Unexpected Combinations of Null Mutations in Genes Encoding the Actin Cytoskeleton Are Lethal in Yeast

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Submitted November 6, 1992; Accepted March 3, 1993

To understand the role of the actin cytoskeleton in cell physiology, and how actin-binding proteins regulate the actin cytoskeleton *in vivo*, we and others previously identified actin-binding proteins in *Saccharomyces cerevisiae* and studied the effect of null mutations in the genes for these proteins. A null mutation of the actin gene (*ACT1*) is lethal, but null mutations in the tropomyosin (*TPM1*), fimbrin (*SAC6*), Abp1p (*ABP1*), and capping protein (*CAP1* and *CAP2*) genes have relatively mild or no effects. We have now constructed double and triple mutants lacking 2 or 3 of these actin-binding proteins, and studied the effect of the combined mutations on cell growth, morphology, and organization of the actin cytoskeleton. Double mutants lacking fimbrin and either Abp1p or capping protein show negative synthetic effects on growth, in the most extreme case resulting in lethality. All other combinations of double mutations and the triple mutant lacking tropomyosin, Abp1p, and capping protein, are viable and their phenotypes are similar to or only slightly more severe than those of the single mutants. Therefore, the synthetic phenotypes are highly specific. We confirmed this specificity by overexpression of capping protein and Abp1p in strains lacking fimbrin. Thus, while overexpression of these proteins has deleterious effects on actin organization in wild-type strains, no synthetic phenotype was observed in the absence of fimbrin. We draw two important conclusions from these results. First, since mutations in pairs of actin-binding protein genes cause inviability, the actin cytoskeleton of yeast does not contain a high degree of redundancy. Second, the lack of structural and functional homology among these genetically redundant proteins (fimbrin and capping protein or Abp1p) indicates that they regulate the actin cytoskeleton by different mechanisms. Determination of the molecular basis for this surprising conclusion will provide unique insights into the essential mechanisms that regulate the actin cytoskeleton.

INTRODUCTION

In vitro studies have identified a wide variety of proteins that bind to actin, and multiple biochemical activities that affect actin assembly and myosin-dependent motility have been attributed to many of these proteins (Stossel *et al.*, 1985; Pollard and Cooper, 1986). In addition, a variety of mechanisms based on regulation by calcium, phosphoinositides, and other molecules have been described *in vitro* (Stossel, 1989; Goldschmidt-Clermont and Janmey, 1991). For most actin-binding

proteins, however, it is unclear which biochemically identified activity or regulatory mechanism, if any, is important in the cell. How the various actin-associated proteins act in concert to produce a functional cytoskeleton is also not known.

We have taken a genetic approach to this problem, and are using yeast to study the cytoskeleton. We are particularly interested, at this point in our analysis, in the question of the level of functional redundancy among elements of the actin cytoskeleton. A number of null mutations in *Dictyostelium* have a minimal phenotype, for which one explanation may be a high degree of functional redundancy among the components

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(Andre *et al.*, 1989; Bray and Vasiliev, 1989; Egelhoff and Spudich, 1991; Hofman *et al.*, 1992; Witke *et al.*, 1992). The array of phenotypes caused by mutations in yeast actin cytoskeletal genes is much more broad. At one extreme, null mutations in the genes for actin (*ACT1*) (Shortle *et al.*, 1982), an actin-related protein (*ACT2*) (Schwob and Martin, 1992), a myosin of the *dilute* family (*MYO2*) (Johnston *et al.*, 1991), and cofilin (*COF1*) (Moon *et al.*, 1993) are lethal. At the other extreme, mutations in the genes for capping protein (*CAP1*, *CAP2*) (Amatruda *et al.*, 1990, 1992) and a novel actin-binding protein (*ABP1*) (Drubin *et al.*, 1990) have a minimal effect on growth and viability, although the morphology of the actin cytoskeleton is changed in capping-protein mutants. In addition, mutations in genes encoding other elements of the actin cytoskeleton fall between these extremes. Mutations in the genes for profilin (*PFY1*) (Haarer *et al.*, 1990), tropomyosin (*TPM1*) (Liu and Bretscher, 1989, 1992), and fimbrin (*SAC6*) (Adams *et al.*, 1991) are viable and have severe, moderate, and mild effects, respectively, on growth.

We were interested, therefore, to make double and triple combinations among those mutations that have only slight phenotypes to determine whether enhancement, or perhaps even lethality, was observed. Strikingly, most combinations showed no enhancement, but two combinations, namely fimbrin with capping protein and fimbrin with *Abp1p*, showed extreme enhancement. To document that these synthetic effects are specific, we examined growth rates and actin distributions for the viable single and combination mutant strains. In some previously reported cases, synthetic-lethal mutations were found to reside in genes whose products are homologues. However, fimbrin, *Abp1p* and capping protein share no structural homology, and when we tested for functional homology by overexpression we found none.

METHODS

Strains, Media, and Genetic Techniques

Diploid yeast strains, for the analysis described in Table 2, are listed in Table 1. Haploid segregants derived from these diploids were used in the analyses described in Figures 1 and 2. Additional haploid strains used in the overexpression studies are listed in Table 1. Media for yeast growth and sporulation, and methods for mating, sporulation, and tetrad analysis were as described by Sherman *et al.*, 1974. Growth of segregants on plates was scored by spotting suspensions of cells in water onto plates using a 32-point inoculator.

Gene Disruptions and Plasmids

Most of the gene disruptions used in this study were described previously: *cap2-1::URA3* and *cap2-Δ1::HIS3* (Amatruda *et al.*, 1990), *cap1-Δ1::TRP1* (Amatruda *et al.*, 1992), *sac6::LEU2* (Adams *et al.*, 1991), *sac6::URA3* (Adams *et al.*, 1989), *tpm1::URA3* (Liu and Bretscher, 1989), *tpm1::LEU2* (Liu and Bretscher, 1992). Previously undescribed deletions are *abp1::URA3* and *abp1::LEU2*. In each of these cases, the *Xho* I-*Pvu*II fragment of *ABP1*, which extends from 227 bp upstream of the start codon to 246 bp upstream of the stop codon [thus leaving the last 82 amino acids at the C-terminus intact (Drubin *et al.*, 1990)],

Table 1. Yeast strains used in this study

Strain	Genotype
Diploids^a	
AAY1205	<i>MATa/MATα tpm1::LEU2/+ abp1::URA3/+ cap2-Δ1::HIS3/+ leu2/leu2 ura3/ura3 his3/his3 lys2/+ ade2/+ trp1/+</i>
AAY1240	<i>MATa/MATα abp1::URA3/+ cap2-Δ1::HIS3/+ ura3/ura3 his3/his3 leu2/leu2 ade2/+ lys2/+</i>
AAY1288	<i>MATa/MATα sac6::LEU2/+ tpm1::URA3/+ leu2/leu2 ura3/ura3 lys2/+ his3/+</i>
AAY1289	<i>MATa/MATα sac6::LEU2/+ abp1::URA3/+ leu2/leu2 ura3/ura3 lys2/lys2 his3/+</i>
AAY1290	<i>MATa/MATα sac6::LEU2/+ cap2-Δ1::HIS3/+ leu2/leu2 his3/his3 ura3/ura3 lys2/lys2 ade2/+</i>
AAY1291	<i>MATa/MATα tpm1::LEU2/+ abp1::URA3/+ leu2/leu2 ura3/ura3 his3/+ ade2/+ lys2/+</i>
YJC121	<i>MATa/MATα sac6::LEU2/+ cap2-1::URA3/+ leu2/leu2 ura3/ura3 his3/+ lys2/+ his4/+</i>
YJC140	<i>MATa/MATα tpm1::URA3/+ cap2-Δ1::HIS3/+ ura3/ura3 his3/his3 ade2/ade2 lys2/lys2 met/+ tyr/+</i>
YJC144	<i>MATa/MATα abp1::LEU2/+ cap2-1::URA3/+ ura3/ura3 leu2/leu2 his4/+ lys2/+ ade2/+</i>
YJC145	<i>MATa/MATα abp1::LEU2/+ cap2-1::URA3/+ ura3/ura3 leu2/leu2 his4/+ lys2/+ ade2/+</i>
YJC240	<i>MATa/MATα sac6::LEU2/+ cap2-Δ1::HIS3/+ leu2/leu2 his3/his3 ade2/ade2 trp1/trp1 ura3/ura3</i>
YJC242	<i>MATa/MATα sac6::LEU2/+ cap2-Δ1::HIS3/+ leu2/leu2 his3/his3 ade2/ade2 trp1/trp1 ura3/ura3</i>
YJC244	<i>MATa/MATα sac6::LEU2/+ cap1-Δ1::TRP1/+ leu2/leu2 ade2/ade2 ura3/ura3 trp1/trp1 his3/his3</i>
YJC245	<i>MATa/MATα sac6::LEU2/+ cap1-Δ1::TRP1/+ leu2/leu2 ade2/ade2 ura3/ura3 trp1/trp1 his3/his3</i>
Haploids^b	
AAY1046	<i>MATα sac6::LEU2 leu2 his3 lys2 ura3 Gal⁺</i>
AAY1048	<i>MATa SAC6⁺ leu2 his3 lys2 ura3 Gal⁺</i>
AAY1200	<i>MATα sac6::URA3 ura3 leu2 his3 trp1</i>

All strains were generated in our laboratories.

^a The diploids listed are those used in the analysis described in Table 2. Haploid segregants derived from these diploids were used in the experiments described in Figures 1 and 2.

^b The haploids listed were used in the overexpression studies described in the text.

was replaced with a 1.1-kB fragment containing *URA3* or a 3.1-kB fragment containing *LEU2*. The gene disruptions were confirmed as described previously (Shortle *et al.*, 1982) using DNA hybridization of genomic DNA and tetrad analysis.

Plasmid pBJ114, a 2-μ plasmid containing both *CAP1* and *CAP2* under the control of the bidirectional *GAL1/10* promoter, has been described previously (Amatruda *et al.*, 1992). pABZ259 is a similar plasmid, lacking *CAP1* and *CAP2* (Amatruda *et al.*, 1992). Plasmid pRB1201, a 2-μ plasmid carrying *ABP1* and *URA3* has been described before (Drubin *et al.*, 1988).

Determination of Growth Rates

Sixty-eight strains, which were segregants derived from the diploids listed in Table 2, were grown in yeast extract, peptone, dextrose (YEPD) liquid medium at 23°C. Samples were taken at ~1 h intervals (for

Table 2. Viability of double and triple mutants

Cross ^a	Segregation patterns ^b			Double or triple mutants Alive:Dead ^c
	PD	TT	NPD	
<i>sac6::LEU2</i> × <i>tpm1::URA3</i> (AAY1288)	0	5	2	9:0
<i>sac6::LEU2</i> × <i>abp1::URA3</i> (AAY1289)	5	8	0	4:4 ^d
<i>sac6::LEU2</i> × <i>cap1-Δ1::TRP1</i> (YJC244 & YJC245)	5	24	4	0:32
<i>sac6::LEU2</i> × <i>cap2-Δ1::HIS3</i> & <i>sac6::LEU2</i> × <i>cap2-1::URA3</i> (YJC240, YJC242, AAY1290, & YJC121)	9	54	12	0:78 ^e
<i>cap2-Δ1::HIS3</i> × <i>tpm1::URA3</i> (YJC140)	4	9	5	19:0
<i>cap2-1::URA3</i> × <i>abp1::LEU2</i> & <i>cap2-Δ1::HIS3</i> × <i>abp1::URA3</i> (YJC144, YJC145, AAY1240)	4	33	8	49:0
<i>abp1::URA3</i> × <i>tpm1::LEU2</i> (AAY1291)	2	11	3	17:0
<i>tpm1::LEU1 abp1::URA3</i> × <i>cap2-Δ1::HIS3</i> (AAY1205)	see footnote ^f			4:0

Crosses were made between strains deleted for the genes shown, and viability of the various segregants was determined. The genetic segregation of each null locus was followed in these crosses by scoring the auxotrophic marker genes used to replace or disrupt each actin-binding protein gene. Numbers of parental ditype (PD), tetratype (TT), and nonparental ditype (NPD) tetrads are shown. In addition, double mutants of *cap2* with disruptions of *sac7* (Dunn and Shortle, 1990), *myo1* (Rodriguez and Paterson, 1990), or the Ts mutation *myo2-66* (Johnston *et al.*, 1991) at permissive temperature were found to be viable.

^a The genotypes of the diploids are listed in Table 1.

^b The segregation patterns of the nutritional markers used in disrupting the various cytoskeletal genes are shown.

^c The genotypes of the dead segregants have been deduced from the genotypes of the live segregants in each tetrad.

^d The 4 live segregants all grew extremely poorly, eventually giving rise to tiny colonies; the 4 "dead" segregants from this cross yielded microcolonies visible under the microscope.

^e Microscopic examination of these dead segregants revealed that the majority of them germinated, and gave rise to variable numbers of cells ranging from 2 to several tens.

^f Because 3 auxotrophic markers are scored in this cross, the tetrads cannot be categorized as PD, TT, or NPD. From 12 tetrads from this cross, 4 segregants were Ura⁺ His⁺ Leu⁺ (and so are deduced to be triple null mutants).

5–8 h), fixed, and sonicated, and cell numbers were determined by using a Coulter Counter, as described previously (Pringle and Mor, 1975). Generation times were calculated for each strain and plotted as a histogram in Figure 1.

Fluorescence Microscopy

Fluorescence microscopy was as described previously (Pringle *et al.*, 1989), using affinity-purified anti-actin and 4,6-diamidino-2-phenylindole (DAPI). To characterize the actin organization of each strain, blind studies were conducted in which 3–11 strains of each genotype were examined by an observer who did not know the identity of the strains. Forty-three of the 68 strains mentioned above were examined in this way.

RESULTS

Genetic Analysis of Mutants Carrying Multiple Cytoskeletal Defects

To look for synthetic phenotypes, mutants lacking different combinations of capping protein, fimbrin, tropo-

myosin, and Abp1p were created by crosses of the various mutants, followed by tetrad dissection (Table 2). A synthetic enhanced phenotype was found for mutants lacking two pairs of proteins among all the combinations.

Double mutants lacking fimbrin and capping protein were dead. Capping protein has two subunits, encoded by the genes *CAP1* and *CAP2*. Disruption of either single gene leads to the loss of both protein subunits, therefore the phenotypes of the two single mutants and the *cap1 cap2* double mutant are the same (Amatruda *et al.*, 1992). Therefore, we first crossed *sac6* with *cap2*, and then confirmed the observed synthetic lethality by also crossing *sac6* with *cap1* (Table 2). Double mutants lacking fimbrin and Abp1p were either dead or grew extremely poorly. The latter segregants were indeed alive and not simply dying gradually because they continued

to grow extremely poorly with subculturing. The inviable *sac6 cap1*, *sac6 cap2*, and *sac6 abp1* double mutants are defective in mitotic growth, and not simply in germination, because microscopic examination of the spores revealed that several cells were often formed (Table 2).

Double mutants of every other possible permutation were recovered as viable strains, as also was the *abp1 cap2 tpm1* triple mutant. All of these viable strains grew significantly better than the viable *abp1 sac6* segregants.

Specificity of the Synthetic Effects

To determine more quantitatively the specificity of the synthetic lethality between the *sac6* and *cap2* and the *sac6* and *abp1* mutations, the effects of the null mutations on growth rates and cytoskeletal organization were determined in the viable single, double, and triple mutants. One simple hypothesis to explain the severe phenotype is that these single mutants have the most severe defects among the collection, and, therefore, their combination has the most severe defect, namely lethality, among the combinations.

Because the various gene disruptions had been generated in four different laboratories over the course of several years, the strains used in the crosses were not isogenic. Although this nonisogenicity could result in variability in the phenotypes of the mutants if differences in strain background had an effect, this potential difficulty could be minimized by characterizing multiple segregants from each cross. Indeed, any genotype that consistently cosegregated with a given phenotype, despite subtle differences in genetic background, could more convincingly be associated with the mutation or combination of mutations of interest. For these reasons, several segregants of each genotype were examined.

Growth Rates. Growth rates were determined for 3–11 segregants of each genotype, a total of 68 different strains (Figure 1). The growth rates of single mutants have been characterized previously (Liu and Bretscher, 1989, 1992; Amatruda *et al.*, 1990, 1992; Drubin *et al.*, 1990; Adams *et al.*, 1991), but we repeated those experiments so that we could directly compare the single, double, and triple mutants. Our observations here on the single mutants are consistent with the published reports. Thus *abp1* mutants are almost indistinguishable from wild-type cells in generation time, *cap2* and *sac6* mutants show a small increase, and *tpm1* mutants show a larger increase (Figure 1). The generation times for all four viable double mutants (*abp1 cap2*, *abp1 tpm1*, *cap2 tpm1*, and *sac6 tpm1*) overlap with those for the *cap2*, *sac6*, and *tpm1* single mutants. Interestingly, none of the three double mutants containing the *tpm1* mutation consistently shows generation times longer than those of *tpm1* alone. Although we note that the *sac6 tpm1* generation times were measured as less than those for *tpm1* alone, the level of variance in the data among

Figure 1. Growth rates of strains carrying various combinations of mutations in the *ABP1*, *CAP2*, *SAC6*, and *TPM1* genes. Calculated growth rates are plotted as histograms for each of the genotypes listed on the left. All strains, including the wild type, are segregants from the diploids listed in Table 2. Scale: smallest bars correspond to single strains.

individual segregants limits the strength of any conclusion that can be drawn from this observation. Segregants of the viable triple mutant *abp1 cap2 tpm1* have generation times slightly longer than those of the viable double mutants.

Given these results, one would have predicted, *a priori*, that the double mutants *sac6 abp1* and *sac6 cap2* would have generation times in the range of those measured here. One would not predict that these two double mutants would fall at the extreme end of these measurements of generation times. The observation that these double mutants are dead or at best grow very poorly indicates that the phenotype represents synergy, as opposed to merely addition of the two single phenotypes.

Actin Distribution and Cell Morphology. For similar reasons, we also examined the distribution of the actin cytoskeleton in the viable single, double, and triple mutants by immunofluorescence with anti-actin. The rationale was to determine whether the single mutants that in combination gave a synthetic-lethal phenotype also had more severe actin defects than mutants that did not. In addition, we wanted to determine whether the actin distributions for the viable double and triple mutants defined a range of defects in which we would have expected to find the synthetic-lethal double mutants.

Forty-three of the segregants studied in the growth rate experiments, comprised of 3–11 representatives for each genotype, were examined. To guard against bias, the experimenter recorded observations without knowing the identity of the samples. In terms of the actin distribution, the presence of cables, the thickness and length of cables, the degree of polarization of cortical patches to the tip of the bud, and the presence of actin bars were noted. Other criteria including cell size, cell shape, fraction of budded cells, and number of nuclei per cell (by DAPI staining) were also examined.

Again, we began with the single mutants. Although the actin distribution of each single mutant has been described previously, we felt it was important to repeat those experiments here, in the hands of one experimenter, to permit direct comparison of the different strains.

Among the single mutants, *abp1* cells have only subtle differences in the organization of the actin cytoskeleton

relative to wild-type cells (Figure 2). The thickness of the actin cables may be slightly decreased. For both wild-type and mutant cells, cortical actin patches are concentrated in the growing bud region, and actin cables are aligned along the mother-bud axis. In addition, cell shape and size are both normal in the mutant cells. The *sac6*, and *cap2* single mutants have slightly more pronounced defects in organization of the actin cytoskeleton (Figure 2). Thus both *sac6* and *cap2* cells have increased numbers of cortical actin patches in the mother cells, and the actin cables are significantly more faint, or absent altogether, when compared with the wild type; in addition, the mutant cells are round, rather than oval, and cell size is more heterogeneous than normal, with some cells being quite large (Figure 2). Finally, the *tpm1* single mutant has the most severe defect in organization of the actin cytoskeleton (Figure 2). As reported previously (Liu and Bretscher, 1989), the most pronounced features are the presence of brightly staining actin bars

Figure 2. Anti-actin immunofluorescence of yeast strains cultured at 25°C. All strains, including the wild type, are segregants from the diploids listed in Table 2. Strains: wild type, strain AAY1299 (A); *abp1::URA3*, strain AAY1261 (B); *cap2-Δ1::HIS3*, strain AAY1297 (C); *abp1::URA3 cap2-Δ1::HIS3* double mutant, strain AAY1301 (D); *tpm1::URA3*, strain AAY1208 (E); and *abp1::URA3 cap2-Δ1::HIS3 tpm1::LEU2* triple mutant, strain AAY1231 (F). Bar, 10 μ .

Table 3. Overexpression of capping protein in a strain lacking fimbrin

Temp	Synthetic selective media				Rich media			
	Glucose		Galactose		Glucose		Galactose	
	[CP]	[Ctrl]	[CP]	[Ctrl]	[CP]	[Ctrl]	[CP]	[Ctrl]
15°	4	4	2	3	4	4	3	3
24°	4	4	2	3	4	4	3	3
30°	4	4	2	3	4	4	4	4
37°	1	1	1	1	0	0	1	1

The *sac6* null strain AAY1200 was transformed with plasmid pBJ114, which expresses *CAP1* and *CAP2* from the *GAL1/10* promoter (denoted [CP]), or plasmid pBJ95, which lacks *CAP1* and *CAP2*. (denoted [Ctrl]). The promoter is repressed in glucose and stimulated in galactose, so cells from 2 independent transformants were patched and streaked on solid media containing glucose or both galactose and raffinose. Growth is recorded here from 0 to 4 (4 representing the best growth). Scores for plates at different temperatures are based on growth for different lengths of time, to optimize detection of differences between strains. To confirm that strains were overexpressing capping protein, parallel liquid cultures were analyzed by immunoblot with anti-capping protein antibodies. Dilution of the overexpression sample showed the level of capping protein to be increased by about 50-fold. In addition, cell shape was characteristically abnormal, with elongated and mis-shaped buds viewed by phase contrast microscopy.

and a lack of normal actin cables. Notably, the *tpm1* mutation has no significant effect on the asymmetric distribution of the cortical actin patches. The size distribution of *tpm1* cells is rather heterogeneous, with many large cells that often contain multiple nuclei.

Based on these results, one might have predicted that the double mutants involving *tpm1* would be inviable because *tpm1* single mutants have defects more severe than those of *cap2*, *sac6*, and *abp1* strains. However, observations on the distribution of the actin cytoskeleton in the viable combination mutants generally agree with the results from the growth rate experiments, namely that the synthetic defects seen in the fimbrin/Abp1p and fimbrin/capping protein double mutants are not simply an additive effect of two severe phenotypes. Indeed, the various combination mutants define a spectrum of defects that one might have predicted would include *sac6 abp1* and *sac6 cap2*. Thus actin organization and cell morphology of the *cap2 abp1* and *tpm1 abp1* double mutants (Figure 2) are similar to those of the *cap2* and *tpm1* single mutants, respectively. The *cap2 tpm1* and the *sac6 tpm1* double mutants show effects that are additive, namely slightly worse than those of either single mutant alone. Many cells are large, actin cables are uniformly absent, actin bars are seen at a low frequency, and the degree of polarization of cortical actin patches is slightly less than in the *tpm1* single mutant. The *tpm1 abp1 cap2* triple mutant shows defects similar to those seen in the *tpm1* single mutants (Figure 2). Interestingly, these triple mutants, although lacking three actin binding proteins, are able to polarize their cortical actin structures and to grow in a polarized manner to form buds: in six different triple mutant strains examined, 42–63% of the cells were budded, and 62–74% of these budded cells had cortical actin patches concentrated in the buds (Figure 2). These results, consistent

with the growth rate results, indicate that the actin distribution phenotypes of the viable double and triple mutants define a range of defects into which one would have expected the synthetic lethal double mutants to fall.

Test for Enhancement of the *sac6* Phenotype by Overexpression of Capping Protein or Abp1p

One of the points in the preceding section is that *tpm1* single mutant strains are more severely affected, based on growth rate and actin distribution, than the other single mutants, and therefore one might have expected lethality in combinations including *tpm1*, which was not observed. Similarly, we had noted in the past that overexpression of Abp1p leads to more severe morphogenetic defects than does a null mutation in *ABP1* (Drubin *et al.*, 1988, 1990), and that overexpression of capping protein also leads to more severe morphogenetic defects than does a corresponding null mutation (Amatruda *et al.*, 1992). Therefore, one might have predicted that overexpression of Abp1p or capping protein would also enhance the defects of the *sac6* mutant and lead to lethality, since the null mutations do so. However, such was not the case (see below), which provides further support for the specificity of the synthetic effect between the *sac6* and *abp1* or *cap2* null mutations.

To overexpress capping protein, we transformed a *sac6* strain with a plasmid that co-expresses *CAP1* and *CAP2* under control of the bidirectional galactose-inducible *GAL1/10* promoter. A similar plasmid lacking *CAP1* and *CAP2* was used as a control. Transformants were plated on solid media, selective for the plasmid, and tested for growth on glucose and galactose (see Table 3 under *Synthetic selective media*).

We found no striking enhancement of the *sac6* phenotype at any temperature, certainly not lethality. As

shown in Table 3, cells on glucose grew well at all temperatures, except for 37°C, as expected for the *sac6* background. There was no difference between the capping protein and control plasmids. In the presence of galactose, strains with either plasmid grew slightly less well than on glucose, which is expected because galactose is a poorer carbon source. However, overexpression of capping protein on galactose caused an additional slight decrease in growth, relative to the control plasmid, consistent with the effects in wild-type strains described previously (Amatruda *et al.*, 1992); synthetic lethality was not observed.

For overexpression of Abp1p, the design of the experiments was slightly different because the *ABP1* overexpression plasmid used the endogenous promoter for *ABP1*, not that of *GAI1/10*. The plasmid has high copy number due to its 2- μ replication origin, expression from this plasmid is not inducible, and previous work showed that it leads to overexpression of Abp1p by approximately five-fold (Drubin *et al.*, 1988). Therefore, as a control, we compared the expression plasmid with a control plasmid and transformed each plasmid into both *sac6* and *SAC6* strains. To determine whether overexpression of Abp1p enhanced the *sac6* growth phenotype, we plated transformants on selective synthetic media. As shown in Table 4 (under *Synthetic selective media*), the presence of the *ABP1* plasmid, as opposed to the control plasmid, did not inhibit growth of the *sac6* strains at any temperature. Again, no synthetic phenotype enhancing the *sac6* defect was observed.

Test for Suppression of the *sac6* Phenotype by Overexpression of Capping Protein or Abp1p

One simple interpretation for synthetic lethality is that the two proteins are homologues or just slightly different forms of essentially the same protein. Because we know the amino acid sequences for Abp1p, fimbrin, and capping protein, we know that they are not simple homologues and indeed share no discernable structural

homology at the level of primary structure. Nevertheless, the tertiary structures of the proteins may have similar regions that lead to functional similarities in their interaction with actin or other cellular components.

To test for functional homology, we reasoned that overexpression of either capping protein or Abp1p might suppress phenotypic traits caused by the fimbrin mutation. Fimbrin null mutants are Ts- for growth at 37°C. This effect is pronounced on rich media but only marginal on synthetic media. Therefore while the data described in the previous section and listed in Tables 3 and 4, under "synthetic selective media," addresses this question, we also tested the same set of strains for growth on rich media. On rich media, the overexpression plasmid may be lost; however, if the overexpression plasmid does suppress the *sac6* growth defect, then the plasmid should be retained by selection.

The results assessing growth at 37°C are listed in Table 3 for capping protein and in Table 4 for Abp1p. On neither rich media nor synthetic selective media was any growth advantage conferred by overexpression. Therefore, these results do not suggest functional homology between fimbrin and either Abp1p or capping protein.

DISCUSSION

Redundancy in the Actin Cytoskeleton

Results from the molecular genetic analysis of the actin cytoskeleton have been paradoxical for several years. On the one hand, actin is clearly very important to cells, because a yeast null mutant of actin is dead (Shortle *et al.*, 1982) and because, in a variety of cell types, cytochalasin, an actin toxin, has severe and dramatic effects on cell shape and motility (Cooper, 1987). The high degree of conservation of the actin amino acid sequence through evolution suggests actin's numerous binding interactions, both in its interior with nucleotide and divalent cation, as well as on its exterior with various

Table 4. Overexpression of Abp1p in a strain lacking fimbrin

Temp	Synthetic selective media				Rich media			
	<i>sac6</i>		<i>SAC6+</i>		<i>sac6</i>		<i>SAC6+</i>	
	[Abp1]	[Ctrl]	[Abp1]	[Ctrl]	[Abp1]	None	[Abp1]	None
24°	3	3	3	3	4	4	4	4
30°	4	4	4	4	4	4	4	4
37°	0	0	0	1	0	0	3	4

The *sac6* null strain AAY1046 was transformed with plasmid pRB1201, a 2- μ plasmid with *ABP1* and *URA3*, (denoted [Abp1]), or pAB123, which is a similar plasmid lacking *ABP1* (denoted [Ctrl]), or no plasmid (denoted None). The *SAC6+* strain AAY1048, a segregant from the same tetrad as AAY1046, was used as a control. Diluted cells were patched and streaked onto solid media. Growth is recorded here from 0 to 4 (4 representing the best growth). Scores for plates at different temperatures are based on growth for different lengths of time, to optimize detection of differences between strains. Plates were examined ≤ 6 d after plating.

actin-binding proteins, are each very important (Wertman and Drubin, 1992). On the other hand, null mutations of several actin-binding proteins in *Dictyostelium* have relatively mild phenotypes (De Lozanne and Spudich, 1987; Andre *et al.*, 1989; Brink *et al.*, 1990; Witke *et al.*, 1992). One possible explanation for the mild phenotypes is that the actin cytoskeleton has a high degree of redundancy (Bray and Vasiliev, 1989), which is supported by the observation that in multicellular organisms there are often multiple genes encoding actin and various actin-binding proteins.

In yeast, null mutations of some actin-binding protein genes are lethal. This includes the genes encoding a dilute myosin (*MYO2*) (Johnston *et al.*, 1991) and cofilin (*COF1*) (Moon *et al.*, 1993), in addition to those for actin (*ACT1*) (Shortle *et al.*, 1982) and an actin-related protein (*ACT2*) (Schwob and Martin, 1992). Deletion of profilin (*PFY1*) has a very severe phenotype, approaching lethality (Haarer *et al.*, 1990). On the other hand, null mutations of several other actin-binding proteins, including those studied here, have moderate to no effects on viability and growth (Watts *et al.*, 1987; Liu and Bretscher, 1989, 1992; Amatruda *et al.*, 1990, 1992; Drubin *et al.*, 1990; Adams *et al.*, 1991). For these components, some degree of "functional redundancy" is likely to exist, but we are interested in understanding the specifics of this rather vague notion. One specific question is how redundant are these proteins? In other words, are there 1, 2, 10, or 100 other components that can each provide viability in the absence of the others? Another specific question is whether "functionally redundant" components, mutations in the genes for which are "synthetically lethal," are simple structural and functional homologues, which implies that the cell carries multiple copies of certain proteins. In the long run, related and more complex questions include (1) whether the actin cytoskeleton contains "pathways," by which we mean subsets of proteins that act coordinately or cooperatively to achieve certain results, (2) which pathways contain which actin-binding proteins, and (3) whether the synthetic effects seen here represent defects in the same or different pathways.

We have addressed the first two questions by making double and triple mutants combining null mutations of four different proteins, namely tropomyosin, fimbrin, Abp1p, and capping protein, for which the single null mutants have relatively mild phenotypes in terms of growth and viability. Remarkably, we find that two of the double mutant combinations do result in lethality, whereas all the other ones do not. To be certain that the synthetic lethality was specific, and not merely the result of the addition of two moderate effects creating a severe one, we studied the single mutants and the viable combinations both in terms of growth rate and the organization of the actin cytoskeleton. The overall conclusion from those data is that the phenotypes of the viable combinations do reflect simply an expected

combination of the features seen in single mutants. The expected phenotype for the combinations found to be synthetically lethal would be well within the range of phenotypes found for the viable combinations. In addition, overexpression of Abp1p or capping protein, which leads to morphogenetic effects more severe than those observed in the respective null mutants, also does not show a synthetic enhancement of the fimbrin mutant phenotype. Therefore, the synthetic lethal phenotype is very specific.

The most important conclusion from these results, in answer to the first question posed above, is that the level of "functional redundancy" in the actin cytoskeleton is not high. Indeed, for fimbrin, Abp1p, and capping protein, the loss of only one additional protein leads to cell death.

How does one rationalize the apparent discrepancy between results in yeast, where deletion of one or two proteins of the actin cytoskeleton can be lethal, and results in *Dictyostelium*, where deletion of one, two, or even three different proteins is compatible with life? First, among actin-binding proteins, only conventional myosin heavy chain has been mutated in both yeast and *Dictyostelium*; therefore, the results may simply reflect the fact that different proteins were selected for study in the two systems. Second, *Dictyostelium* is clearly a more complex organism than yeast, especially considering the actin cytoskeleton. *Dictyostelium* displays a wide range of motile behaviors not seen in yeast, including development of multicellular structures. Perhaps it does require a level of redundancy greater than does yeast (Bray and Vasiliev, 1989). Third, since *Dictyostelium* are haploid, and yeast are diploid with the ability to sporulate to haploids, lethal phenotypes and suppressors thereof are more readily identified in yeast than in *Dictyostelium*.

Interpretation of Synthetic Lethality

A second conclusion from the results reported here, in response to the second question posed earlier relating to homology, is that these other components, which are in some sense "functionally redundant" are not simple homologues of each other, as seen for example with histone 2B (Rykowski *et al.*, 1981) and HMG-CoA reductase (Basson *et al.*, 1987) in yeast. None of these three actin-binding proteins shares any structural homology, at the level of amino acid sequence, with each other. Also, we found that overexpression of neither Abp1p nor capping protein was able to suppress the temperature-sensitive growth phenotype of the fimbrin null mutant, which argues that neither Abp1p nor capping protein can functionally substitute for fimbrin. Furthermore, the *in vitro* mechanisms of action of fimbrin and capping protein on actin polymerization have no common features.

How does the combinatorial loss of these proteins lead to cell death? In other cases, synthetic lethality was

seen between mutations in pairs of genes encoding proteins that associate as subunits in complexes such as nucleosomes (Morgan *et al.*, 1991), and ribosomes (Haffter *et al.*, 1991) or that participate in common steps of the secretion pathway (Salminen and Novick, 1987; Kaiser and Schekman, 1990; Nair *et al.*, 1990; Walworth *et al.*, 1992). In this regard, fimbrin, Abp1p, and capping protein are all located in the cortical patches of the actin cytoskeleton (Drubin *et al.*, 1988; Amatruda and Cooper, 1992), although fimbrin is also found along actin cables. Perhaps the function of the cortical patch is essential, which agrees with the finding that a single null mutation in another patch component, cofilin, is lethal (Moon *et al.*, 1993). Since the three proteins can be purified independently of each other under nondenaturing conditions (unlike the two subunits of capping protein, which always co-purify), the proteins are not bound very tightly to each other in the patch complex. Since each protein binds actin filaments, and actin filaments are also a component of cortical patches, we suspect that the three actin-binding proteins are in patches due to their interaction with actin as opposed to each other. Alternative models are suggested by the fact that synthetic-lethal effects have also been observed among proteins whose sequences and genetics imply that they regulate the binding or assembly of other proteins in the development of yeast polarity (Bender and Pringle, 1991).

In this regard, one possibility is that two proteins share some regulatory function. For example, even though fimbrin acts by binding to the sides of actin filaments (Bretscher, 1981; Glenney *et al.*, 1981), and capping protein acts by binding to the ends of actin filaments (Caldwell *et al.*, 1989; Amatruda and Cooper, 1992) both mechanisms of action may prevent actin depolymerization and thereby stabilize filaments *in vivo* (Broschat, 1990; Cano *et al.*, 1992; Zigmond *et al.*, 1992). A single actin filament may have both proteins bound to it, and the loss of both proteins leads to total depolymerization of the filament. Again, the finding that all three proteins are found in cortical actin patches supports this model.

On the other hand, we have little or no information about the location, polarity and dynamics of individual actin filaments in yeast. Therefore, another reasonable alternative, which is more complex, is that each actin-binding protein binds to and affects only a subset of actin filaments in the cell and that these subsets are not overlapping. In this model, the disfunction of two subsets of actin filaments results, via some complex mechanism, in cell death. The localization of the proteins is also consistent with this model since fimbrin is a component of cables and cortical patches, whereas Abp1p and capping protein are components only of patches. The molecular mechanism of the synthetic effect is, of course, unclear; however, the significance of these results is to direct our attention to understanding the roles

of these actin-binding proteins, especially how their effects on actin may be complementary or interactive.

ACKNOWLEDGMENTS

We thank Doug Holtzman for the *abp1::LEU2* strain, Haoping Liu and Anthony Bretscher for the *tpm1* disruption, Julie Hoover for help with tetrad dissection, and Sharon Jaffe for assistance in measuring growth rates. We are especially grateful to Roy Parker, John Pringle, and colleagues in our labs for helpful discussions and comments on the manuscript. This work was supported by grants from the NIH (GM45288 to A.A., GM38542 to J.A.C., and GM42759 to D.D.), the Searle Scholars Program/The Chicago Community Trust (D.D.), and the PEW Scholars Program (A.A.). J.A.C. is a Lucille P. Markey biomedical scholar.

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