed to germinate at 24 °C. Germination was poor with 29 surviving colonies from the maximum of 300 possible. None of these contained GG actin alone in the absence of Sac6p (Table II), although cells were isolated with an ACT1/sac6 genotype (his2, ura-, leu1). When DNA from these cells were subjected to PCR using primers within the SAC6 coding region, no amplification of DNA occurred, whereas with both diploid and haploid cells containing a functional SAC6 gene, the expected band was observed. These results indicated that in terms of the ability to germinate and seed a colony, deletion of SAC6 is synthetically lethal in the presence of GG actin alone.

We next tested the ability of vegetative cells expressing only Sac6p coding sequences from strain yPAR266-2 and selected for the desired disrupted diploid cells by the ability to grow on leu- medium. Deletion of one of the genes was confirmed by PCR as shown in Fig. 10. Three sets of primers were used. Two sets included primers within the LEU2 coding sequence and sequences outside of both ends of the SAC6 coding region. Each gave the fragment expected if the appropriate deletion had occurred. PCR using two primers within the SAC6 coding region confirmed the presence of one active SAC6 gene in the diploid cells. Following sporulation, 100 tetrads were dissected, and the spores were allowed to germinate at 24 °C. Germination was poor with 29 surviving colonies from the maximum of 300 possible. None of these contained GG actin alone in the absence of Sac6p (Table II), although cells were isolated with an ACT1/sac6 genotype (his2, ura-, leu1). When DNA from these cells were subjected to PCR using primers within the SAC6 coding region, no amplification of DNA occurred, whereas with both diploid and haploid cells containing a functional SAC6 gene, the expected band was observed. These results indicated that in terms of the ability to germinate and seed a colony, deletion of SAC6 is synthetically lethal in the presence of GG actin alone.

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**Fig. 5.** Temperature dependence of Sac6p-facilitated polymerization of GG-actin. Sac6p (0.95 μM) was combined with GG-actin (9.5 μM) in F-buffer at 4 °C, and the light scattering was recorded as a function of time. As indicated, the temperature was raised to 10, 15, 20, and 25 °C, with 30-min intervals. No increase in light scattering was observed prior to 25 °C.

**Fig. 6.** Temperature dependence of Sac6p-dependent GG-actin repolymerization. Sac6p (0.95 μM) and GG-actin (9.5 μM) were combined in F-buffer at 25 °C and allowed to polymerize. At the completion of polymerization, the temperature was lowered to 4 °C, and the bundles were allowed to disassemble over about a 2-h period until a plateau was reached. The temperature was then raised in steps to a final temperature of 25 °C. At each step, the temperature was maintained for about 1 h until a plateau was reached. The arrows indicate the time when each temperature change was initiated. Actin assembly was monitored by the change in light scattering as described under “Experimental Procedures.”

**Fig. 7.** EM images of GG-actin/Sac6p assemblies at different temperatures during the repolymerization experiment described in Fig. 6. Samples were removed following attainment of equilibrium at the temperatures indicated, negatively stained, and examined by electron microscopy as described under “Experimental Procedures.” A, depolymerized sample at 4 °C; B, after repolymerization at 15 °C; C, following elevation of the sample to 25 °C. The bar represents 0.1 μm.
GG-actin to survive in the absence of Sac6p. The SAC6 gene was deleted from haploid cells expressing either WT or GG-actin as its sole actin using homologous recombination with the LEU2 gene as a marker, and transformants were selected on leucine-deficient medium. Leu+ transformants were readily obtained when WT actin cells were utilized. However, no leu+ transformants were obtained with the GG cells. Control transformations with an unrelated plasmid demonstrated that these GG cells were competent. This experiment suggested that not only was Sac6p required in the presence of GG-actin for germination but also for vegetative growth.

DISCUSSION

GG-actin, due to decreased hydrophobicity in a subdomain 3/4 hydrophobic plug, will not polymerize in vitro but will allow growth and proliferation of yeast when it is the only actin in the cell. Although phalloidin and beryllium fluoride will both restore GG polymerization in vitro, neither agent is present in a yeast cell. Since Sac6p will cause formation of stable filament bundles, it is a candidate for a protein in the cell that will allow GG-actin to function. The goal of this study was thus 2-fold: 1) to determine if Sac6p, the yeast homologue of fimbrin, could restore GG-actin polymerization in vitro, and 2) if so, to determine whether the interaction was necessary for GG-actin function in vivo. Our work has provided new insight into what interactions are required for formation of a stable actin filament, and it has demonstrated that Sac6p can induce the polymerization of GG-actin in vitro, that this protein is necessary for GG-actin function in vivo, and that this requirement is specific. Finally, it has led to a new model to explain the basis for the necessity of Sac6p in a GG-actin background.

In Vitro Studies—We have demonstrated for the first time that Sac6p can not only bind to F-actin and induce filament cross-linking but that it can actually induce polymerization of G-actin. Sac6p restores the ability of this mutant actin to polymerize. It therefore serves as a filament-stabilizing protein in vitro by virtue of its ability to lower the critical concentration of this mutant actin for polymerization. Furthermore, at room temperature, this interaction drives the filaments into bundles since few if any free filaments are observed. Fimbrin binds between adjacent actin monomers along the filament axis and is believed to strengthen monomer-monomer contacts along this axis (30). Previous studies have shown that bundling of actin will occur with an actin:fimbrin ratio of 8:1 (31). In comparison, our cosedimentation studies show that at sub-saturating amounts of fimbrin, a molar ratio of at least 4:1 is required for filament formation that leads to bundling. Thus, on the average, each alternating pair of monomers must be bound to half of a Sac6p for a stable filament to form. The interaction of Sac6p with actin at a density sufficient to bundle WT actin will not develop sufficient longitudinal stabilization of the filament to overcome the defect introduced by decreasing the hydrophobicity of the plug. Interaction of the second actin-
Interestingly, not every actin-binding protein can rescue polymerizability of this mutant. Rabbit skeletal myosin S1, which will polymerize actin, even in low salt conditions, was unable to affect the polymerization of this mutant.²

We previously demonstrated that the rescue of GG-actin polymerization by BeFx, but not phalloidin, was cold-sensitive. Here we demonstrated cold sensitivity for rescue by Sac6p as well. No polymerization was observed de novo at temperatures below 25 °C, and exposure of pre-formed GG-actin/Sac6p bundles to temperatures below 25 °C resulted in their disassembly. However, following disassembly, even at 4 °C, residual bundle fragments remained. When the temperature was again raised following depolymerization, repolymerization began at 15 °C instead of the 25 °C previously observed, and the extent of polymerization increased until at 25 °C complete assembly was achieved. Treatment of the depolymerized material with calcium to inactivate the fimbrin and subsequent removal of part of the Ca²⁺ with EGTA delayed the onset of re-polymerization until 25 °C, similar to what we observed for polymerization de novo.

The difference in temperature required for de novo Sac6p/ GG-actin assembly and reassembly following cold-induced depolymerization is consistent with predictions of the Holmes’ model for F-actin. The hydrophobic plug-pocket interaction should play a much more significant role in facilitating formation of a nucleus rather than in filament elongation. This hypothesis derives from the fact that in the nucleus each monomer participates in fewer contacts with other monomers than in the filament. Previous work suggested that stabilization of the filament depends on the sum of the energy provided by both longitudinal and perpendicular contacts. Hydrophobic interactions are cold-sensitive, and in GG-actin, the hydrophobicity of the plug has been severely compromised. In this situation, the further decrease in stabilization of the plug-pocket interaction caused by lowered temperatures should lead to a much greater cold sensitivity for nucleus formation than for addition of a monomer to a stabilized filament bundle. This is precisely the result we obtained. Rescue of GG-actin polymerization by Sac6p thus seems to derive from its ability to capture and stabilize these nuclei or transient oligomers thereby providing a scaffold to which subsequent monomers can more easily add.

Our EM results at 4 and 15 °C provide additional insight into the elements determining filament stabilization by Sac6p. We observed small amounts of assembled material of different morphologies following depolymerization, and after raising such a sample to 15 °C, we observed a mixture of individual filaments and incipient bundles. If interaction with Sac6p stabilizes the filament by strengthening monomer-monomer contacts along the filament, then a filament in which these monomer-monomer contacts are initially stronger should lead to a stronger Sac6p-actin interaction. In terms of the Holmes’ F-actin model, decreased strength of the residual plug-pocket interaction at lowered temperatures may alter the actin-Sac6p interaction in a way that unequally affects the interaction of the actin with the two actin binding domains on Sac6p leading to filament stabilization but decreased cross-linking. Although the two ABDs of Sac6p are very similar, they are not identical, and their position in the polypeptide may also differentially affect their ability to interact with an actin filament with altered topology.

In Vivo Studies—Our work in vitro clearly establishes that Sac6p can restore polymerizability of GG-actin. To determine whether this ability of Sac6p was required in cells expressing only GG-actin, we determined whether a Sac6 deletion was

² B. Kuang and P. A. Rubenstein, unpublished results.
synthetically lethal in a GG-actin background. Previously, we showed that expression of GG-actin from a centromeric plasmid was sufficient for cell viability in the presence of Sac6p. However, it is possible that multiple copies of the plasmid in the same cell might have produced enough actin to overcome sufficiently the defect in the protein to allow vegetative growth. Our results here demonstrate that even when the GG coding sequence is integrated in the chromosome thereby limiting gene expression, the actin produced is compatible with viability. We also showed that diploids simultaneously expressing both WT and GG actins from single genes are able to produce viable spores expressing only the mutant actin.

Although Sac6 null diploids will not sporulate, sporulation and germination will occur in Sac6 hemizygotes, producing viable ΔSac6 spores (19). In our study with diploid cells hemizygous for SAC6 and containing single copies of both WT and GG-actin genes, we dissected 100 tetrads. Only about one-tenth of the expected number of viable haploids was obtained, and of these, there were none with a ΔSac6/GG-actin phenotype. The presence of GG-actin was not detrimental per se in a ΔSac6 background since equal numbers of viable spores with a GG/WT and WT phenotype were obtained. Furthermore, among surviving cells, roughly half were ΔSac6. Thus, with the proper actin content present, the SAC6 gene appeared to segregate normally. These results indicate that in a GG-actin background, deletion of the SAC6 gene is synthetically lethal in terms of the ability of the cells to sporulate and germinate successfully.

Since cytoskeletal requirements for sporulation and germination might be more demanding than for vegetative growth, we attempted to delete the SAC6 gene from haploid cells expressing GG-actin as their only actin. Again, no cells with a ΔSac6/GG-actin phenotype were obtained. Overall, these results strongly indicate that both for vegetative growth and for sporulation and germination, sole expression of GG-actin requires the presence of Sac6p for cell viability.

We have shown that not every actin-binding protein, myosin for example, can restore GG-actin polymerization in vivo. However, our results reported here raise the possibility that any actin-binding protein capable of restoring GG-actin polymerization might be required for cell viability in a GG-actin background. This is not the case, however. Unpublished work from our laboratory has shown that tropomyosin will rescue GG-actin polymerization in vitro and that it results in the formation of individual filaments as opposed to bundles. However, contrary to the case with Sac6p, deletion of the TPM1 gene, which accounts for 85% of the tropomyosin in the cell, is not synthetically lethal with GG-actin.

A Model for Rescue of GG-Actin Function by Sac6p—Actin functions in vivo generally are performed by the filamentous and not the bundled form of the protein. The absence of an absolute requirement for Sac6p in a WT actin background indicates that its bundling functions are not necessary. Sac6p will rescue the polymerizability of GG-actin, but this rescue results in bundle formation and not the generation of individual filaments. Thus, its removal from the GG-filament, necessary for the interaction of the filament with other proteins such as myosin, would likely result in filament disassembly. It is therefore not immediately apparent why Sac6p is required in a GG-actin background. Our previous studies (5) demonstrated that this mutation results in an enhanced rate of nucleotide exchange from the interior of the actin and a significantly lower thermal denaturation point. These results suggest that this mutation, besides affecting polymerization, results in a significantly destabilized monomer. A turnover of monomers faster than they can be replaced biosynthetically will lead to a decrease in total actin. The ability of Sac6p to sequester GG-actin in stable filament bundles would, in essence, create a more stable reservoir of potential actin monomers in the cell. This situation would prolong the lifetime of the GG-actin while allowing the monomer pool to be replenished via the normal equilibrium that exists between monomers, filaments, and bundles. It has already been demonstrated that deletion of Sac6p from a wild-type background causes a significant increase in the monomeric actin pool (20). With GG-actin and its inherent instability, the result of a Sac6p deletion would be an accelerated rate of monomer turnover leading to cytoskeletal compromise and ultimately cell death.

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REFERENCES

1. Ng, R., and Abelson, J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3912–3916
2. Holmes, K. C., Popp, D., Gebhardt, W., and Kabasch, W. (1990) Nature 347, 44–49
3. Lorenz, M., Popp, D., and Holmes, K. C. (1993) J. Mol. Biol. 234, 826–836
4. Kuang, B., and Rubenstein, P. A. (1997) J. Biol. Chem. 272, 1237–1247
5. Kuang, B., and Rubenstein, P. A. (1997) J. Biol. Chem. 272, 4412–4418
6. Steinmetz, M. O., Stoffler, D., Muller, S. A., Jahn, W., Wolpensinger, B., Golde, K. N., Engel, A., Fauth, H., and Aebl, U. (1998) J. Mol. Biol. 276, 1–6
7. Combeau, C., and Carlier, M.-F. (1988) J. Biol. Chem. 263, 17429–17436
8. Orlowa, A., and Egelman, H. E. (1992) J. Mol. Biol. 227, 1043–1053
9. Pollard, T. D., and Cooper, J. A. (1986) Annu. Rev. Biochem. 55, 897–1035
10. Bretscher, A., and Weber, K. (1986) J. Cell Biol. 106, 335–340
11. De Arruda, M. V., Watson, S., Lin, C. S., Leavitt, J., and Matsudaira, P. (1990) J. Cell Biol. 111, 1069–1079
12. Lin, C. S., Shen, W., Chen, Z. P., Tu, Y. H., and Matsudaira, P. (1994) Mol. Cell. Biol. 14, 2457–2467
13. Matsudaira, P. (1991) Trends Biochem. Sci. 16, 87–92
14. Hanein, D., Volkmann, N., Goldsmith, S., Michon, A. M., Lehman, W., Craig, R., DeRosier, D., Almo, S., and Matsudaira, P. (1996) Nat. Struct. Biol. 3, 787–792
15. Adams, A. E., Botstein, D., and Druh, D. G. (1991) Nature 354, 404–408
16. Adams, A. E., Botstein, D., and Druh, D. G. (1989) Science 243, 231–233
17. Druh, D. G., Miller, R., and Botstein, D. (1988) J. Cell Biol. 107, 2551–2561
18. Kühler, E., and Riezman, H. (1993) EMBO J. 12, 2655–2662
19. Adams, A. E., Shen, W., Lin, C. S., Leavitt, J., and Matsudaira, P. (1995) Mol. Cell. Biol. 15, 69–75
20. Karpova, T. S., Tatchell, K., and Cooper, J. A. (1995) J. Cell Biol. 131, 1483–1493
21. Sherman, F. (1991) Methods Enzymol. 194, 3–20
22. Cook, R. K., and Rubenstein, P. A. (1992) in Practical Approaches in Cell Biology (Carraway, K., and Carraway, C. C., eds) pp. 99–122, IRL Press, Oxford
23. Kron, S. J., Druh, D. G., Botstein, D., and Spudich, J. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4466–4470
24. Pollard, T. D. (1986a) J. Cell Biol. 103, 2747–2754
25. Sandrock, T. M., O'Dell, J. L., and Adams, A. E. M. (1997) Genes 147, 1635–1642
26. Hentschel, T., Dotrock, T. S., Royser, M., and O’Dell, J. L., Adams, A. E. M. (1994) J. Cell Biol. 123, 413–422
27. Butler, G., and McConnell, D. J. (1988) Curr. Genet. 14, 405–412
28. Glenny, J. R., Jr., Kauflis, P., Matsudaira, P., and Weber, K. B. (1981) J. Biol. Chem. 256, 9283–9288
29. Prasalov, J., Stocks, S., Mattiot, G., Heidecker, M., and Gerisch, G. (1997) Mol. Biol. Cell 8, 83–95
30. Hanein, D., Matsudaira, P., and DeRosier, D. J. (1997) J. Cell Biol. 139, 357–369
31. Namba, Y., Itu, M., Zo, Y., Shigesada, K., and Maruyama, K. (1992) J. Biochem. (Tokyo) 112, 505–507
32. Orlowa, A., and Egelman, E. H. (1992) J. Mol. Biol. 227, 1043–1053