Actin Filaments in Yeast Are Unstable in the Absence of Capping Protein or Fimbrin

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Abstract. Many actin-binding proteins affect filament assembly in vitro and localize with actin in vivo, but how their molecular actions contribute to filament assembly in vivo is not understood well. We report here that capping protein (CP) and fimbrin are both important for actin filament assembly in vivo in *Saccharomyces cerevisiae*, based on finding decreased actin filament assembly in CP and fimbrin mutants. We have also identified mutations in actin that enhance the CP phenotype and find that those mutants also have decreased actin filament assembly in vivo. In vitro, actin purified from some of these mutants is defective in polymerization or binding fimbrin. These findings support the conclusion that CP acts to stabilize actin filaments in vivo. This conclusion is particularly remarkable because it is the opposite of the conclusion drawn from recent studies in *Dictyostelium* (Hug, C., P. Y. Jay, I. Reddy, J. G. McNally, P. C. Bridgman, E. L. Elson, and J. A. Cooper. 1995. *Cell.* 81:591–600). In addition, we find that the unpolymerized pool of actin in yeast is very small relative to that found in higher cells, which suggests that actin filament assembly is less dynamic in yeast than higher cells.

In *Saccharomyces cerevisiae*, actin is essential for viability (35), and important for polarized secretion (28) and endocytosis (19). The functional state of actin is presumed to be a helical filament, assembled from monomeric subunits. The assembly and activity of actin filaments can be regulated in vitro by a large number of proteins that interact with actin. We wish to understand assembly of actin filaments in vivo and to achieve a molecular view of how actin-binding proteins function to control actin filament assembly. In vitro, monomers add to and leave from filaments at their ends, both barbed and pointed. Therefore, filament assembly in vivo can theoretically be controlled by actin-binding proteins that bind to monomers or filaments and inhibit, enhance, or bias this exchange reaction.

In vitro, capping protein (CP)[1] binds to the barbed end of actin filaments, which inhibits both assembly (actin monomer addition) and disassembly (actin monomer loss) of the filament (for review see 30). CP is found in all eukaryotes, including *S. cerevisiae*, where the heterodimeric protein is encoded by the genes *CAP1* and *CAP2* (3, 4). CP colocalizes with filamentous actin in yeast and other cells (3, 31, 32). In developing muscle, the actin-binding activity of CP is necessary for proper assembly of the actin filaments of the sarcomere (34). In muscle, CP probably helps to determine the proper location and polarity of the actin filaments as well as to prevent their growth into adjacent sarcomeres. In *Dictyostelium*, CP functions to limit or inhibit growth at barbed ends, and the level of CP determines the level of actin filament assembly (15).

One might imagine that mechanisms governing actin filament assembly in yeast are very different from those that operate in striated muscle, *Dictyostelium*, and animal cells in general, based simply on the observation that the actin content of yeast is 100 times less than that in other cells. In most animal cells, the concentration of actin is very high (200 μM) relative to the critical concentration for polymerization (0.1 μM), and these cells most likely use actin monomer–binding proteins, such as thymosin and profilin, to create a buffered pool of actin monomers available for rapid assembly. Yeast, however, have no apparent need for rapid and dynamic actin assembly. Although they clearly need to create actin filaments as they grow and divide, there is no evidence that filaments ever disassemble. In addition, there are no reports of a buffered pool of actin monomers in yeast, and our experiments here find that the soluble monomer pool is indeed very small. Therefore, we hypothesize that in yeast actin filaments are inherently unstable due to the low actin concentration and therefore actin-binding proteins function to create, assemble, and stabilize actin filaments.

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1. *Abbreviations used in this paper*: CP, capping protein; wt, wild type.

Here we report experiments to test this hypothesis. We measured actin filament assembly by two complementary assays in yeast mutants lacking CP and fimbrin, another actin-binding protein that interacts genetically with CP and has also been hypothesized to stabilize actin filaments (1). We find that actin filament assembly is decreased in vivo in these mutants. We also searched for actin mutations that enhance the phenotype of CP mutations, a genetic interaction similar to the effect of the loss of fimbrin. We identified a set of actin mutations that do enhance the loss of CP and find that these actin mutations also cause decreased actin filament assembly in vivo. To investigate the molecular basis for this observation, we purified actin from these mutants and found that the mutant actins often show either decreased polymerization or poor binding to fimbrin in vitro. These observations also support the hypothesis that CP and fimbrin act to stabilize actin filaments. As predicted, these conclusions are the opposite of the ones found in *Dictyostelium* (15).

Materials and Methods

Materials, Media, and Culture Conditions

Unless stated otherwise, chemicals, materials and solvents were from Fisher Scientific Co. (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO). Restriction endonucleases and other enzymes were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Yeast rich medium (YEPD), sporulation medium (MSPO) and synthetic minimal medium with different metabolites omitted for selection (DOBA), were from BIO 101, Inc. (La Jolla, CA). Medium containing 0.1% 5-fluoroorotic acid (PCR Inc., Gainesville, FL) was prepared as described (16). Presporulation medium was YEPD with adenine (200 μg/ml). Strains were grown at 30°C unless specified otherwise.

Molecular and Genetic Techniques

Strains used in this work are listed in Table I. Tetrad dissection, random spore analysis, and yeast LiCl transformation were as described (16). DNA manipulations were performed by standard methods (25). Yeast genomic DNA was prepared as described (11). Mutant *act1* alleles were cycle sequenced (9) from both PCR-amplified genomic DNA and gap-repair plasmids using [γ-^{33}P] ATP to end-label a set of oligonucleotide primers that covered the gene.

Proteins

Two-dimensional gel electrophoresis and immunoblotting were performed as described (33), using goat anti-yeast actin antibodies (17). Actin was purified as described (18), except that a MonoQ column was substituted for the DEAE column. Also, the pool from the MonoQ column was further purified by a cycle of polymerization–depolymerization (14). Purified wild-type (wt) and mutant actins showed minimal proteolysis on a heavily loaded SDS gel. To exclude contamination or reversion, we also sequenced the relevant region of each *act1* gene by PCR amplification and cycle sequencing, using yeast cells obtained at the end of the preparation as template.

To test actin polymerization, each mutant actin was polymerized at 6 μM for 2 h at room temperature in 1× MKEI (2 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 20 mM imidazole/HCl, pH 7.0), then diluted to 3, 1, and 0.5 μM in the same buffer and incubated for an additional 2 h. Samples were periodically mixed gently during the incubation, to accelerate the approach to steady state. 25 of 100 μl was removed, for the sample "Total," and added to 25 μl of 2× SDS sample buffer. The remaining 75 μl was spun at 150,000 *g* for 30 min at 22°C (70,000 rpm in a rotor (TLA 100.1, Beckman Instruments, Inc., Fullerton, CA) in a centrifuge (TL100; Beckman Instruments, Inc.)). 25 μl was removed from the meniscus, for the sample "Supernatant," and added to 25 μl of 2× SDS sample buffer. The remaining liquid was removed, avoiding the pellet, and the pellet was dissolved in 150 μl of 1× SDS sample buffer. Equal vols of the three samples, which represent equivalent fractions of the total sample, were analyzed on 10% SDS–polyacrylamide gels.

Yeast fimbrin, Sac6p, was purified as described (14) and kindly provided by T. S. Sandrock and A. E. M. Adams (University of Arizona, Tucson, AZ). Sedimentation assays to determine binding of fimbrin to the mutant actins were performed as described (14) with the following modifications: (*a*) the fimbrin (Sac6p) concentration was 0.75 μM; and (*b*) samples were sedimented at 90,000 *g* for 60 min at 22°C (50,000 rpm in a TLA100.1 rotor in a TL100 centrifuge). Fimbrin alone partially sedimented under these conditions, even with prior clarification; therefore, we analyzed the fimbrin in the supernatant, comparing each mutant actin with wt actin and no actin.

For actin polymerization and fimbrin-binding assays, 10% SDS–polyacrylamide gels stained with Coomassie blue were scanned and the intensity of the relevant bands was quantitated with NIH Image 1.54, (available by ftp from zippy.nimh.nih.gov). Intensities of actin bands were converted to absolute amounts by comparison with internal standards.

Cell Actin Assembly Assays

In each assay, cultures were grown in YPD to 10^7 cells/ml, and total protein was determined by Bradford assay (6) for normalization of the actin values.

To determine total actin, cells were completely disrupted with glass beads for SDS-PAGE. Actin was measured by a quantitative immunoblot using either affinity-purified goat antiyeast actin (17) or the mouse antiactin mAb C4 (20) as the first Ab and ^{125}I-labeled secondary Abs as described (15). Five aliquots of cells were assayed for each strain, with three serial twofold dilutions.

To determine G-actin levels, the amount of soluble actin released from

Table I. Yeast Strains Used in This Study

Name	Relevant genotype	Source
KWY201	MATa/α *act1::LEU2/ACT1 ura3/ura3 leu2/leu2 his3/his3*	K. Wertman, University of California, Berkeley
KT1277	MATa *cap2-1::URA3 ura3 leu2 his3*	This study
KT1278	MATα *cap2-1::URA3 ura3 leu2 his3*	This study
YJC 450	MATα *cap2::HIS3 ade2 ade3 lys2 ura3 leu2*	17
YJC 472	MATa *cap2::HIS3 ade2 ade3 lys2 ura3 leu2 trp1 his3* [*pBJ 198 - CAP2 ADE3 URA3*]	17
YJC 536	MATa *slc1-66 cap2::HIS3 ade2 ade3 ura3 leu2 trp1* [*pBJ 198 - CAP2 ADE3 URA3*]	17
YJC 542	MATa *slc1-87 cap2::HIS3 ade2 ade3 ura3 leu2 trp1* [*pBJ 198 - CAP2 ADE3 URA3*]	17
YJC 960	MATa/α *slc1-66/slc1-66 cap2::HIS3/cap2::HIS3 ade2/ade2 ade3/ade3 ura3/ura3 leu2/leu2 trp1/TRP1 lys2/LYS2 his3/his3* [pBJ 416 - *ADE3 LEU2 CAP2*]	This study
YJC 963	MATa *slc1-87 cap2::HIS3 ade2 ade3 ura3 leu2 trp1 lys2* [*pBJ 414 - CAP2 ADE3 LEU2*]	This study
YJC 1080	MATα *slc1-87 cap2::LEU2 ade2 ade3 ura3 leu2 trp1* [*pBJ 198 - CAP2 ADE3 URA3*]	This study
YJC 1096	MATa *act1-101::HIS3 his3 ura3 leu2 tub2*	This study
YJC 1104	MATa *act1-108::HIS3 his3 ura3 leu2 tub2*	This study
YJC 1107	MATα *act1-113::HIS3 his3 ura3 leu2 tub2*	This study
YJC 1117	MATα *act1-120::HIS3 his3 ura3 leu2 tub2*	This study
YJC 1133	MATa *act1-133::HIS3 his3 ura3 leu2 tub2*	This study

permeabilized unfixed cells was measured. Cells were permeabilized by flash freezing and saponin treatment in an F-actin stabilizing buffer MKEI, essentially as described (21). This procedure provides permeability for actin monomers and 70 kD dextran. The permeabilized cells were sedimented in a microfuge for 15 min, and the supernatant was removed for determination of actin and protein. In wt cells, the actin level was very low, therefore the volume loaded onto the gel was maximized. In this experiment, error was propagated from the error in the determination of actin level from the blot and the error in the protein assay.

To determine F-actin levels in fixed cells, a rhodamine–phalloidin-binding assay was performed (22). A control with unlabeled phalloidin (Molecular Probes Inc., Eugene, OR) was performed for each sample to determine nonspecific binding. Four aliquots of fixed cells were assayed for each strain, with three serial twofold dilutions.

The level of F-actin could not be determined as the pelletable fraction in the latter, permeabilized cell, experiment because only a fraction (≥65%) of the cells are permeabilized by this procedure (21). Attempts at a similar analysis with a more aggressive method of permeabilization (bead-beating) gave inconsistent results, presumably due to variable disruption of F-actin–containing cell structures.

Cloning of SLC1/ACT1

A synthetic lethal screen with *cap2Δ* yielded two alleles, *slc1-66* and *slc1-87* (17), of a gene that we now identify as *act1*. Strain YJC 963 carrying *slc1-87* and *cap2Δ* and a plasmid with *CAP2* was transformed with a cDNA library under control of the *GAL1* promoter (23), kindly provided by Anthony Bretscher (Cornell University, Ithaca, NY) and screened for rescue of synthetic lethality by colony color sectoring. The frequency of transformation was estimated by plating aliquots on Ura$^-$ medium. Aliquots of the transformation, each containing at least 25 transformants, were grown in 96-well microtiter plates in −Ura/glucose and then plated on −Ura/galactose. Approximately 9,000 independent transformants were screened. Each independent transformant was represented by at least four colonies. After 5 d at 30°C, red/white sectoring was examined, and colonies with white sectors, indicating loss of pBJ 414, were tested for Leu$^-$, Ura$^-$/glucose, and Ura$^+$/galactose. Plasmids were recovered and transformed back into the original strain to confirm their ability to rescue.

Based on restriction endonuclease maps, three different types of plasmids were recovered that complemented *slc1*. One plasmid type carried *CAP2*, based on Western and Southern analysis and DNA sequence. A second plasmid type carried *ACT1*, based on DNA sequence. Three nonidentical clones (pBJ 476, 477, and 481) were recovered. A third group of plasmids with insert size ~1 kb showed weak rescue on retransformation, and DNA sequencing and database searching with BLAST (2) did not identify the cDNA. These plasmids were not analyzed further.

The *ACT1* plasmids, pBJ 476 and 477, rescued the synthetic lethality of both alleles, *slc1-66* and *slc1-87*, upon transformation into strains YJC 960 and 963, respectively. In addition, a CEN plasmid carrying *ACT1* under control of its own promoter (pBJ 508) provided partial rescue of synthetic lethality for *slc1-87* and complete rescue for *slc1-66*.

Overexpression of *ACT1* is lethal in some strains, but not in the wt background used here or in certain *act1* strains (23). We suspect that overexpression was important for the success of cloning. Several different genomic libraries produced no clones that rescued, and *ACT1* with its own promoter on a CEN plasmid provided only partial rescue for one allele. Both alleles were previously observed as semidominant, with *slc1-87* more so than *slc1-66* (17). Therefore, the levels of the rescuing wt Act1p presumably needed to be in excess of those of the mutant Act1p.

Recombinational Allelism Test for slc1 and ACT1

To determine whether *slc1* was allelic to *ACT1*, a recombinational allelism test was performed on a heterozygous diploid carrying one copy of *slc1-87* and one copy of *ACT1* marked by *LEU2*. The integrating plasmids pBJ 486 and 489 contain a promoterless fragment of *ACT1* because they were constructed from the SalI/NotI inserts of cDNA clones pBJ 476 (1.4 kb) and pBJ 477 (1.8 kb), respectively. Integration of pBJ 486 or 489 at the genomic locus of *ACT1* results in two copies of *ACT1*, only one of which is expressed. pBJ 486 and 489 carry *LEU2* as a marker, which was chosen to allow detection of plasmid integration at *LEU2*, instead of at *ACT1*, by testing for linkage to MAT and the centromere. Constructs were verified by restriction digestion.

The strain YJC 542, which carries *slc1-87*, *cap2*, and a *CAP2 URA3* plasmid was transformed with the integrating plasmid that had been cut at a unique HindIII site in *ACT1*. As a control, a similar plasmid lacking *ACT1* sequences was used. Stable transformants were mated with the *cap2* strain YJC 450, and the resulting diploids were subjected to tetrad analysis. Segregants were tested for (*a*) synthetic lethality by testing growth on 5-fluoroorotic acid to lose the *CAP2 URA3* plasmid, (*b*) the *ACT1* marker *LEU2*, (*c*) mating type (MAT), and (*d*) the centromere-linked marker *TRP1*. These tests permit discrimination between integration at *ACT1* as opposed to integration at *LEU2*.

slc1 was linked to *ACT1* at a distance <0.49 cM, ~1.43 kbp. Allele numbers 66 and 87 have not been used for *act1*, to our knowledge. We now therefore refer to *slc1-66* as *act1-66* and to *slc1-87* as *act1-87*.

Recovery of Mutant Alleles with Gapped Plasmids

Replicating plasmid pBJ 508, which carries an EcoRI genomic clone of *ACT1* in pRS 314 (36), was cut with NdeI to produce a gap that included the entire *ACT1* coding region plus 460 bp upstream and 300 bp downstream. The remaining arms were 660 and 1,000 bp. The gapped plasmid was transformed into strains YJC 536 and 1080. Two independent plasmids were recovered for *act1-66* and three for *act1-87*.

The plasmids were retransformed into the mutant strain to confirm that they could not rescue the synthetic lethal phenotype of the mutant. This was the case. However, this test does not exclude *act1* loss-of-function mutations that might be incidental to *slc1*. To show that the plasmids provide *act1* function, they were transformed into an *act1Δ* strain. A plasmid carrying *ACT1* (pBJ 505 − *ACT1 URA3 CEN*), *act1-66* (pBJ 576 − *act1-66 URA3 CEN*), or *act1-87* (pBJ 603 − *act1-87 HIS3 CEN*) was transformed into the diploid KWY 201, which carries one disruption of *act1*. On tetrad analysis, Leu$^+$ (*act1::LEU2*) segregants were recovered for both *act1-66* and *act1-87*, but only in the presence of the plasmid carrying the mutant actin gene. These results indicate that the recovered *slc1/act1* alleles function in the sense of compensating for the disruption of *ACT1*, and are not simply coincidental loss-of-function mutations.

Screen of act1 Collection for Synthetic Lethality with cap2

A collection of *act1* mutants (38) was screened in two ways. First, to reveal synthetic lethality, tetrad dissection was performed on diploids heterozygous for *act1* alleles marked by adjacent *HIS3* integration and for a *cap2::URA3* gene disruption. These diploids were prepared by crossing haploid *act1* segregants derived from Wertman collection diploids to the *cap2::URA3* strains KT1277 or KT1278, depending on mating type. Synthetic lethality was revealed by the absence of *act1::HIS3 cap2::URA3* segregants. This approach also detects weaker *act1 cap2* interactions by comparing the growth of *act1::HIS3 cap2::URA3* segregants with that of single mutants. 15 four-spored tetrads were dissected onto YPD. After incubation for 4–5 d at 24°C, the spore clones were replica plated to medium lacking uracil or histidine to score the *cap2::URA3* and *act1::HIS3* genotypes, respectively, and also replica plated to YPD and incubated at 15, 24, and 37°C to score for temperature-sensitive growth. Before replica plating, we microscopically examined those spores that had not grown to macroscopic colonies to determine whether germination and cell division had occurred. Spore viability was generally very good, except in cases of synthetic lethality, and in a few cases where the *act1* allele showed reduced viability (*act1-132*, *act1-136*).

Second, because synthetic lethals detected above could be deficient in spore germination or logarithmic growth, we determined whether double mutants covered with a *CAP2* plasmid could lose the plasmid during logarithmic growth. The *act1* haploids above were crossed with strains YJC 1082 or 1084, depending on mating type, which carried a *cap2::LEU2* disruption, covered by a *CAP2 URA3* plasmid (pBJ 198). Tetrad dissection was performed, and all spore types germinated. The ability to lose the plasmid was tested by growth on 5-fluoroorotic acid, and *cap2::LEU2* and *act1::HIS3* genotypes were determined by growth on −Leu and −His, respectively. In this background, spore viability was poor, so data for different ascospores derived from the same diploid were combined, as in a random spore analysis. Note also that *slc2*, which is synthetic lethal with *cap2*, is segregating in this analysis. The analysis of *slc2* is beyond the scope of this paper and is not discussed per se; however, the synthetic lethal segregants are present and identified to allow consideration of the *act1 cap2* phenotype.

Structural Analysis

The structure of the actin monomer and filament was viewed and analyzed with InsightII on an IRIS computer, using subunit coordinates and

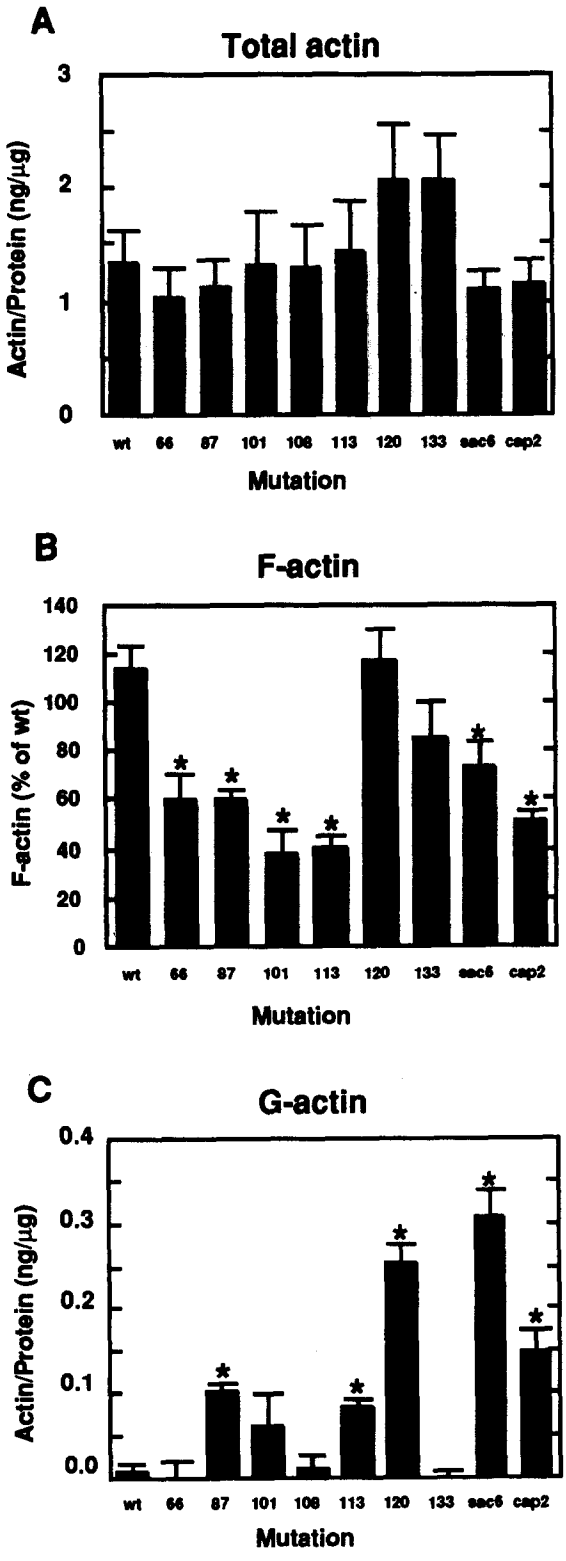

Figure 1. Assays of cellular actin in *act1*, *sac6*, and *cap2* strains. (*A*) Total actin content. The value is the mean and the error bar is the standard error. No strain is significantly different from wt. (*B*) F-actin content by rhodamine-phalloidin binding. The value is the mean of bound rhodamine fluorescence normalized to total protein for each strain, then normalized to wt. The error bar is the standard error. Asterisks mark cases where the difference between the mutant and wt is statistically significant ($P < 0.05$). (*C*) G-actin content by determination of soluble actin released from permeabilized cells. Values are actin normalized to total protein in the supernatant after permeabilization. Again, asterisks mark cases where the difference between the mutant and wt is statistically significant ($P < 0.05$).

Table II. Recombinational Allelism Test for Linkage of slc1-87 (Foa⁻) and ACT1::LEU2

	Foa⁻ and *LEU2*			*LEU2* and *TRP1*			*LEU2* and *MAT*		
ACT1 plasmids	P	N	T	P	N	T	P	N	T
pBJ 489	11	0	0	1	3	7	2	3	6
pBJ 489	15	0	0	2	2	11	1	2	11
pBJ 486	12	0	0						
pBJ 486	13	0	0	2	1	10	0	4	10
Total	51	0	0	5	6	28	3	9	27
Control plasmids	P	N	T	P	N	T	P	N	T
pBJ 487	3	2	3	4	2	2	2	1	5
pBJ 487	3	2	7	2	2	8	2	5	5
Total	6	4	10	6	4	10	4	6	10

The number of tetrads of different classes derived from independent transformants is listed. *P*, parental ditype; *N*, nonparental ditype; *T*, tetratype. The predicted ratio of tetrads for absence of linkage is 1P: 1N: 4T. χ-squared statistical analysis indicates linkage ($P < 0.05$) for *slc1-87* (Foa⁻) and *ACT1::LEU2* at <0.49 cM (~1.4 kbp), and no linkage ($P > 0.05$) in any other case. For the control plasmid that lacks *ACT1*, integration should have occurred at the *LEU2* chromosomal locus, giving linkage to both MAT and the centromere (*TRP1*). This linkage is observed in the slight deficiency of tetratypes, which is not statistically significant due to the number of tetrads examined.

helical filament parameters provided by Dr. Michael Lorenz (Max Planck Institute, Heidelberg, FRG) (24).

Results

CP and Fimbrin Promote Actin Filament Assembly In Vivo

Previously, CP and fimbrin mutations were found to be synthetic lethal (1, 17). This enhanced phenotype is specific for this combination; combinations with many other actin-binding protein mutations did not show enhancement (1), and a screen for mutations synthetic lethal with a CP null mutation did not uncover other actin-binding protein genes (17 and herein). An hypothesis to explain this observation, based on the biochemical properties of CP and fimbrin in vitro, was that both proteins are capable of stabilizing actin filaments in different ways (1). CP binds to barbed ends and prevents subunit loss; fimbrin binds to filament sides and also should prevent subunit loss from ends. Therefore, the loss of two proteins both acting to stabilize actin filaments would lead to a severe loss of actin filaments.

We tested this hypothesis by determining the level of actin filament assembly in yeast strains lacking CP (*cap2* mutants) or fimbrin (*sac6* mutants). We used two complementary assays that have been used extensively in other cell systems. We performed both assays because each has certain advantages and disadvantages. First, F-actin was measured by a rhodamine-phalloidin–binding assay. The advantage of this assay is that the cells are chemically fixed at the outset; therefore, actin structures are preserved during the assay. The disadvantage is the assumption that all actin filaments bind rhodamine-phalloidin equally well.

Table III. Interactions between act1::HIS3 Alleles and cap2::URA3 by Tetrad Analysis of Heterozygous Diploids

act1 allele		Number of viable segregants: Ura⁻ His⁻	Ura⁺ His⁻	Ura⁻ His⁺	Ura⁺ His⁺	Phenotype of Ura⁺His⁺	No. of tetrads (PD/ TT/ NPD)		
wt	Obs	14	10	14	13	wt	2	10	2
	Exp	14	14	14	14				
101	Obs	13	15	15	0	Microscopic colonies*	4	7	3
	Exp	13	15	15	13				
102	Obs	17	11	11	17	wt	1	9	4
	Exp	17	11	11	17				
104	Obs	13	17	17	13	Slight ts	3	11	1
	Exp	13	17	17	13				
105	Obs	16	12	6	13	Small colonies	1	10	3
	Exp	16	12	12	16				
108	Obs	14	14	12	0	Inviable	2	10	2
	Exp	14	14	14	14				
111	Obs‡	15	13	2	0	Inviable§	2	9	3
	Exp	15	13	13	15				
113	Obs	14	14	13	0	Inviable	2	10	2
	Exp	14	14	14	14				
115	Obs	7	21	21	9	ts	7	7	1
	Exp	9	21	21	9				
116	Obs	20	8	10	19	ts	2	6	7
	Exp	20	10	10	20				
117	Obs	16	13	14	16	Slightly ts	3	8	4
	Exp	16	14	14	16				
119	Obs	16	12	14	0	Inviable	3	8	4
	Exp	16	14	14	16				
120	Obs	13	15	15	0	Microscopic colonies*	1	13	0
	Exp	13	15	15	13				
121	Obs	15	13	12	15	cs and ts	1	11	2
	Exp	15	13	13	15				
122	Obs	13	17	17	13	ts	3	4	1
	Exp	13	17	17	13				
123	Obs	13	13	13	14	wt	2	10	2
	Exp	14	14	14	14				
124	Obs	16	14	13	0	Small colonies*	3	8	4
	Exp	16	14	14	16				
125	Obs	16	12	11	0	Inviable	0	12	2
	Exp	16	12	12	16				
129	Obs	14	15	16	7	Small colonies	5	6	4
	Exp	14	16	16	14				
132	Obs	17	13	8	0	Inviable§	0	13	2
	Exp	17	13	13	17				
133	Obs	16	14	13	0	Inviable	3	8	4
	Exp	16	14	14	16				
135	Obs	14	16	16	14	Slight ts	2	12	1
	Exp	14	16	16	14				
136	Obs	11	19	10	0	Inviable§	4	11	0
	Exp	11	19	19	11				

The total number of observed (*Obs*) and expected (*Exp*) viable haploid segregants of each genotype is listed, along with the phenotype of the *act1 cap2* segregants, and the number of different types of tetrads.

§These *act1* alleles had low viability in this experiment, reflected in the number of Ura⁻His⁺ segregants. Also, these alleles were not synthetic lethal in a test of logarithmic growth (Table IV).

‡10 small colonies could not be analyzed for markers.

*The genotype of the Ura⁺ His⁺ segregants was inferred from those of macroscopic colonies that could be tested for markers. The phenotype listed describes how those spores were able to grow.

Actin-binding proteins or actin mutations may influence the efficiency of phalloidin binding. Second, G-actin was measured as the amount of actin that was released in a soluble form from permeabilized cells. The advantage here is that the quantitation of the actin by immunoblot is unambiguous, and the disadvantage is that the permeabilization procedure may cause the solubilization of some F-actin. We also measured total cell actin by immunoblot, to determine whether changes in the amount of F- and G-actin were reflective of the total actin pool or independent effects.

Different results with the two different assays were sometimes found and are to be expected. Actin may exist in a form detected in both or neither assays. For example, some actin filaments may depolymerize rapidly and become soluble on permeabilization, so this pool would be measured in both assays. An increase in this pool of actin would lead to increased G-actin and unchanged F-actin in

Table IV. Plasmid Loss Analysis of Interactions between act1 Alleles and cap2 during Logarithmic Growth

Allele of *act1*	Foa$^+$ Leu$^+$	Foa$^+$ Leu$^-$	Foa$^-$ Leu$^+$	Foa$^-$ Leu$^-$*	Ratio Foa$^+$Leu$^+$/total
wt	9	16	8	2	0.26
4	14	6	5	2	0.52
101	0	11	14	1	0.00
102	10	29	6	0	0.22
104	14	29	11	0	0.26
105	14	22	11	0	0.30
108	0	9	10	0	0.00
111	2	6	4	0	0.17
113	0	5	5	0	0.00
115	11	15	10	0	0.31
116	10	12	4	0	0.38
117	9	14	4	0	0.33
119	0	8	9	0	0.00
120	0	7	6	0	0.00
121	1	2	0	2	‡
122	2	3	3	0	‡
123	13	15	6	1	0.37
124	3	10	8	0	0.14
125	0	6	4	0	0.00
129	3	8	10	0	0.14
132	9	9	1	0	0.47
133	0	8	2	0	0.00
135	11	13	7	2	0.33
136	16	15	4	3	0.42
Predicted results for four segregants, considering segregation at the *CAP2* and *SLC2* loci					
Synthetic lethality					
none	1	2	1	0	0.25
act1 cap2	0	2	2	0	0
act1 slc2	1	1	0	0	0.5
act1 slc2 and *act1 cap2*	0	1	1	0	0.0

The genotype of the diploids used in this analysis was *ACT1/act1::HIS3 cap2-Δ1::LEU2/CAP2 slc2/SLC2* with a plasmid [*CAP2 URA3*]. Since only *act1* segregants are relevant for this analysis, only His$^+$ segregants are included in the table. Note that *slc2*, which is synthetic lethal with *cap2*, is segregating in this analysis and does show allele-specific interactions with *act1*. The analysis of *slc2* is beyond the scope of this paper and is not discussed per se, however, the synthetic lethal segregants are present and identified here by necessity. The key parameter to examine to determine whether an *act1* allele is synthetic lethal with *cap2* is the number of Leu$^+$ (*cap2*) Foa$^+$ (plasmid loss viable) segregants. In the case of synthetic lethality, the number should be zero. In the absence of synthetic lethality, the number of Leu$^+$ Foa$^+$ segregants should be either one-half or one-quarter of the total number of segregants, depending on whether the *act1* allele is or is not synthetic lethal with *slc2*, respectively.

**CAP2* strains should not be Foa$^-$, therefore, these segregants must have poor growth on FOA for some other reason and presumably represent strains with marginal growth properties overall.

‡These strains had low viability in this experiment, so no ratio is calculated and no conclusion drawn.

Table V. Summary of Interactions between cap2 and act1

act1 allele	Amino acid substitution	Phenotypes of double mutants: *act1 cap2*	*act1 sac6**
wt		NE	NE
3	P32L	ND	NE
4	E259V	NE	NE
66	M16I	SynLet	NE
87	E292K	SynLet	NE
101	D363A, E264A	SynLet	Mod
102	K359A, E361A	NE	Mod
104	K415A, E316A	Mod	NE
105	E311A, R312A	Mod	NE
108	R256A, E259A	SynLet	SynLet
111	D222A, E224A, E226A	Mod‡	ND
113	R210A, D211A	SynLet	SynLet
115	E195A, R196A	Mod	SynLet
116	D187A, K191A	Mod	Mod
117	R183A, D184A	Mod	Mod
119	R116A, E117A, K118A	SynLet	SynLet
120	E99A, E100A	SynLet	NE
121	E83A, K84A	Mod	SynLet
122	D80A, D81A	Mod	ND
123	R68A, E72A	NE	NE
124	D56A, E57A	Mod	SynLet
125	K50A, D51A	SynLet	SynLet
129	R177A, D179A	Mod	SynLet
132	R37A, R39A	Mod	ND
133	D24A, D25A	SynLet	Mod
135	E4A	NE	NE
136	D2A	Mod	SynLet

The results in Tables III and IV are compiled and summarized here.

NE, no effect; *Mod*, modified phenotype (slow or temperature-sensitive growth); *SynLet*, synthetic lethal; *ND*, not determined.

*The data in this column are reproduced from the work of Holtzman and colleagues (13), except for alleles 66 and 87, which were described by us previously (17).

‡Low viability reduces the significance of this interpretation.

the assays. In support of this possibility, permeabilization leads to loss of actin cables (21). On the other hand, denatured and precipitated actin would neither bind rhodamine-phalloidin nor be soluble, which would lead to decreased F-actin and unchanged G-actin in the assays. Many actin and actin-binding protein mutations have this effect on actin.

Both CP and fimbrin mutants showed less F-actin, more G-actin, and no change in total actin compared with wt (Fig. 1). Therefore, the results with the two different assays are consistent and support the hypothesis that both actin-binding proteins promote actin filament assembly. For CP, this result is especially interesting because it is the opposite of the one found in *Dictyostelium*, where less CP led to more F-actin (15). This difference is possible because CP binding to barbed ends stops both the addition and loss of actin subunits. Therefore, in yeast, CP prevents the loss of subunits that otherwise occurs from free barbed ends, while in *Dictyostelium* CP prevents the addition of subunits.

Pool of Actin Monomers in Yeast

This difference between *Dictyostelium* and yeast is most likely due to a difference in the level of actin monomers available for addition to free barbed ends. We hypothesize that the level is low in yeast and high in *Dictyostelium*. The total actin content of *Dictyostelium* is ~100 times that of yeast, ~10% vs ~0.1% of total cell protein (12, 15, 27, confirmed by Fig. 1 *A* here). About 50% of actin in *Dictyostelium* is soluble, and therefore exists as either free or buffered monomers (15). In contrast, we report here that very low levels of actin are soluble in yeast.

The soluble actin released from permeabilized yeast cells is normalized in Fig. 1 *C* to the level of protein released, to account for possible differences in the percentage of cells permeabilized in different strains. However, if one normalizes to total protein in a whole-cell extract, soluble actin is 2 pg/μg of protein in wt cells. The fraction of cells permeabilized in the assay is ≥60% (21). Even assuming that only 50% of cells are permeabilized, then at most 4 pg/μg is soluble, and the soluble fraction of actin is

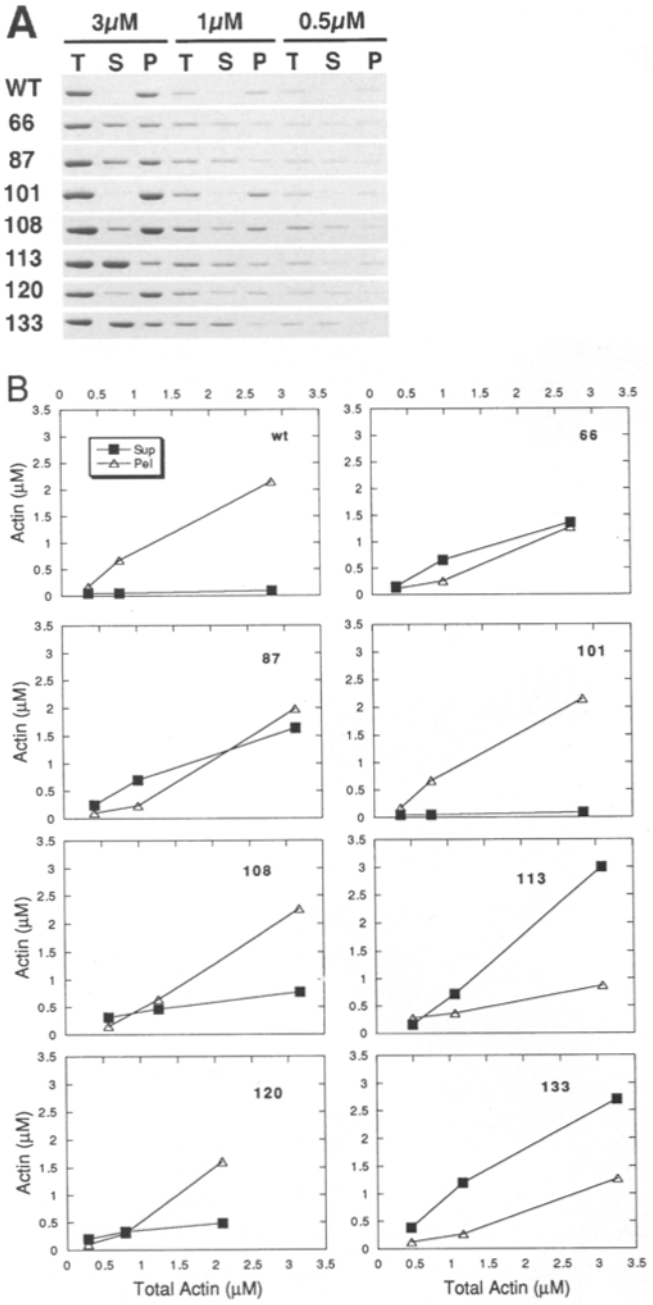

Figure 2. Assay of actin polymerization by sedimentation. (*A*) The actin band of SDS gels stained with Coomassie blue is shown for the total sample (*T*) before sedimentation and the supernatant (*S*) and pellet (*P*) after sedimentation. The experiment was performed at three different total actin concentrations, listed across the top. The *act1* allele numbers are indicated at left. (*B*) Graphs of the concentration of actin (μM) in the supernatant (*filled squares*) and pellet (*open triangles*) fractions vs. total actin (μM). The intensities of the bands in *A* were quantified to produce these data. The *act1* allele numbers are indicated at the upper right of each panel.

at most 4 pg/1.3 ng = 0.3%. This value is 160 times less than the comparable value of 50% in *Dictyostelium.*

Actin Mutations Enhanced by the Loss of CP

As an alternative approach to investigate the in vivo role of CP on actin filament assembly, we tested whether mutations in actin that are synthetic lethal with the loss of CP, as is the fimbrin mutation, would also lead to actin that did not polymerize well in vivo. Our hypothesis predicts that actin mutants whose phenotype is aggravated by the loss of CP should also have less F-actin in vivo. We tested actin mutants from two different sources. First, a screen for mutations synthetic lethal with the loss of CP (*cap2*) identified two alleles of a gene *slc1* (17), which we here identify as novel alleles of *act1*, the gene for actin. Second, we screened existing *act1* mutations, including the alanine-scanning collection of Wertman et al. (38).

slc1 Is act1

Plasmids carrying *ACT1* cDNA's were recovered by library transformation to complement the *cap2 slc1* synthetic lethal phenotype. A recombinational allelism test showed tight linkage between *slc1* and *ACT1* (Table II).

Sequence of act1-66 and act1-87

To confirm the assignment of *slc1* as *act1* and to determine the amino acid sequence of the mutant actins, we sequenced PCR-amplified genomic DNA and gap-repair plasmids carrying the recovered alleles. *act1-66* has a single base change (ATG to ATA in codon 16) that converts Met 16 to Ile. *act1-87* has a single base change (GAA to AAA in codon 292) that converts Glu 292 to Lys. As predicted from these amino acid changes, the electrophoretic mobility of Act1-87p shifted to a more basic position and that of Act1-66p did not change, by two-dimensional gel electrophoresis and immunoblots of whole-cell extracts with antiactin (data not shown).

Existing act1 Mutants

We also searched for synthetic lethality among the existing collection of viable *act1* alleles. Synthetic lethality was tested in two ways. First, tetrad analysis was performed on diploids heterozygous for *act1* alleles and a *cap2::URA3* gene disruption (Table III). An *act1* allele categorized as synthetic lethal in this test may be defective in spore germination or outgrowth, but not be inviable during logarithmic growth. To test this possibility and to independently confirm the results from the tetrad analysis, we performed a plasmid loss assay in growing cells (Table IV). These results largely confirm those of the first approach. Some alleles are inviable in the first approach and viable in the second, as expected. A compilation and summary of all the results is presented in Table V.

Alleles 66, 87, 101, 108, 113, 119, 120, 125, and 133 were inviable in combination with *cap2.* Spores failed to grow into macroscopic colonies, and microscopic examination revealed that most divided only a few times, but 101 and 120 gave rise to a few hundred cells. Alleles 104, 105, 111, 115, 116, 117, 121, 122, 124, 129, 132, 135, and 136, showed some enhancement of their phenotype in the presence of the *cap2* mutation. They were more temperature or cold sensitive, grew more slowly at the permissive temperature, or were defective in germination, representing a continuum of effects. Alleles 4, 102, and 123 exhibited no synthetic phenotype.

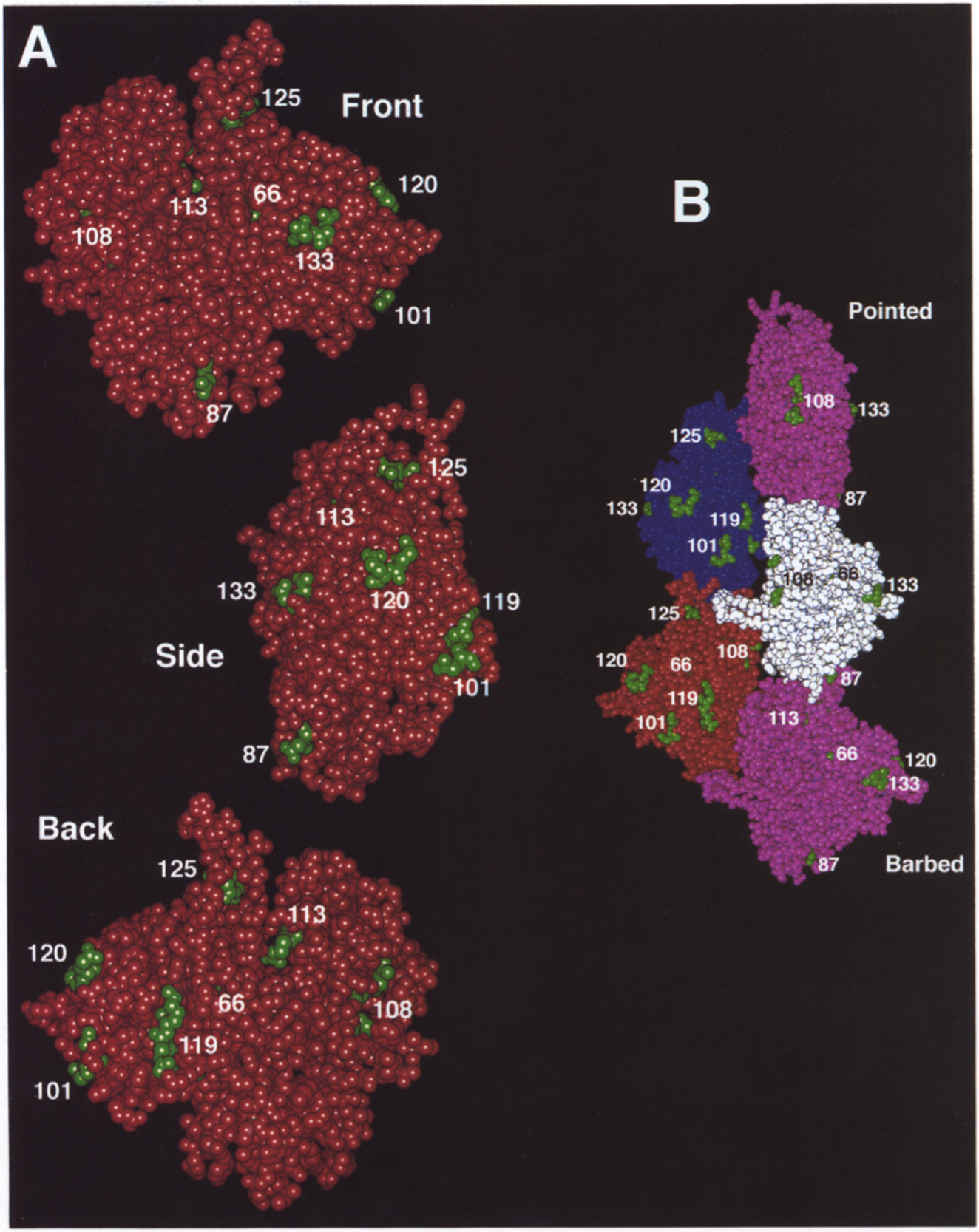

Figure 3. Structural model of actin, indicating location of mutations. (*A*) Three views of an actin monomer, in red, with the synthetic lethal allele residues indicated in green. The view labeled Front is similar to the one conventionally shown, with the exception that the subunit is rotated slightly counterclockwise about an axis perpendicular to the page, placing it in an orientation similar to that seen in the filament. (*B*) A filament of five subunits, with the subunits having different colors—purple, red, white, blue, and purple again—as one proceeds from barbed to pointed end. Again, the synthetic lethal residues are indicated in green.

Defective Actin Assembly In Vivo in Actin Mutants

Our hypothesis then predicts that the set of synthetic lethal actin mutants will include ones that have impaired actin filament assembly in vivo, as seen for the CP and fimbrin null mutants. We assayed the mutants for actin filament assembly in vivo by the two complementary methods discussed above, independent measurements reflecting F-actin and G-actin. Total actin was not changed significantly for any *act1* mutant, as also seen for the CP and fimbrin mutants.

For nearly all the *act1* mutants, in at least one of the assays, we detected a defect similar to that seen in CP and fimbrin mutants: less F-actin or more G-actin. For alleles 87 and 113 changes in the predicted direction were seen in both assays. The predicted effect was seen in one but not the other assay for alleles 66, 101, and 120. As discussed above, different results in the two assays are not unexpected. In the F-actin assay, allele 108 was not tested because of poor growth, and 133 showed a change in the predicted direction, but without statistical significance. Both 108 and 133 were normal in the G-actin assay.

Biochemical and Structural Analysis of Actin Mutants

Our hypothesis predicts two obvious molecular mechanisms to explain defective actin assembly in vivo in these actin mutants. The mutant actin could polymerize poorly or it might bind fimbrin poorly. To test further our hypothesis and discriminate between these possibilities, we purified actin from the mutants and assayed polymerization and fimbrin binding in vitro. We hypothesized that these tests might also reveal defects in actin filament function not seen in the in vivo assays. In addition, using the structural model of the actin filament, we were able to make predictions and correlate results in the biochemical assays with the locations of the mutations.

First, we tested the ability of the mutant actins to polymerize (Fig. 2). Most alleles did show defective polymerization, manifested by less actin in the pellet and more in the supernatant in a sedimentation assay. Alleles 66, 87, 113, and 133 showed substantial defects. Alleles 108 and 120 showed mild defects, and 101 was similar to wt. Structural analysis provided interesting correlations (Fig. 3). First, amino acid residue 292, altered in allele 87, which shows a severe polymerization defect in vitro, is a predicted contact between actin subunits along the long axis of the filament. 292 lies within 4 Å of residues 45 and 46 of an adjacent subunit. Second, in allele 108, two amino acid residues are altered (256 and 259), and they lie just above the loop connecting the protofilament strands. Although residues 256 and 259 are not within 6 Å of any other subunit, they may be important for the structure of that loop. Third, the amino acid residues altered in alleles 66 and 113 occupy positions at or near the cleft where nucleotide binds, and nucleotide binding has been shown to be important for subunit and filament stability (7, 8).

Second, we tested the ability of the mutant actins to bind fimbrin. The binding site for fimbrin on actin has been characterized, and allele 120 shows the best agreement to the criteria (13, 14, 26). As expected, allele 120 also showed decreased fimbrin binding here (Fig. 4). Other alleles that change residues at and near the fimbrin-binding

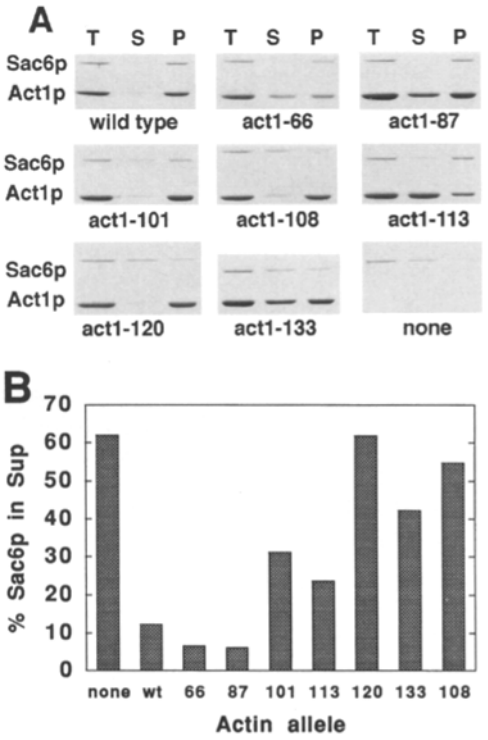

Figure 4. Binding of fimbrin to actin. (*A*) The fimbrin (*Sac6p*) and actin bands of SDS gels stained with Coomassie blue are shown for the total sample (*T*) before sedimentation and the supernatant (*S*) and pellet (*P*) after sedimentation. The *act1* allele numbers are given below each panel. The panel in the bottom right is a control without actin. (*B*) Binding of fimbrin (*Sac6p*) to mutant actins. The intensities of the fimbrin bands in *A* were quantified. The amount of fimbrin (*Sac6p*) in the supernatant is plotted as a percentage of the total fimbrin. In a control experiment without actin, some fimbrin does sediment, despite prior clarification.

site are 125, 101, 119, and possibly 133 (Fig. 4). In our assays of fimbrin binding, allele 108 was severely impaired, and alleles 101, 113, and 133 showed moderate defects in fimbrin binding (Fig. 4). The explanation for decreased fimbrin binding by alleles 108 and 113, located outside of the previously defined fimbrin-binding site, might be either long-range allosteric effects, seen with other actin mutations (10, 29, 37), or interaction of fimbrin with additional sites not revealed in the previous analysis.

Therefore, among the collection of actin mutants are some that show poor polymerization, some that show poor fimbrin binding, and some that show both. No alleles are normal for both properties. As examples of the first case, consider alleles 66 and 87. They show poor polymerization (Fig. 2 *B*), but they bind fimbrin well (Fig. 4 *B*). This makes sense with their localization in the structure (Fig. 3) because they are not at the fimbrin-binding site, rather they are near the nucleotide-binding site (66) and a subunit–subunit contact site (87). For the second case, allele 120 polymerizes well (Fig. 2 *B*) but binds fimbrin poorly (Fig. 4 *B*). Again, the structural location for this mutant make sense. Others have documented well that this region is part of the fimbrin-binding site (13, 14, 26), and this site would not be expected to affect polymerization, which is

the result here. For the third case, allele 133 is poor at both polymerization (Fig. 2 *B*) and fimbrin binding (Fig. 4 *B*). In this case, one might not have predicted these effects based on the structural location. The affected region is close to but not part of the recognized sites for fimbrin binding or subunit contacts. This discrepancy may reflect a limitation in the structural analysis or allosteric effects.

Another possible molecular model to explain synthetic lethality is that another actin-binding protein is essential in the absence of CP. Mutations in the binding site for such a protein might be identified as ones with normal actin assembly and fimbrin binding in vitro and in vivo. The set of *act1* mutations does not include exactly such a case, however, we did observe a broad range of effects in the assays and were not always able to include all strains in every assay. One potential candidate is *slc2*, recovered in a synthetic lethal screen with *cap2* (17). *slc2* is the mannoprotein synthesis gene *MNN10* (Dean, N., and J. B. Poster, manuscript submitted for publication, and 5) which encodes a protein with sequence similarity to galactosyl transferase (Karpova, T. S., and J. A. Cooper, unpublished results) (These sequence data are available from GenBank under accession number L42540).

Discussion

Mechanisms for Actin Assembly

The most important conclusions from this study are that, in yeast, actin filaments are unstable without either of two major actin-binding proteins. The loss of either CP or fimbrin leads to decreased F-actin and increased G-actin. This hypothesis was proposed based on several observations. First, the loss of both CP and fimbrin is lethal. Second, the loss of actin is lethal. Third, the filament is presumed to be the functional state of actin. Fourth, the biochemical activities of CP and fimbrin in vitro have in common the ability to stabilize actin filaments, i.e., to prevent depolymerization. However, the biochemical activities of CP also include preventing filament assembly by inhibiting growth at barbed ends. Therefore, it was uncertain which biochemical activity of CP would be relevant in vivo in yeast.

For CP, this conclusion was supported by additional experiments in which mutations in actin that are also synthetic lethal with the loss of CP were found to have unstable actin in vivo. As expected, some of these actin mutants bound fimbrin poorly, in biochemical experiments with purified proteins. This would be the equivalent of the loss of fimbrin. In addition, biochemical studies showed that some of these mutant actins polymerized poorly on their own. Therefore, the actin mutants are either unstable in their own polymerization or in binding fimbrin, which contributes to stability. Coupled with the loss of filament stability that accompanies the loss of CP, these mutations are now lethal because filament stability is severely compromised.

Contrasting Yeast and Dictyostelium

These conclusions in yeast present a striking contrast to the case in *Dictyostelium*, where the opposite conclusions were reached (15). In *Dictyostelium*, the loss of CP leads to increased F-actin. There, actin is poised to assemble, and CP, by binding to filament barbed ends, prevents growth.

CP can act in these opposing fashions in the two systems because of its biochemical properties. When bound to the barbed end, it prevents both the addition and loss of actin subunits, preventing the filament from growing or shrinking, respectively. Which activity is relevant in a given case depends on the concentration of free monomers present in solution and available for polymerization. If the actin monomer concentration is high, then free barbed ends tend to polymerize and CP prevents filament growth. On the other hand, if the actin monomer concentration is low, then free barbed ends tend to depolymerize and CP prevents filament shrinkage. In agreement with this prediction, the actin monomer concentration in *Dictyostelium* is high, and we report here that in yeast it is low.

The biology of actin in *Dictyostelium* vs. yeast supports these conclusions. *Dictyostelium* is highly motile, using a high concentration of actin filaments as a basis for motility. These filaments are probably highly dynamic and require local bursts of actin polymerization and depolymerization for coordinated assembly. Therefore, the requisite high concentrations of monomeric actin needed for bursts of assemble must be held in check by actin-binding proteins. Yeast, on the other hand, are not motile and show no analogous need for dynamic actin filament assembly. While yeast show dramatic rearrangements of the actin cytoskeleton during the cell cycle and in response to certain external stimuli, there is no evidence for substantial increase or decrease in the total cellular content of actin filaments during any of these changes. While actin filaments are essential for viability of yeast, they are present at comparatively low concentrations and therefore tend not to assembly spontaneously. In this system, the actin-binding proteins may not be needed to hold actin polymerization in check, but rather to induce and stabilize actin filament formation at the proper location and time.

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Note Added in Proof. *SLC2* and *MNN10* are also identical to *BED1*, described in Mondesert and Reed (Mondesert and Reed. 1996. *J. Cell Biol.* In press.).

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