Suppressor Analysis of Fimbrin (Sac6p) Overexpression in Yeast

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Manuscript received August 18, 1998
Accepted for publication December 18, 1998

ABSTRACT

Yeast fimbrin (Sac6p) is an actin filament-bundling protein that is lethal when overexpressed. To identify the basis for this lethality, we sought mutations that can suppress it. A total of 1326 suppressor mutations were isolated and analyzed. As the vast majority of mutations were expected to simply decrease the expression of Sac6p to tolerable levels, a rapid screen was devised to eliminate these mutations. A total of 1324 mutations were found to suppress by reducing levels of Sac6p in the cell. The remaining 2 mutations were both found to be in the actin gene and to make the novel changes G48V (*act1-20*) and K50E (*act1-21*). These mutations suppress the defect in cytoskeletal organization and cell morphology seen in *ACT1* cells that overexpress *SAC6*. These findings indicate that the lethal phenotype caused by Sac6p overexpression is mediated through interaction with actin. Moreover, the altered residues lie in the region of actin previously implicated in the binding of Sac6p, and they result in a reduced affinity of actin for Sac6p. These results indicate that the two mutations most likely suppress by reducing the affinity of actin for Sac6p *in vivo*. This study suggests it should be possible to use this type of suppressor analysis to identify other pairs of physically interacting proteins and suggests that it may be possible to identify sites where such proteins interact with each other.

OVEREXPRESSION of actin and many actin-associated proteins is lethal to the cell, suggesting that the correct stoichiometry of cytoskeletal components is critical. For example, in yeast, actin (encoded by *ACT1*), Abp1p, and fimbrin (encoded by *SAC6*) are lethal under at least some conditions when overexpressed (Drubin *et al.* 1988; Magdolen *et al.* 1993; this study). However, little is known about why overexpression of these genes is lethal.

In this study, we set out to address the reason for the lethality when the actin filament-bundling protein Sac6p is overexpressed. To better understand why *SAC6* overexpression is lethal and to identify proteins that may interact with, or compensate for, an excess of Sac6p, a genetic screen was devised to isolate genes that, when mutated, can suppress the growth defect caused by *SAC6* overexpression. Overexpression of *SAC6* from the *GAL1* promoter inhibits cell growth, so suppressors could be isolated on galactose plates that selected for the p*GAL1-SAC6* plasmid. However, as there were numerous ways in which the levels of Sac6p in the cell could be reduced to tolerable levels, it was essential to have a rapid way of eliminating such mutations from the analysis. We therefore devised a screen that simply involved plate tests. Using this screen, we were able to rapidly eliminate all but 2 of 1326 suppressors isolated. The remaining two mutations were both found to be in the *ACT1* gene, indicating that the lethality resulting from overexpression of Sac6p occurs via the specific interaction of this protein with actin. These mutations make novel changes in actin (G48V and K50E) and, thus, further increase the collection of mutant actin alleles that are available for analysis of actin cytoskeleletal function in yeast. In addition, these two mutations change residues in a region of actin previously implicated in the binding of Sac6p, suggesting that they suppress the overexpression lethality by reducing the affinity of actin for Sac6p. Biochemical analysis of the interaction between the mutant actins and Sac6p confirms that both *act1-20* and *act1-21* result in a decreased affinity of actin for Sac6p, providing a molecular explanation for our *in vivo* findings. These studies suggest that it may be possible to map unknown sites of interactions of proteins through analysis of mutations that suppress lethality (or some other phenotype) caused by overexpression of an interacting protein.

These studies are important in showing clearly that overexpression phenotypes, which are often considered suspect (largely because of the possibility that they result from nonspecific interactions) can arise from a highly specific and relevant interaction. Moreover, they demonstrate that such phenotypes, which are often viewed as a poor starting point for genetic analysis of interacting proteins, can lead to the identification of normal binding partners through suppressor analysis.

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TABLE 1

Yeast strains used in this study[a]

Strain	Genotype
IGY105	*MAT*α *act1-21::HIS3 bar1*Δ*::LYS2 ura3-52 leu2-3,112 ade2 his3*Δ*200 lys2-801 [pAAB364]*
IGY123	*MAT***a**/*MAT*α *bar1*Δ*::LYS2/bar1*Δ*::LYS2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2/ade2 his3*Δ*200/his3*Δ*200 lys2-801/lys2-801*
IGY169	*MAT*α *bar1*Δ*::LYS2 ura3-52 leu2-3,112 ade2 his3*Δ*200 lys2-801 [pAAB121]*
IGY170	*MAT*α *act1-20::HIS3 bar1*Δ*::LYS2 ura3-52 leu2-3,112 ade2 his3*Δ*200 lys2-801 [pAAB121]*
IGY171	*MAT*α *act1-20::HIS3 bar1*Δ*::LYS2 ura3-52 leu2-3,112 ade2 his3*Δ*200 lys2-801 [pAAB364]*
IGY172	*MAT*α *bar1*Δ*::LYS2 ura3-52 leu2-3,112 ade2 his3*Δ*200 lys2-801 [pAAB364]*
IGY173	*MAT*α *ade2 his3*Δ*200 leu2-3,112 lys2*Δ*::HIS3 ura3-52 act1-21 [pAAB416, pAAB329].*
IGY174	*MAT*α *ade2 his3*Δ*200 leu2-3,112 lys2::HIS3 ura3-52 act1-21 [pAAB329].*
IGY181	*MAT*α *ade2 his3*Δ*200 leu2-3,112 lys2*Δ*::HIS3 ura3-52 [pAAB416, pAAB329].*
IGY182	*MAT***a** *ade2 his3*Δ*200 leu2-3,112 lys2*Δ*::HIS3 ura3-52 [pAAB416, pAAB329].*
IGY184	*MAT*α *ade2 his3*Δ*200 leu2-3,112 lys2*Δ*::HIS3 ura3-52 [pAAB328].*
IGY185	*MAT***a** *ade2 his3*Δ*200 leu2-3,112 lys2*Δ*::HIS3 ura3-52 act1-20 [pAAB329, pAAB416].*
IGY186	*MAT***a** *ade2 his3*Δ*200 leu2-3,112 lys2*Δ*::HIS3 ura3-52 act1-20 [pAAB328].*
AAY1947	*MAT***a** *his3*Δ*200 leu2-3,112 lys2-801 ura3-52 [pAAB328].*

[a] All strains are Gal^+. All IGY strains are isogenic, differing from each other in only the markers and/or plasmid indicated. They are all derived from strain DBY4975 (Botstein lab) by transformation or spontaneous mutation, and they are in the S288C background. AAY1947 is congenic, but not isogenic, to the IGY strains. All strains were generated in our lab.

MATERIALS AND METHODS

Yeast strains, plasmids, and media: The yeast strains used in this study are listed in Table 1. Plasmids used in this study are listed in Table 2. Media for yeast growth and sporulation and the methods for mating, sporulation, and tetrad dissection are described in Sherman *et al.* (1974). Rich medium containing glucose is referred to as YEPD; rich medium containing galactose is referred to as YEP-Gal. Galactose plates contained 2% galactose. Osmotic sensitivity was determined by growth of cells on rich medium (YEPD or YEP-Gal) containing 900 mm NaCl. Growth on plates was determined by placing cell cultures onto plates using a 32-prong inoculator.

Construction of plasmids containing *SAC6* under the control of the *GAL1* promoter: A p*GAL1-SAC6* overexpression plasmid pAAB157 was constructed in two steps. The first step placed a portion of the 5′ end of the *SAC6* gene behind a *GAL1* promoter. The vector pAAB121, a centromere-containing plasmid carrying the yeast *URA3* gene and *GAL1-10* promoter (Table 2), was prepared by cutting with *Bam*HI (two sites in the polylinker, adjacent to the *GAL1* promoter), filling the ends, and then cutting with *Xba*I (in the polylinker, distal to the *GAL1* promoter). A fragment containing a blunt-ended *Eco*RI site, followed by the 5′ end of the *SAC6* gene (from 25 bp 5′ of the start codon) to the *Xba*I site within the coding sequence, was obtained from a cDNA construct described previously (Adams *et al.* 1991). This fragment was ligated into the blunt-ended *Bam*HI and *Xba*I sites, respectively, of pAAB121 (above). The blunt-ended *Bam*HI sites of pAAB121 and the *Eco*RI site of the fragment were destroyed in the cloning process. The second step involved fusing the remaining 3′ portion of *SAC6* from the *Xba*I site, resulting in the generation of plasmid pAAB157 containing the entire *SAC6* coding region. To this end, the pAAB121-derived construct described above was cut with *Sph*I (distal to the *Xba*I site in the polylinker), the ends were made blunt, and the linearized plasmid was cut with *Xba*I. The 3′ end of *SAC6* (from a wild-type genomic clone described in Adams *et al.* 1991), from the *Xba*I site in the coding sequence to a blunt-ended *Bam*HI site (engineered, overlapping with the *Nco*I site 1245 bp 3′ of the *SAC6* stop codon), was inserted into the *Xba*I and blunt-ended *Sph*I sites of the plasmid described above to generate pAAB157 (the *Bam*HI site was regenerated while the *Sph*I site was destroyed in the process). The cloning junctions at the 5′ end of the gene and the *Xba*I site were sequenced. The first part of the construct was derived from a cDNA clone, and, thus does not contain the *SAC6* intron (Adams *et al.* 1991).

The entire p*GAL1-SAC6* fragment from pAAB157, flanked by *Eco*RI (5′ of the *GAL1-10* promoter) and the regenerated *Bam*HI site described above, was inserted into the *Eco*RI-*Bam*HI sites of pAAB107 (*LEU2 CEN6*) and pAAB149 (*LYS2 CEN*) to create pAAB328 and pAAB329, respectively.

Isolation of suppressors: Spontaneous suppressors of the growth inhibition caused by *SAC6* overexpression were isolated as follows. A *SAC6* strain of mating-type **a** or α (IGY182 or IGY181, respectively) carrying a *SAC6* overexpression plasmid pAAB329 (p*GAL1-SAC6 LYS2 CEN*) and reporter plasmid pAAB416 (p*GAL1-LacZ URA3 CEN*) was plated onto synthetic-glucose or synthetic-raffinose media lacking uracil and lysine (to select for maintenance of both plasmids) at ~100–200 cells per plate and grown for 4–10 days. Unless otherwise indicated, cells were grown at 30°. Individual colonies were resuspended in sterile water and spread onto synthetic-galactose plates lacking uracil and lysine, such that one colony from the glucose or raffinose plate was put on one entire galactose plate. Cells were grown on galactose plates for 3–7 days. Isolates were streaked for single colonies on synthetic-galactose medium lacking uracil and lysine. To ensure the isolation of independent suppressors, only one colony from each galactose plate was usually selected; in those cases where more than one colony per plate was chosen, only one isolate from any one plate was subsequently retained unless it was clear that additional isolates were genotypically or phenotypically distinct. A total of 1326 colonies from 1173 plates were analyzed. For the isolation of most suppressors, 60-mm plates were used instead of the more standard 100-mm plates because they required much less media and still usually yielded at least one colony per plate.

Genetic analysis of suppressors: Suppressors containing

TABLE 2

Plasmids used in this study

Plasmid	Markers	Alias	Source/reference
pAAB4	*HIS3 URA3 CEN4*	YCp404	Ma *et al.* (1987)
pAAB62	*ACT1 URA3* 2μ	pRB155	Botstein lab
pAAB107	*LEU2 CEN6*	pRS415	Sikorski and Hieter (1989)
pAAB121	p*GAL1-10 URA3 CEN*	pRP23	Parker lab
pAAB132	*URA3 ADE3* 2μ	pTSV31A	Pringle lab
pAAB144	*SAC6 URA3 ADE3* 2μ		This study[a]
pAAB149	*LYS2 CEN*	pRS317	Hieter lab
pAAB157	p*GAL1-SAC6 URA3 CEN*		This study[b]
pAAB328	p*GAL1-SAC6 LEU2 CEN6*		This study[b]
pAAB329	p*GAL1-SAC6 LYS2 CEN*		This study[b]
pAAB364	p*GAL1-SAC6 URA3 CEN*		This study[c]
pAAB416	p*GAL1-LacZ URA3 CEN4*	pRP127	Parker lab
pAAB508	*ACT1*		This study[d]

[a] pAAB144 was constructed by cutting pAAB132 with *Sac*I and *Bam*HI and inserting an ~4-kb *Sac*I-*Nco*I *SAC6*-containing fragment engineered to contain a *Bam*HI site overlapping with the *Nco*I site (Adams *et al.* 1991).

[b] pAAB157, pAAB328, and pAAB329 were constructed as described in materials and methods.

[c] pAAB364 is similar to pAAB157, except that it contains an *Sph*I site just 3′ of the stop codon of *SAC6* and contains only 399 bp 3′ of the stop codon (instead of 1245 bp in pAAB157, as described in materials and methods). The construction of this plasmid has been described (Toenjes 1998).

[d] pAAB508 was constructed by inserting an ~4-kb *Eco*RI *ACT1*-containing fragment from pAAB62 into the *Eco*RI site of pUC119, as described in Wertman *et al.* (1992).

plasmid pAAB329 [p*GAL1-SAC6 LYS2*] were analyzed to determine whether the mutations were plasmid linked or genomic and dominant or recessive. As we expected the majority of mutants to be trivial (see results), a rapid approach was used to eliminate them (see Figure 1). Thus, revertants were crossed to a wild-type strain of opposite mating type (AAY1947 or IGY184) that contained plasmid pAAB328 [p*GAL1-SAC6 LEU2*], and diploids were selected on synthetic-glucose plates lacking lysine and leucine. The resulting strains, containing two p*GAL1-SAC6* plasmids, were tested for growth on plates containing galactose and lacking leucine or lysine. As described in results, for most revertants, it was possible to determine whether the suppressor mutation was plasmid linked or genomic, and recessive or dominant, by simply scoring growth on these plates. However, this analysis was critically dependent on the ability of the various suppressor strains to mate. Some 51 of the 1326 suppressors were unable to mate on plates; of these, 6 could be mated in liquid cultures, and the remaining 45 were analyzed by transformation rather than by mating. In these cases, the cells were transformed with fresh [p*GAL1-SAC6 LEU2*] plasmid and tested for growth on synthetic galactose plates lacking leucine. Growth indicated that suppression was not caused by a plasmid-linked mutation, and these isolates were analyzed further.

Sequence analysis of the *act1-20* and *act1-21* mutations: As described in results, preliminary evidence indicated the two suppressor mutations were in *ACT1*. The presence of an *act1* mutation in each case was confirmed by isolating genomic DNA from each mutant, and by amplifying and cycle sequencing their *ACT1* genes, as described previously (Honts *et al.* 1994).

Generation of strains containing *act1-20::HIS3* or *act1-21::HIS3*: To confirm that the two *act1* mutations identified were sufficient for suppression, strains carrying mutant actin genes with just these mutations were generated by a method similar to that described by Wertman *et al.* (1992). Thus, genomic DNA was isolated from strains IGY186 (*act1-20*) and IGY173 (*act1-21*). The mutant actin genes were isolated via PCR with exo$^+$ *pfu* DNA polymerase, using the primer set AAO119 (5′ ATTTTTCACGCTTACTGC 3′) and AAO120 (5′ ACATAAACATACGCGCAC 3′). In each case, this generated a 1522-bp fragment, from 56 bp 5′ of the coding sequence to 1466 bp within the coding sequence of actin. The fragments were cut with *Xho*I and *Bsm*I (which cut within the coding region at nucleotides 128 and 1300, respectively) and were cloned into pAAB508 (Table 2) cut with *Xho*I and *Bsm*I in the coding region of *ACT1*. This manipulation simply replaced the wild-type actin sequence, from *Xho*I to *Bsm*I, with the same stretch from the mutants. The presence of each mutation was verified through complete DNA sequencing of one strand. Plasmids were then transformed into an adenine methylase-deficient bacterial strain, GM48. Plasmids isolated from this strain were cut with *Bcl*I (just 3′ of the mutant actin genes), and in each case, a 1.8-kb *Bam*HI fragment containing *HIS3* from pAAB4 (Table 2) was inserted. These plasmids were cut with *Eco*RI (flanking the *act1::HIS3* fragment) and transformed into the *ACT1*$^+$/*ACT1*$^+$ diploid strain IGY123. His$^+$ transformants were selected and sporulated, and tetrads were dissected. His$^+$ segregants were isolated, and the presence of just the *act1-20* or *act1-21* mutation in a haploid segregant from each diploid was verified by isolating genomic DNA, amplifying the coding region of the mutant actin genes by PCR (using oligonucleotides AAO119 and AAO120), and then sequencing from the *Xho*I to *Bsm*I site.

X-Gal overlays: X-Gal overlays were conducted by the method used in Roy Parker's lab. Briefly, a 1% solution of agarose/H_2O was prepared and mixed 1:1 with 1 m $NaPO_4$, pH 7.0. One milliliter of 10% SDS was added per 100-ml final volume. Two-hundred microliters of 2% X-Gal in DMF was added per 10 ml. Strains containing the p*GAL1-LacZ URA3* and p*GAL1-SAC6* plasmids were streaked onto galactose plates lacking uracil and lysine, selecting for maintenance of both plasmids, and grown for 3–4 days at 30°. Molten X-Gal mix was poured directly on top of the patches of cells on the plate. Color development was assayed after incubation for 1–2 hr at 37°.

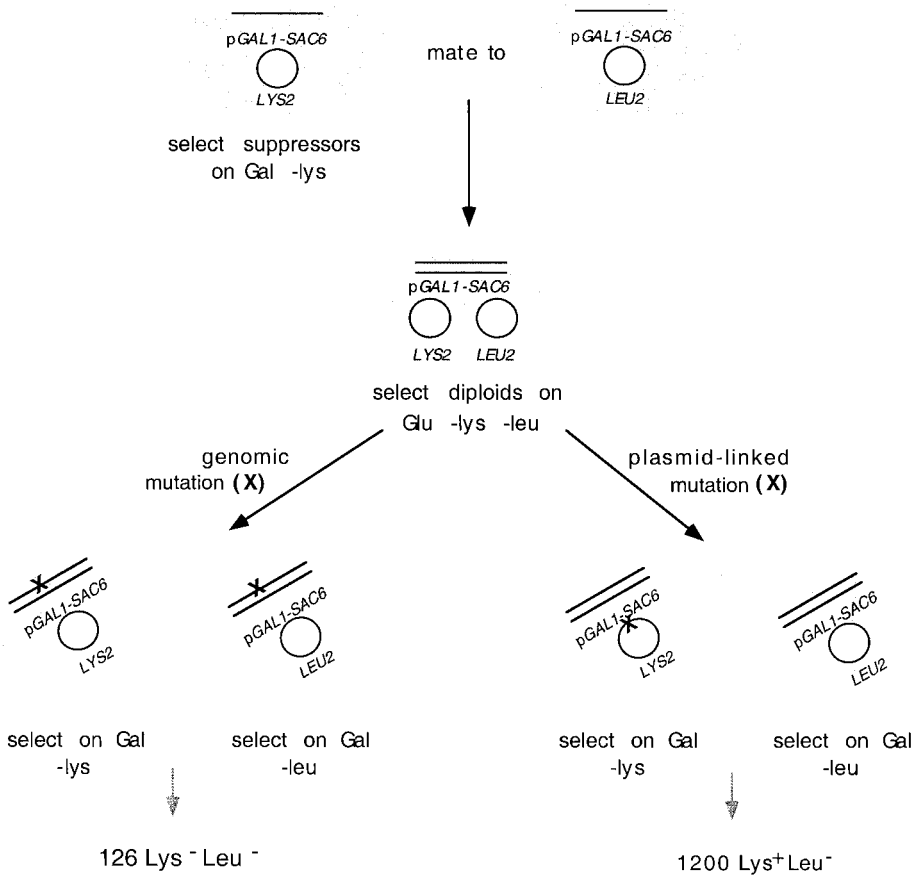

Figure 1.—Identification of suppressors of the lethality resulting from Sac6p overexpression. Each straight line inside the cells represents a haploid genome, and each circle represents a particular plasmid whose selectable marker is shown. For simplicity, the p*GAL1-lacZ* plasmid has been omitted from each cell. **X** represents either a genomic or plasmid-linked mutation. Suppressor strains carrying a plasmid containing p*GAL1-SAC6* and *LYS2* are selected on galactose-containing plates lacking lysine. These strains are crossed to wild-type cells carrying a *LEU2* p*GAL1-SAC6* plasmid. Diploids are selected on glucose-containing plates lacking lysine and leucine, and are then tested for growth on galactose-containing plates lacking lysine or leucine. Some 126 recessive genomic suppressor mutations result in no growth on either plate; ~1200 recessive, plasmid-linked suppressor mutations give growth on plates lacking lysine and no growth on plates lacking leucine.

Purification of actin and Sac6p, and actin filament-binding and cross-linking assays: Actin was purified from wild-type and mutant cells, as described previously (Honts *et al.* 1994), using strains IGY181 (*ACT1*), IGY185 (*act1-20*), and IGY173 (*act1-21*); see Table 1. Sac6p was purified from a strain overexpressing Sac6p, as described previously (Sandrock *et al.* 1997). Actin filament-binding and cross-linking assays were performed as described previously (Honts *et al.* 1994).

RESULTS

Genetic screen to identify suppressors of the growth defect caused by overexpression of Sac6p: To identify suppressors of the lethality caused by Sac6p overexpression, we devised a screen that would allow us to rapidly identify mutants of interest. Cells of the *SAC6* strain IGY181 (mating type α) or IGY182 (mating-type **a**) carrying a plasmid containing *SAC6* under the control of the *GAL1* promoter were plated on galactose (conditions that lead to overexpression of *SAC6*), and spontaneous suppressors that could grow under these conditions were isolated. A total of 1326 suppressor strains (most of which were independent isolates, as described in materials and methods) were obtained and characterized further.

Identification of recessive genomic suppressor mutations: We expected to obtain several classes of suppressors, including (i) plasmid-linked mutations that no longer result in overproduction of wild-type Sac6p, through mutation of either the *GAL1* promoter or the *SAC6* gene; (ii) *lys2::HIS3* gene convertants, in which the *lys2::HIS3* genomic locus is replaced by *LYS2* (thus enabling cells to grow without lysine in the absence of the p*GAL1-SAC6 LYS2* plasmid); (iii) *trans*-acting genomic mutations that decrease levels of expression from the *GAL1* promoter; and (iv) genomic suppressor mutations of interest. The first three classes of suppressors were not of interest, but were expected to be the most common. It was, therefore, necessary to eliminate them rapidly. To this end, the following approach was taken (see Figure 1). Suppressor strains carrying a [p*GAL1-SAC6 LYS2*] plasmid were crossed to a *SAC6* strain of opposite mating type containing a fresh [p*GAL1-SAC6-LEU2*] plasmid. Diploids were selected on synthetic glucose plates lacking lysine and leucine. The resulting diploids, carrying two different *SAC6* overexpression plasmids, were purified and tested for their ability to grow on galactose plates lacking lysine or leucine (see Figure 1 and Table 3). As outlined below, from these tests, we were able to distinguish between genomic and plasmid-linked mutations and between dominant and recessive mutations:

Plasmid-linked mutations: If the suppressor was caused

TABLE 3

Initial genetic analysis of suppressor mutations

Type of suppressor	Growth on -Lys Gal	Growth on -Leu Gal	No. identified
Recessive plasmid linked or conversion of *lys2::HIS3* to *LYS2*[a]	+	−	~1200
Dominant plasmid linked[b]	+	±	0
Recessive genomic	−	−	126
Dominant genomic	+	+	0

Suppressors carrying pAAB329 (p*GAL1-SAC6 LYS2 CEN*) were mated to a wild-type strain of the opposite mating type carrying pAAB328 (p*GAL1-SAC6 LEU2 CEN*). Diploids were selected on glucose plates lacking lysine and leucine. Individual colonies were tested for growth on galactose plates lacking lysine or leucine. Growth is indicated by +; no growth is indicated by −.

[a] A subset of these mutants was tested to determine the relative frequencies of plasmid-linked mutations *vs. lys2::HIS3* gene conversion events. Out of 314 mutants tested, 298 were plasmid linked (*i.e.*, His^+), and 16 were gene convertants (*i.e.*, His^-).

[b] The symbol ± indicates that growth on -Leu Gal is dependent on the presence of the *LYS2* p*GAL1-SAC6*-containing plasmid.

by a recessive mutation in the [p*GAL1-SAC6 LYS2*] plasmid, then the resulting diploid should be able to grow on galactose plates lacking lysine, but not lacking leucine (Figure 1). As shown in Table 3, the vast majority of mutants were of this type. If the suppressor was caused by a dominant mutation in the [p*GAL1-SAC6 LYS2*] plasmid, *i.e.*, a mutation that can suppress even in the presence of a fresh plasmid, the resultant diploid should be able to grow on galactose plates lacking either lysine or leucine. However, growth on the latter would be dependent on the presence of the [p*GAL1-SAC6 LYS2*] plasmid. None of the revertants fell into this class (Table 3).

lys2::HIS3 gene convertants: If the suppressor was caused by replacement of the *lys2::HIS3* locus by the *LYS2* gene from the plasmid, then diploids resulting from a cross to wild-type carrying the [p*GAL1-SAC6 LEU2*] plasmid would be able to grow on synthetic galactose lacking lysine but not lacking leucine and, therefore, would appear similar to strains carrying recessive, plasmid-linked mutations (see above and Table 3). As neither category of mutation was of interest to us, the distinction was unimportant, but could be made by analysis of such strains on synthetic galactose plates lacking histidine (see Table 3).

Genomic suppressor mutations: The class of suppressor mutations that we wished to isolate in this screen were those that were genomic. Such mutations could be identified in the crosses described above. Thus, if a suppressor mutation were genomic and recessive, it would be masked by the wild-type copy in the resulting diploid strain. The diploid would then be unable to grow on synthetic galactose plates lacking either lysine or leucine (Figure 1 and Table 3). Some 126 suppressor mutations were of this class (Table 3). If the suppressor mutation were genomic and dominant, however, the diploid should be able to suppress the lethality caused by either the [p*GAL1-SAC6 LYS2*] or the [p*GAL1-SAC6 LEU2*] plasmid and, thus, should grow on synthetic galactose plates lacking either lysine or leucine. In contrast to the dominant plasmid mutations (above), however, such suppressors would not be dependent on the presence of the *LYS2*-containing plasmid. No suppressors of this type were obtained.

Identification of genomic suppressor mutations that act by reducing the levels of expression from the *GAL1* promoter: Among the genomic suppressor mutations, we expected to obtain mutations in the galactose regulatory genes which could result in decreased expression of *SAC6* to tolerable levels. To rapidly identify such mutations, a reporter plasmid, pAAB416, containing p*GAL1-LacZ URA3*, was included in the initial strain (IGY181 or IGY182). This plasmid allowed assessment of the relative levels of induction from the *GAL1* promoter. Expression was measured using an X-Gal overlay assay, as described in materials and methods. Suppressors were evaluated on the basis of the relative level of blue color development (resulting from levels of β-galactosidase activity). Only two mutants developed blue color as dark as the controls. The remaining mutants presumably grow in the presence of galactose because of reduced levels of expression of *SAC6* from the *GAL1* promoter. Indeed, consistent with this expectation, of 23 mutants that had reduced levels of expression and that were analyzed, 20 carried mutations in either *SPT5* or *GAL11*, two genes known to be involved in transcriptional regulation (Suzuki *et al.* 1988; Swanson *et al.* 1991). The genes defective in the remaining three mutants of this group were not identified and were not studied further.

Identification of *act1* mutant alleles: The two mutants that were dark blue on X-Gal indicator plates were analyzed further. Preliminary phenotypic analysis suggested these mutations might be in the *ACT1* gene. Thus, in

the presence of the p*GAL1-SAC6* plasmid, both mutants were found to have temperature-sensitive and osmotic-sensitive phenotypes reminiscent of many *act1* mutant strains (Botstein *et al.* 1997): the cells grew more poorly at 37° than at 23° on either YEPD or YEP-Gal plates, and they failed to grow on media containing 900 mm salt. Therefore, we tested whether the two suppressors isolated in this study were in the *ACT1* gene. In each case, we obtained preliminary evidence from complementation and/or linkage analyses that the mutations were in *ACT1* (not shown). Therefore, we isolated genomic DNA from each mutant (strains IGY186 and IGY173) and amplified and cycle sequenced their *ACT1* genes as described previously (Honts *et al.* 1994). In each case, the entire coding region was sequenced on one strand, and the segment containing the mutation was sequenced on both strands. For one (hereafter referred to as *act1-20*), a single nucleotide change in the *ACT1* coding region was found which resulted in a Gly-to-Val change at amino acid 48. For the other (hereafter referred to as *act1-21*), a single change was found which resulted in a Lys-to-Glu change at residue 50. Interestingly, an actin mutation was isolated in a screen for endocytosis mutants (Munn *et al.* 1995). This allele, designated *end7-1*, alters the same residue as *act1-20*, except *end7-1* results in a glycine to aspartic acid substitution (Munn *et al.* 1995).

Demonstration that *act1-20* or *act1-21* is sufficient for suppression of the lethality caused by overexpression of *SAC6*: We confirmed that these mutations were sufficient to cause the suppression phenotype by replacing the wild-type *ACT1* gene with mutant actin genes (linked to *HIS3*) harboring either *act1-20* or *act1-21* and showing that, in each case, the resulting cells were viable when *SAC6* was overexpressed (Figure 2). To this end, *ACT1*/*ACT1* diploid strains were first transformed with *act1::HIS3* (see materials and methods). Heterozygous *act1-20::HIS3*/*ACT1* or *act1-21*::*HIS3*/*ACT1* diploids were then transformed with a *URA3*-containing plasmid carrying p*GAL1-SAC6* (pAAB364). Transformants were selected on synthetic dextrose plates lacking uracil, cells were sporulated, and tetrads dissected. In the case of *act1-20*, 14 out of 18 tetrads dissected on YEPD yielded 4 viable spores, and His^+:His^- segregated 2:2. Some 24 of 56 segregants were Ura^+ and, therefore, carried the p*GAL1-SAC6* plasmid. Of these, 12 were viable on galactose and were His^+ (*act1-20*), whereas 12 were inviable on galactose and were His^- (*ACT1*). These results indicate that the *act1-20* mutation is necessary and sufficient to suppress the lethality caused by *SAC6* overexpression.

In the case of *act1-21*, genetic analysis revealed that *act1-21* not only suppresses the lethality caused by overexpression of *SAC6*, but that it also makes the cells dependent on overexpression of *SAC6* for viability (see below and Figure 2). Heterozygous *act1-21*::*HIS3*/*ACT1* diploids transformed with the *URA3*-containing plasmid carrying *SAC6* (pAAB364) were selected on synthetic dextrose media lacking uracil, cells were sporulated, and tetrads were dissected on galactose. (Tetrads were dissected on galactose-containing plates because *act1-21* cells grew best when they carried the p*GAL1-SAC6* plasmid and were grown on galactose; see below.) Two or fewer viable spores were obtained per tetrad. A total of 32 viable segregants were obtained. Of these, 12 were Ura^+ and, therefore, carried the p*GAL1-SAC6* plasmid (the Ura^- segregants are discussed below). All 12 of these Ura^+ segregants were His^+ (*act1-21*), confirming that overexpression of *SAC6* is lethal in *ACT1* cells, and showing that the *act1-21* mutation is necessary and sufficient for suppression of the lethality caused by overexpression of *SAC6*.

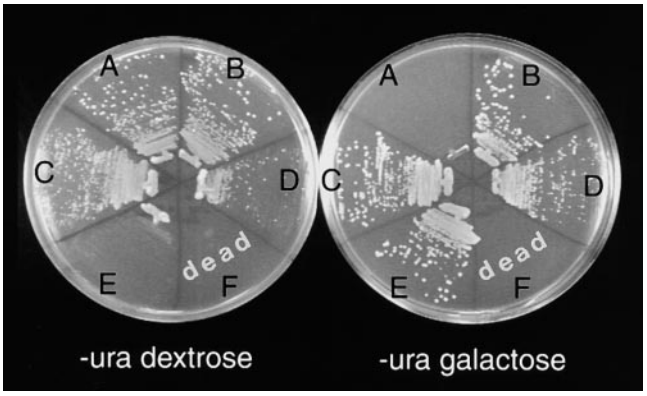

Figure 2.—Suppression of Sac6p overexpression lethality by *act1-20* or *act1-21* mutant alleles. Growth of *ACT1* (A and B), *act1-20* (C and D), or *act1-21* (E and F) cells containing a p*GAL1-SAC6* plasmid (pAAB364; A, C, and E) or control plasmid (pAAB121; B, D, and F) on -Ura dextrose or -Ura galactose after 3 days at 30°. Strains shown are (A) IGY172 (*ACT1* strain containing pAAB364), (B) IGY169 (*ACT1* strain containing pAAB121), (C) IGY171 (*act1-20* strain containing pAAB364), (D) IGY170 (*act1-20* strain containing pAAB121), and (E) IGY105 (*act1-21* strain containing pAAB364). *act1-21* cells containing the vector pAAB121 and, therefore, expressing normal levels of Sac6p are inviable (F). *act1-21* cells containing the p*GAL1-SAC6* plasmid are viable even on glucose, most likely because of low (but nevertheless elevated) levels of Sac6p expression from the *GAL1* promoter on glucose.

Overexpression of *SAC6* suppresses the lethality caused by the *act1-21* mutation: Analysis of the Ura^- segregants from the *act1-21::HIS3*/*ACT1* diploid above revealed that 20 out of 20 were His^- (*ACT1*). As none were His^+ (*act1-21*), *act1-21* cells are viable only in the presence of the p*GAL1-SAC6* plasmid. *act1-21* cells carrying the p*GAL1-SAC6* plasmid are viable on either glucose or galactose, indicating that there must be enough expression of *SAC6* from the *GAL1* promoter, even on glucose, to suppress the lethality caused by *act1-21*. Indeed, the *act1-21* mutant originally had to go through a period of growth under noninducible conditions before its identification on galactose (see materials and methods); any mutants that were dependent on higher

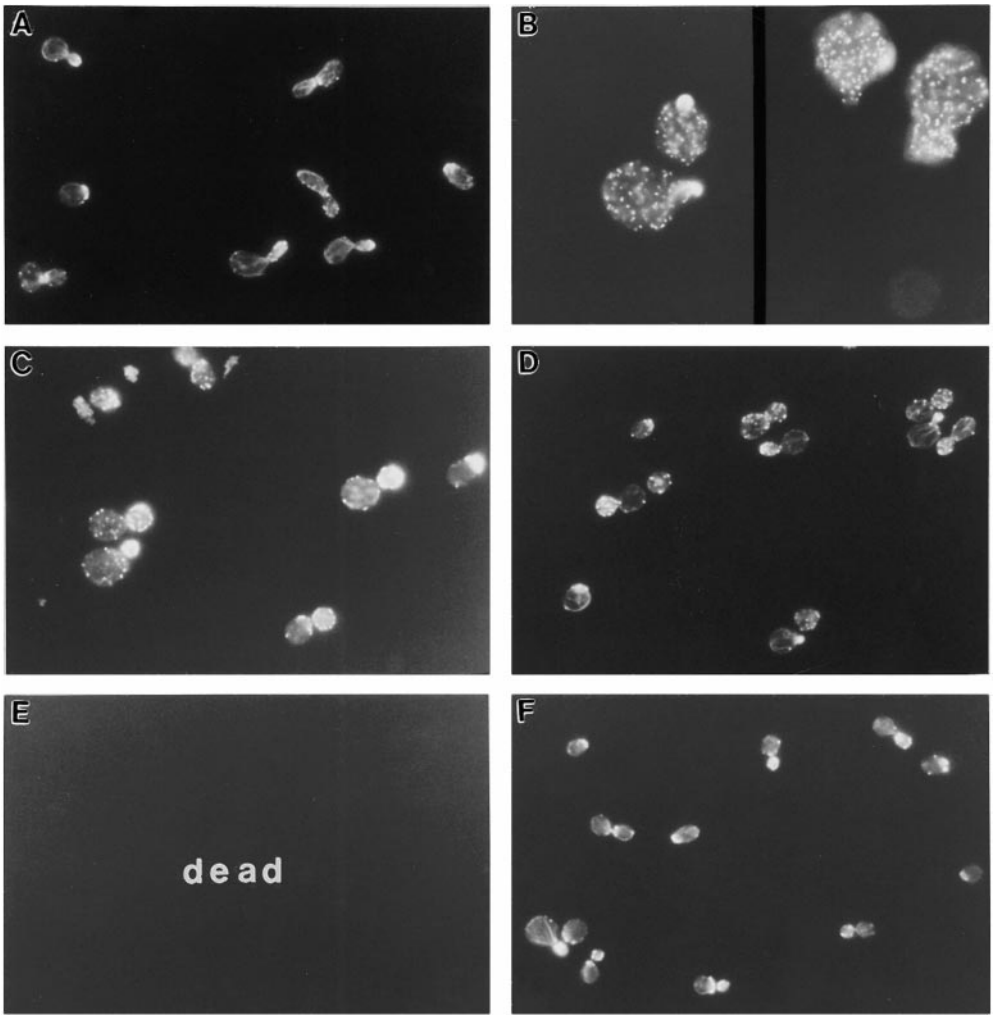

Figure 3.—Actin organization in *ACT1* wild-type (A and B), *act1-20* mutant (C and D), or *act1-21* mutant (F) cells expressing normal levels of *SAC6* (A and C) or overexpressing *SAC6* under the control of the inducible *GAL1* promoter (B, D, and F). Cells carrying the *SAC6*-containing plasmid (pAAB364, B, D, and F) or vector control (pAAB121, A and C) were grown on minimal medium lacking uracil (selective conditions) and containing 2% glucose (repressing conditions) to midlog, and were then shifted to galactose (to induce expression from the *GAL1* promoter) for ~16 hr. Cells were then fixed and stained with rhodamine-phalloidin, as described previously (Chowdhury *et al.* 1992), and examined under a Zeiss Axioskop using a 100× objective. All prints are at the same magnification. Strains are as described in Figure 2. *act1-21* cells containing the vector pAAB121 (*i.e.*, expressing normal levels of Sac6p) are inviable, so E does not contain any cells.

levels of expression of *SAC6* would not have been isolated in this scheme. Growth on glucose, however, is much weaker than on galactose, suggesting that higher levels of *SAC6* are optimal (Figure 2).

Additional evidence confirmed that overexpression of *SAC6* suppresses the lethality caused by the *act1-21* mutation. Thus, IGY174 (*act1-21*) mutant cells containing the [*LYS2* p*GAL1-SAC6*] plasmid are unable to lose the plasmid unless they have first been transformed with (i) a [*LEU2* p*GAL1-SAC6*] plasmid (pAAB328), or (ii) a high-copy-number (2μ) plasmid carrying *URA3 SAC6* (pAAB144), which allows the cells to grow on medium containing glucose. (In the presence of the *LEU2* or *URA3* control plasmid [pAAB107 or pAAB132, respectively], the cells are still not able to lose the [*LYS2* p*GAL1-SAC6*] plasmid.)

***act1-20* and *act1-21* suppress the defects in actin organization seen in cells overexpressing *SAC6*:** To gain insight into the underlying basis of the suppression by the mutant actin alleles, cells were stained with rhodamine-labeled phalloidin, and the intracellular distribution of actin in wild-type or mutant cells overexpressing *SAC6* was examined by fluorescence microscopy (Figure 3). As reported previously (Adams and Pringle 1984; Kilmartin and Adams 1984), wild-type cells expressing normal levels of Sac6p have a polarized distribution of actin, with patches concentrated in regions of growth and cables extending through the cytoplasm in a direction roughly parallel to the axis of polarized growth (Figure 3A). Wild-type cells overexpressing *SAC6*, however, have an abnormal distribution of actin. In particular, cells show delocalized actin patches and a loss of actin cables (Figure 3B). [If *ACT1* cells overexpressing *SAC6* are stained with anti-actin antibodies instead of phalloidin, ~27% of the cells are seen to have densely stained actin cables or bars. Similar bars have been seen previously in cells stained with antiactin antibodies, rather than phalloidin, but the nature of these bars is unknown (Haarer *et al.* 1990). In the present case, in which *SAC6* is overexpressed, these bars likely reflect an increase in actin filament cross-linking activity.] In addition, wild-type cells overexpressing *SAC6* are often larger and rounder than normal. *act1-20* or *act1-21* mutant cells overexpressing *SAC6*, however, show a distribution of actin and cell size and morphology very similar to wild-type cells expressing normal levels of Sac6p (Fig-

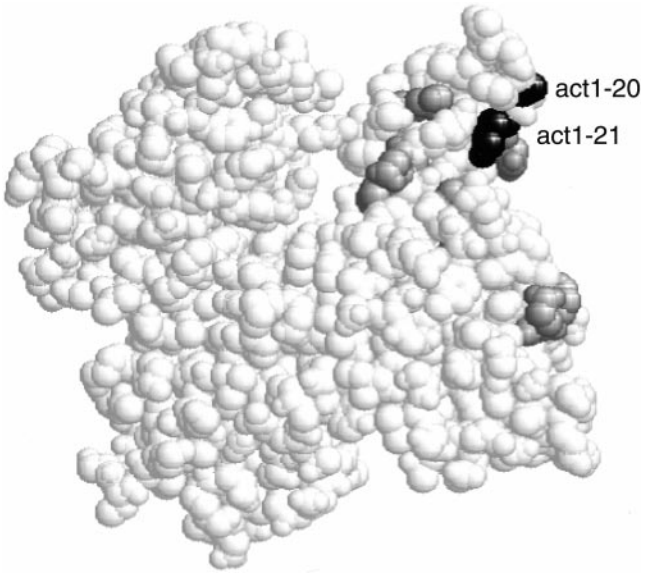

Figure 4.—Location in a model of rabbit muscle G-actin of residues altered by two novel actin alleles. A model of the rabbit muscle actin molecule was displayed from the atomic coordinates of the rabbit muscle actin, as determined by Kabsch *et al.* (1990). Location of residues previously identified as important for Sac6p interaction with actin are indicated by gray shading (Honts *et al.* 1994). Residues altered by the suppressor mutations are indicated by black shading.

ure 3, D and F). These results indicate that the *act1* suppressor mutations restore function to cells overexpressing *SAC6* by directly affecting the way in which Sac6p organizes actin.

Actin mutations that suppress the overexpression of Sac6p map to a defined region of the actin monomer and reduce the affinity of actin for Sac6p *in vitro*: The two new *act1* alleles lie in the region of actin that was previously implicated in the binding of Sac6p (Figure 4). Indeed, *act1-21* changes one of the residues altered in *act1-125* (K50A, D51A, Wertman *et al.* 1992), which has been shown to encode actin with a lower affinity for Sac6p than wild-type actin (Honts *et al.* 1994). This finding suggests that the mechanism of suppression of the lethality caused by overexpression of Sac6p may be simply by reducing the affinity of Sac6p for actin. To test this idea, we purified actin from *ACT1* wild-type and *act1-20* and *act1-21* mutant cells, and we asked whether the mutant actins are defective in (i) binding to wild-type Sac6p and (ii) forming bundles of actin filaments in the presence of Sac6p. Wild-type or mutant actins were mixed with Sac6p under polymerization conditions, and filaments of actin and bound Sac6p were sedimented by high-speed centrifugation. Under these conditions, nonpolymerized actin and unbound Sac6p remain in the supernatant. As reported previously and shown in Figure 5A, most wild-type actin is found in the high-speed pellet with Sac6p, indicating that most actin forms filaments to which Sac6p is bound. In the case of Act1-20p and Act1-21p, most mutant actin is similarly found in the high-speed pellet, but in each case (and most dramatically in the case of Act1-21p), a significant fraction of Sac6p remains in the supernatant (Figure 5, B and C). These findings indicate that both Act1-20p and Act1-21p are able to form filaments, but the mutant filaments are defective in binding to Sac6p.

As Sac6p is an actin filament-bundling protein, we tested whether defects in binding of Sac6p to mutant actin result in defects in bundle formation. To this end, mixtures of actin and Sac6p were subjected to low-speed centrifugation—conditions under which cross-linked actin filaments sediment but individual filaments do not. As shown in Figure 5A, in the case of wild-type, most actin and bound Sac6p are found in the pellet, indicating the formation of bundles of actin filaments. In the case of Act1-20 and Act1-21 mutant actins, however, a significant fraction of actin and Sac6p is found in the supernatant, indicating a defect in actin filament bundle formation. As expected, (i) in the absence of actin, most Sac6p is found in the supernatant after either high- or low-speed centrifugation (Figure 5D), and (ii) in the absence of Sac6p (Figure 5, A–C), most wild-type or mutant actin is found in the high-speed pellet (indicating the presence of F-actin) and in the low-speed supernatant (indicating the absence of bundles).

Together, these results show that Act1-20 and Act1-21 mutant actins are defective in binding to Sac6p, and that they have a defect in Sac6p-induced actin filament bundle formation. Therefore, we conclude that these mutations suppress the Sac6p overexpression lethality by reducing the affinity of actin for Sac6p. Moreover, as the lethal phenotype of Sac6p is mediated through an interaction with actin, it is highly specific.

DISCUSSION

In yeast, numerous proteins are lethal when overexpressed, and the overexpression phenotype can frequently provide insight into the functions of the overexpressed protein (Phizicky and Fields 1995). The use of the overexpression phenotype as a starting point for genetic analysis is, therefore, likely to lead to the identification of proteins that interact with the overexpressed protein; indeed, there are several cases where this has proven to be so. For example, several studies with yeast have identified high-copy suppressors of the overexpression phenotype (*e.g.*, Meeks-Wagner and Hartwell 1986; Weinstein and Solomon 1990; Magdolen *et al.* 1993; Archer *et al.* 1995; Phizicky and Fields 1995; Alvarez *et al.* 1998; Smith *et al.* 1998). Rarely, however, have genomic mutations that suppress the overexpression phenotype been sought [although there are examples, *e.g.*, suppressors of the phenotype caused by overexpression of the GAL4-VP16 fusion protein (Berger *et al.* 1992) and suppressors of the phenotype caused by overexpression of the *Schizosaccharomyces pombe cdc7* protein (Fankhauser and Simanis 1994)]. Indeed, in

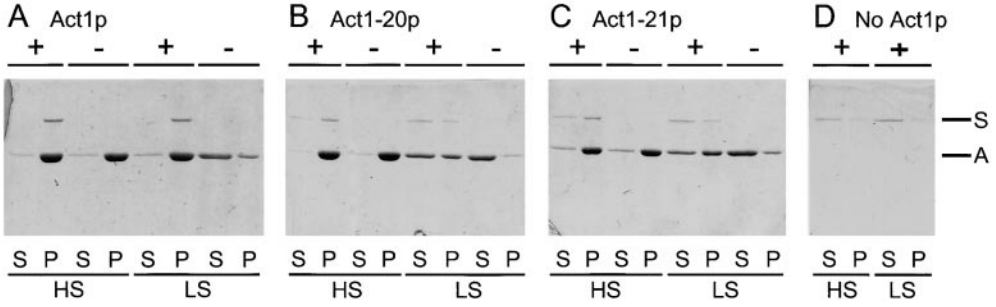

Figure 5.—Actin-binding and cross-linking assays for wild-type and mutant actins with wild-type Sac6p. SDS-PAGE of supernatants (S) or pellets (P) obtained by high-speed (HS) or low-speed (LS) centrifugation of wild-type or mutant actins polymerized in the presence (+) or absence (−) of Sac6p, as described in materials and methods. The positions of yeast actin (A) and Sac6p (S) bands in the gels are indicated. The proteins were resolved in a 10% SDS-polyacrylamide gel and stained with Coomassie blue R-250. The data are representative of multiple experiments.

recent reviews of genetic methods used to study the yeast cytoskeleton (Botstein *et al.* 1997) or other aspects of biology (Phizicky and Fields 1995), this particular brand of suppressor analysis was not even mentioned. The reason for this may lie in the suspicion that overexpressed proteins might interfere with cellular functions through nonspecific mechanisms or through altered specificity and, therefore, may identify proteins that do not interact with the protein of interest under normal conditions. However, dosage suppressors that ameliorate a phenotype caused by mutation have been identified in numerous studies with yeast (*e.g.*, Francisco *et al.* 1994; Kirkpatrick and Solomon 1994; Vallen *et al.* 1994; Machin *et al.* 1995; Prendergast *et al.* 1995; Glerum *et al.* 1996; Lapinskas *et al.* 1996; Sapperstein *et al.* 1996; Hermann *et al.* 1997; Hovland *et al.* 1997; Huang and Elledge 1997; Kagami *et al.* 1997; Schmidt *et al.* 1997; Webb *et al.* 1997; Desrivieres *et al.* 1998; Grandin *et al.* 1998; Zhang *et al.* 1998). As such suppressors often identify proteins of interest, it should be similarly possible to identify mutations that suppress an overexpression phenotype. In this study, we used suppressor analysis to examine the nature of the lethality caused by overexpression of the actin filament-bundling protein fimbrin, which is encoded by the *SAC6* gene (Adams *et al.* 1991). We found a very low frequency of mutations of interest, but we showed that those that were obtained were highly specific and were novel mutations in the actin gene.

Utility of the suppressor screen devised in this study to identify physically interacting proteins: We have described a suppressor screen that should be useful in identifing suppressors of other genes that are lethal when overexpressed. The suppressor screen devised in this study is likely to be of general use in identifying interacting proteins, as (i) it led to the identification of a protein known to physically interact with Sac6p, and (ii) the screen was effective in identifying just mutations of interest (all the trivial mutations were rapidly eliminated early in the analysis). It is striking that in a screen of the entire genome, the only mutations identified as suppressors of the lethality caused by Sac6p overexpression were those that changed residues in a protein known to interact with Sac6p *in vivo*. However, it is probable that screens such as the one described here will not always identify just physically interacting proteins, as there are multiple ways in which the activity of proteins can potentially be downregulated.

Utility of the approach in mapping sites of interaction of proteins: The observation that the actin mutations *act1-20* and *act1-21* suppress Sac6p overexpression is explained by the finding that the mutant actins have a decreased affinity for Sac6p. Both mutations are within the DNase I-binding loop in subdomain 2 of actin (see Figure 4), and they may effect local or more global changes. As they alter residues important for Sac6p binding, however, it is likely that these mutations further define the site of interaction of actin with Sac6p and fimbrins in general. This finding suggests that it may be possible to use this approach to map where, on a particular protein, an overexpressed protein binds. For example, if overexpression of a protein causes a lethal (or any other) phenotype, it may be possible to map where that protein binds to another protein through analysis of mutations that suppress the overexpression phenotype. Moreover, it may be possible to map sites of interaction of individual domains if overexpression of just that domain has a phenotype.

***act1-20* and *act1-21* are novel actin alleles:** Previous studies have identified a variety of mutant actin alleles (*e.g.*, Shortle *et al.* 1984; Adams and Botstein 1989; Dunn and Shortle 1990; Wertman *et al.* 1992; Munn *et al.* 1995). These alleles have provided a collection that has been invaluable in the analysis of both the interactions and functions of actin (Botstein *et al.* 1997). In this study, we describe two additional alleles that further increase this collection. One of these alleles, *act1-20*, changes the same residue altered by the *end7-1* mutation. As *end7-1* results in a defect in endocytosis (Munn *et al.* 1995), and *sac6* null mutant cells are defective in endocytosis (Kubler and Riezman 1993), it is likely that *end7-1* is defective in endocytosis because of a defect in binding to Sac6p.

The other allele, *act1-21*, is particularly interesting, as it has the novel phenotype of being dependent on overexpression of an actin-binding protein for viability.

The underlying biochemical basis for this dependence is unknown, but it will likely prove interesting and may shed light on cytoskeletal dynamics. Interestingly *act1-125* (K50A, D51A; Wertman *et al.* 1992), which changes the same residue as *act1-21* (K50E), is also suppressed by overexpression of Sac6p (Honts *et al.* 1994), but in the case of *act1-125*, the cells are viable at 23° without extra Sac6p, and it is only the growth defect at 37° that is suppressed.

Overexpression of Sac6p leads to a highly specific phenotype that is more severe than that of the *sac6* null mutation: We previously found that *sac6* null mutant cells are viable at room temperature but inviable at 37° (Adams *et al.* 1991). In contrast, we found that overexpression of Sac6p is lethal, even at 23°. This finding indicates that overexpression of Sac6p is more toxic to the cells than is a complete absence of this protein. There are several possible explanations for this observation, including that excess Sac6p (i) interferes nonspecifically with cellular function; (ii) causes excess bundling of actin, which is more deleterious to the cells than is a deficiency of bundling; (iii) titrates out some factor that is essential for cell growth; and (iv) competes with an essential actin-binding protein(s) for sites of interaction on actin. However, the finding that actin mutations suppress the overexpression lethality indicates that lethality is not simply through nonspecific interaction, but rather, through a specific association of Sac6p with actin. It is possible that excess Sac6p hyperstabilizes actin, making it less dynamic, and consistent with this idea is the observation that mutations of *SAC6* (*e.g.*, *sac6-19*) that result in an increased affinity of Sac6p for actin are also more deleterious to the cell than is the *sac6* null mutation (Sandrock *et al.* 1997). However, it is equally likely that either mutant Sac6-19 protein or excess Sac6p outcompetes some other actin-binding protein whose interaction with actin is essential for cell viability. Alternatively, Sac6p may be titrating out some factor other than actin, but if so, binding of Sac6p to actin must be required for that factor to bind to Sac6p.

We thank Roy Parker, Ted Weinert, and members of our lab for helpful discussions during the course of this work; and Johanna O'Dell and Ted Weinert for comments on the manuscript. We are grateful to Skip Vaught and his staff in the DNA Sequencing Service at the University of Arizona for assistance with DNA sequencing and to Roy Parker for plasmids. This work was supported by a grant from the National Institutes of Health (GM45288), a predoctoral National Research Service Award (CA09213) to T.M.S., and an NSF Predoctoral Minority Fellowship to K.A.T.

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Communicating editor: F. Winston