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) 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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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 (YPED), 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 act*/* alleles were cyclic sequenced (9) from both PCR-amplified genomic DNA and gap-repair plasmids using [γ-32P] 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 act* 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 MgCl2, 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 antiactin actin (17) or the mouse antiactin mAb C4 (20) as the first Ab and 125I-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

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**Table 1. Yeast Strains Used in This Study**

| Name    | Relevant genotype | Source                  |
|---------|-------------------|-------------------------|
| KWy201  | MATa act1::LEU2/ACT1 ura3/1 leu2/2 his3/1 | K. Wertman, University of California, Berkeley |
| KT1277  | MATa cap2-1::URA3 ura3 leu2 his3 | This study |
| KT1278  | MATa cap2-1::URA3 ura3 leu2 his3 | This study |
| YJC450  | MATa cap2::HIS3 ade2 ade3 lys2 ura3 leu2 | This study |
| YJC472  | MATa cap2::HIS3 ade2 ade3 lys2 ura3 leu2 trp1 his3 | [pBJ 198 - CAP2 ADE3 URA3] |
| YJC536  | MATa slc1-66 cap2::HIS3 ade2 ade3 ura3 leu2 trp1 | [pBJ 198 - CAP2 ADE3 URA3] |
| YJC542  | MATa slc1-87 cap2::HIS3 ade2 ade3 ura3 leu2 trp1 | [pBJ 198 - CAP2 ADE3 URA3] |
| YJC960  | MATa slc1-66 slc1-66 cap2::HIS3 cap2::HIS3 ade2 ade3 ade3 ura3 ura3 leu2/2 trp1/2/2 trp1/2/2 lys2/2/2 his3/3/3 [pBJ 416 - ADE3 LEU2 CAP2] | This study |
| YJC963  | MATa slc1-87 cap2::HIS3 ade2 ade3 ura3 leu2 trp1/2/2 his3/3 [pBJ 414 - ADE3 LEU2 URA3] | This study |
| YJC1080 | MATa slc1-87 cap2::LEU2 ade2 ade3 ura3 leu2 trp1/2/2 [pBJ 198 - CAP2 ADE3 URA3] | This study |
| YJC1096 | MATa act1-101::HIS3 ura3 leu2 trp2 | This study |
| YJC1104 | MATa act1-108::HIS3 ura3 leu2 trp2 | This study |
| YJC1107 | MATa act1-113::HIS3 ura3 leu2 trp2 | This study |
| YJC1117 | MATa act1-120::HIS3 ura3 leu2 trp2 | This study |
| YJC1133 | MATa act1-133::HIS3 ura3 leu2 trp2 | 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 cap2A yielded two alleles, slcl-66 and slcl-87 (17), of a gene that we now identify as act1. Strain YJC 963 carrying slcl-87 and cap2A 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. 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 plBl41, were tested for Leu+, Ura+galactose. Approximately 9,000 independent transformants were recovered that complemented both alleles, slcl and actl.

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 strain 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 (act1-12, 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 strain 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-fluoro-orotic 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 slcl, which is synthetic lethal with cap2, is segregating in this analysis. The analysis of slcl2 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.

Recombinational Allelism Test for slcl and ACT1

To determine whether slcl was allelic to ACT1, a recombinational allelism test was performed on a heterozygous diploid carrying one copy of slcl-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 Sall/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, 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 tetrad dissection for large MAT and the centromere. Constructs were verified by restriction digestion.

The strain YJC 542, which carries slcl-87, cap2, and a CAP2 URA3 plasmid was transformed with the integrating plasmid that had been cut at both alleles, slcl and actl.
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. 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).

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

| act1 allele | Ura− Hist | Ura+ Hist | Uraby Hist | Ura+ Hist | Phenotype of Uraby Hist | No. of tetrads (PD/TT/NPD) |
|-------------|-----------|-----------|------------|-----------|-----------------------|--------------------------|
| wt          | Obs 14    | 10        | 14         | 13        |                       | 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        |                       | 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        |                       | 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        | 16         | 16        |                       |                          |
| 119         | Obs 16    | 12        | 14         | 14        | Inviable              | 3/8/4                    |
|             | Exp 16    | 14        | 16         | 16        |                       |                          |
| 120         | Obs 13    | 15        | 15         | 13        | 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        | 14         | 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         | 14        |                       |                          |
| 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).

*0 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
Alleles and cap2 during Logarithmic Growth of act1

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

| Allele of act1 | Foa+ | Foa- | Ratio |
|----------------|------|------|-------|
|                | Leu+ | Leu- | Leu+ | Leu- | Leu+ / total |
| wt             | 9    | 16   | 1    | 0.62 |
| 4              | 14   | 6    | 2    | 0.52 |
| 101            | 0    | 11   | 1    | 0.91 |
| 102            | 10   | 29   | 6    | 0.22 |
| 104            | 14   | 29   | 11   | 0.26 |
| 105            | 14   | 22   | 11   | 0.30 |
| 108            | 0    | 9    | 10   | 0.00 |
| 111            | 2    | 6    | 4    | 0.17 |
| 113            | 0    | 5    | 5    | 0.00 |
| 115            | 11   | 15   | 10   | 0.31 |
| 116            | 10   | 12   | 4    | 0.38 |
| 117            | 9    | 14   | 4    | 0.33 |
| 119            | 0    | 8    | 9    | 0.00 |
| 120            | 0    | 7    | 6    | 0.00 |
| 121            | 1    | 2    | 0    | 0.50 |
| 122            | 2    | 3    | 1    | 1.00 |
| 123            | 13   | 15   | 6    | 0.37 |
| 124            | 3    | 10   | 8    | 0.14 |
| 125            | 0    | 6    | 0    | 0.00 |
| 129            | 3    | 8    | 10   | 0.14 |
| 132            | 9    | 9    | 1    | 0.47 |
| 133            | 0    | 8    | 2    | 0.00 |
| 135            | 11   | 13   | 7    | 0.33 |
| 136            | 16   | 15   | 4    | 0.12 |

*Predicted results for four segregants, considering segregation at the CAP2 and SLC2 loci.

**Synthetic lethality**

none 1 2 1 0 0.25
act1 cap2 1 2 2 0 0 0
act1 slc2 1 1 1 0 0.5
act1 slc2 and act1 cap2 0 1 1 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 shows 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 allele is synthetic lethal with cap2 is the number of Leu+ segregants. In the case of synthetic lethality, the number should be zero. In the absence of synthetic lethality, the number of Leu+ segregants should be either one-half or one-quarter of the total number of segregants, depending on whether the allele is or is not synthetic lethal with slc2, respectively.

**Ne**

The soluble actin released from permeabilized yeast cells is soluble, and therefore exists as either free or bound monomers (15). In contrast, we report here that the soluble actin released from permeabilized yeast cells is soluble, and therefore exists as either free or bound monomers (15). In contrast, we report here that very low levels of actin are soluble in yeast.

**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 bound 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 22 μg/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 μg/μg is soluble, and the soluble fraction of actin is

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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 actl mutant, as also seen for the CP and fimbrin mutants.

For nearly all the actl 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 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 actl 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 can be common 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 assembly 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 assemble 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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