Mapping Actin Surfaces Required for Functional Interactions In Vivo

Douglas A. Holtzman, Kenneth F. Wertman, and David G. Drubin

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

Abstract. An in vivo strategy to identify amino acids of actin required for functional interactions with actin-binding proteins was developed. This approach is based on the assumption that an actin mutation that specifically impairs the interaction with an actin-binding protein will cause a phenotype similar to a null mutation in the gene that encodes the actin-binding protein. 21 actin mutations were analyzed in budding yeast, and specific regions of actin subdomain 1 were implicated in the interaction with fimbrin, an actin filament-bundling protein. Mutations in this actin subdomain were shown to be, like a null allele of the yeast fimbrin gene (*SAC6*), lethal in combination with null mutations in the *ABP1* and *SLA2* genes, and viable in combination with a null mutation in the *SLA1* gene. Biochemical experiments with *act1-120* actin (E99A,E100A) verified a defect in the fimbrin-actin interaction.

Genetic interactions between mutant alleles of the yeast actin gene and null alleles of the *SAC6*, *ABP1*, *SLA1*, and *SLA2* genes also demonstrated that the effects of the 21 actin mutations are diverse and allowed four out of seven pseudo-wild-type actin alleles to be distinguished from the wild-type gene for the first time, providing evidence for functional redundancy between different surfaces of actin.

THE assembly and function of actin are influenced by numerous protein–protein interactions (Stossel et al., 1985; Pollard and Cooper, 1986; Welch et al., 1994). The development of molecular models for monomeric (Kabsch et al., 1990) and filamentous (Holmes et al., 1990) actin has created unprecedented opportunities to determine, at the atomic level, how these protein–protein interactions mediate such varied processes as cytokinesis and cell locomotion. However, while structural studies can reveal the spatial relationships of atoms within proteins, complementary approaches are required to determine how protein topology accounts for biological function.

Chemical cross-linking studies have been used to identify regions of actin that are part of or are proximal to the binding sites of specific actin-binding proteins. For example, profilin can be crosslinked to a glutamic acid (residue 364) of actin (Vandekerckhove et al., 1989). In addition, cross-linking studies have provided evidence for interactions between the acidic NH_2-terminus of actin (residues 1–12) and several actin-binding proteins, including depactin (Sutoh and Mabuchi, 1986), fragmin (Sutoh and Hatano, 1986), and the heavy chain of myosin (Sutoh, 1982). Depactin and the myosin light chain can also be cross-linked to residues in the COOH terminus of actin (Sutoh, 1982; Sutoh and Mabuchi, 1986). These results are consistent with the fact that the NH_2 and COOH termini of actin are in close proximity to each other in the actin monomer (Kabsch et al., 1990).

An alternative approach to the identification of the contacts between actin and actin-binding proteins is to perform structural studies on complexes containing actin and associated proteins. For example, the location of tropomyosin (Milligan et al., 1990) and scruin (Owen and DeRosier, 1993) on the surface of the actin filament have been modeled through cryoelectron microscopy of tropomyosin- or scruin-coated actin filaments followed by image reconstruction. In other examples, the atomic structure of the gelsolin segment 1:actin complex has recently been solved (McLaughlin et al., 1993), and determination of the atomic structure of the myosin head and development of a model for myosin complexed to actin filaments has identified amino acids of actin that are likely to be important for the acto-myosin cross-bridge cycle (Rayment et al., 1993; Schröder et al., 1993).

While these various forms of structural analysis provide opportunities for developing deep mechanistic understandings of the interactions between actin and various actin-binding proteins, each approach also has inherent limitations. For example, studies using electron microscopy and image reconstruction do not achieve atomic resolution. Furthermore, chemical cross-linking studies that attempt to define the residues important for actin–actin-binding protein interactions are limited by the chemical reactivity of amino acids. For example, several amino acid residues in the alpha-helix at positions 341–349 of the actin monomer are part of a major contact site for myosin (Rayment et al., 1993; Schröder et al., 1993), but because of their hydrophobic

Address all correspondence to David G. Drubin, 455 Life Sciences Addition, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720. The present address for Kenneth F. Wertman is Selecticide Corporation, Department of Molecular Pharmacology, 1580 East Hanley Boulevard, Tucson, AZ 85737. The present address for Douglas A. Holtzman is ICOS Corporation, 22021 20th Avenue S.E., Bothell, WA 98021.

character, they are refractory to reaction with cross-linking molecules. In such a situation, the chemical cross-linker might either fail to react with a residue on myosin, or might even react with a remote yet highly reactive amino acid of myosin. Moreover, while data derived from these various structural approaches can show that a particular residue of actin lies near a particular residue of an actin-binding protein, and can suggest molecular mechanisms, such data do not test whether a residue of actin is directly involved in the actin–actin-binding protein interaction. For these various reasons, the development of approaches that can identify actin residues that are functionally important for interactions with actin-binding proteins in vivo is imperative.

Site-directed mutagenesis can be used to determine the importance of specific actin residues for interactions with actin-binding proteins (reviewed by Wertman and Drubin, 1992; Hennessey et al., 1993). This methodology is particularly powerful in *Saccharomyces cerevisiae* because the high frequency of homologous recombination allows precise replacement of the wild-type actin gene by a single copy of any mutant gene engineered in vitro. This allows the effects of the mutation to be examined in vivo. Since *ACT1* encodes the sole source of conventional actin in budding yeast, it is possible to isolate biochemical quantities of a pure mutant actin, provided that the mutant actin can supply a sufficient level of actin function in vivo. Furthermore, these mutant actins can be used in in vitro assays to determine the importance of specific residues for filament assembly (Chen et al., 1993), actin-myosin interactions (Kron et al., 1992; Cook et al., 1993), and interactions with various other actin-binding proteins.

A systematic "charged-to-alanine" mutational analysis of *ACT1* designed to bias mutations to the surface of the monomer generated a large collection of mutations in *ACT1* (Wertman et al., 1992). Of the 36 *act1* mutations constructed, 16 are conditional-lethal alleles, and an additional seven alleles show no phenotype when expressed as the sole source of actin in yeast cells (Wertman et al., 1992). Because most of the charged amino acid residues that were mutated reside on the surface of the actin monomer, and because many different regions of actin were mutated, it is likely that the mutations affect a variety of the different interactions made by actin. Indeed, the mutations cause a variety of different phenotypes, and one mutation abolishes binding to the mushroom toxin phalloidin (Wertman et al., 1992; Drubin et al., 1993).

We sought to develop a genetic approach to determine which, if any, of the charged-to-alanine *ACT1* mutations affect the interaction between actin and actin-binding proteins. We hypothesized that such a mutation in the actin gene might cause a phenotype similar to a mutation in the gene that encodes the actin-binding protein. For example, one hallmark of mutations in the fimbrin (*SAC6*) gene is synthetic lethality with mutations in *ABP1* and *SLA2*, genes that encode components of the actin cytoskeleton (Adams et al., 1993; Holtzman et al., 1993). By synthetic lethality, we mean that each single mutant is viable, but the combination of the two mutations results in inviability, presumably because of redundancy in the functions provided by the products of the two genes. Thus, a mutation in the actin gene that impairs the interaction with fimbrin would be predicted to show synthetic lethality with null alleles of the *ABP1* and *SLA2* genes, but not with null alleles of the *SLA1* and *SAC6* genes (see Results). Taking advantage of the large collection of charged-to-alanine *act1* alleles, and knowledge of the previously decribed synthetic lethal interactions involving the *SAC6* gene, we have genetically implicated specific regions of actin subdomain 1 in the interaction between actin and fimbrin. Biochemical experiments verified our conclusions. The findings reported here demonstrate that the combination of genetic and structural data can create functional maps for the surfaces of proteins.

Materials and Methods

Yeast Methods

Yeast media and genetic manipulations were performed as described (Rose et al., 1990). The strains used in this study are listed in Table I. For the experiments in Fig. 2, tetrads were dissected on rich media plates and grown for 3 d at 25°C before photography. Cells were replica plated (see Fig. 2 *B*) using a multipronged replica-plating device, and the growth properties of viable spores were examined at a variety of temperatures, as well as on selective plates, to follow segregation of the marked *act1* (*HIS3*), *abp1*Δ (*LEU2*), *sac6*Δ (*LEU2*), *sla1*Δ, (*URA3*), and *sla2*Δ (*URA3*) alleles. The plasmids used in this study are YCp50 (Mann, C., unpublished data; see Ma et al. [1987] for a restriction map) and YCp50 containing wild-type *SAC6* (AAB117) (Adams et al., 1991), or otherwise identical plasmids containing the *sac6-6* (AAB122), *sac6-15* (AAB125), and *sac6-19* (AAB163) suppressor alleles (Adams and Botstein, 1989). For the experiments shown in Fig. 2 *C*, plasmids were transformed into diploid strains using lithium acetate/polyethylene glycol as described (Ito et al., 1983), and the transformed strains were subsequently sporulated.

ACT1 and act1-120 Actin Purification

Yeast actins were purified as described (Kron et al., 1992). Briefly, cultures (OD_{600} ~8–10, 6 liters) of DDY354 (*ACT1*) and DDY347 (*act1-120*) were harvested at 4°C at 4,000 rpm. The pellet was washed 1× in 10 mM imidazole/HCl (pH 7.4) and the cells were then resuspended in lysis buffer (10 mM imidazole/HCl, pH 7.4/0.5 mM $CaCl_2$/0.5 mM ATP with an aqueous/ethanol protease inhibitor cocktail (1,000 × aqueous inhibitors = 0.5 mg/ml each antipain, leupeptin, pepstatin A, chymostatin, and aprotinin; 100× ethanol inhibitors = 0.17 g PMSF, 1.6 mg benzamidine HCl, and 1 mg phenanthroline in 10 ml EtOH). Cells were lysed using a glass bead disruption device with 30 cycles of 15 s agitation followed by a 45-s cooling interval. Disruption of cells was monitored by phase contrast microscopy and was typically >90%. The lysate was cleared with a low speed spin (12 krpm, 60 min, 4°C) followed by a high-speed spin (42 krpm in a rotor [45Ti; Beckman Instruments, Inc., Fullerton, CA], 120 min, 4°C). The cytosol was diluted 1:1 with DNAse column buffer (10 mM imidazole/HCl, pH 7.4/0.5 mM $CaCl_2$/0.5 mM ATP) and loaded at 0.5 ml/min onto a 2-ml column containing Sepharose-bound DNAse I (Worthington Biochemical Corp., Freehold, NJ). The DNAse column was washed with several volumes of DNAse column buffer, followed by 10 ml each of 10% formamide in column buffer, 10% formamide/0.2 M NH_4Cl in column buffer, and then column buffer alone. The outlet of the DNAse column was then connected to the inlet of a G-25 column (2.5 × 40 cm) equilibrated with DNAse column buffer. Actin was eluted from the DNAse column with 10 ml of 50% formamide in column buffer, and protein-containing fractions collected from the G-25 column were pooled and dialyzed overnight in G buffer (5 mM Tris/HCl, pH 8.0/0.2 mM $CaCl_2$/0.2 mM ATP/0.2 mM DTT). Actin was concentrated using a 5 × 50-mm (1 ml) Mono Q column using a fast protein liquid chromatography control system (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and eluted with a linear gradient of 0–0.4 M KCl in G buffer.

Actin-Sac6p/Cofilp Copelleting Assays

Actin (3 μM) was assembled by the addition of one-tenth volume of 10 × polymerization-inducing buffer (10× polymerization-inducing buffer is 20 mM $MgCl_2$/5 mM ATP, pH 7.0) in a final volume of 100 μl. Cofilp was a generous gift of Anne Moon (Department of Molecular and Cell Biology, University of California, Berkeley, CA) and was purified as described (Moon et al., 1993). Sac6p (yeast fimbrin), a generous gift of Tanya Sand-

Table I. Yeast Strains Used in this Study

Name	Genotype*
DDY318	*MATα his3-Δ200, leu2-3,112, lys2-801am, ura3-52 sac6Δ::LEU2*
DDY319	*MATα his3-Δ200, leu2-3,112, lys2-801am, ura3-52 sac6Δ::LEU2*
DDY321	*MATα his3-Δ200, leu2-3,112, ura3-52, abp1-Δ2::LEU2*
DDY322	*MATα his3-Δ200, leu2-3,112, ura3-52, abp1-Δ2::LEU2*
DDY332	*MATα his3-Δ200, ura3-52, sla1-Δ1::URA3*
DDY333	*MATα his3-Δ200, ura3-52, sla1-Δ1::URA3*
DDY545	*MATα his3-Δ200, leu2-3,112, ura3-52, sla2-Δ1::URA3*
DDY546	*MATα his3-Δ200, leu2-3,112, lys2-801am, ura3-52, sla2-Δ1::URA3*
DDY334	*MATα ade2-101, ura3-52, act1-4*
DDY335	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3*
DDY336	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, cry1, can1-1, act1-133::HIS3*
DDY337	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, cry1, act1-108::HIS3*
DDY338	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, cry1, can1-1, act1-101::HIS3*
DDY339	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, cry1, can1-1, act1-102::HIS3*
DDY340	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, act1-104::HIS3*
DDY341	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, cry1 act1-111::HIS3*
DDY342	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, act1-113::HIS3*
DDY343	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, can1-1, act1-115::HIS3*
DDY344	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, act1-116::HIS3*
DDY345	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, cry1, act1-117::HIS3*
DDY346	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, cry1, act1-119::HIS3*
DDY347	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, cry1, act1-120::HIS3*
DDY348	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101. can1-1, cry1, act1-123::HIS3*
DDY349	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-124::HIS3*
DDY350	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-125::HIS3*
DDY351	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, act1-129::HIS3*
DDY352	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, can1-1, cry1, act1-132::HIS3*
DDY353	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade2-101, act1-135::HIS3*
DDY354	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, cry1, ACT1::HIS3*
DDY355	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, ade4, cry1, act1-112::HIS3*
DDY356	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, act1-105::HIS3*
DDY357	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-136::HIS3*
DDY654	*MATα his3-Δ200, leu2-3,112, ura3-52, tub2-201, can1-1, ade4, act1-121::HIS3*

* All strains are derived from the S288C background.

rock (Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ), is the first (and the most pure) of two Sac6p peaks that elute from a hydroxyapatite column (Sandrock, T. S., and A. E. M. Adams, manuscript in preparation). Sac6p and Cofilp were added to the reaction at ~0.1 μM and 1.0 μM, respectively, before addition of polymerization salts. Polymerization was carried out at 22° for 1 h. Filaments were sedimented at 90 krpm for 40 min at 22°C in a Beckman TLA table-top ultracentrifuge using a TLA 100 rotor. To better separate the supernatants and pellets, actin filaments were sedimented through a 50-μl cushion of 50% sucrose in 1× polymerization buffer. For Sac6p pelleting reactions, supernatants were concentrated by TCA precipitation (Drubin et al., 1988). Pellets and supernatants were brought to the same total volume in 2× SDS sample buffer (2× SDS sample buffer is 125 mM Tris, pH 6.8/2% SDS/20% glycerol/10% beta-mercaptoethanol/0.1% bromophenol blue), boiled for 3 min, and

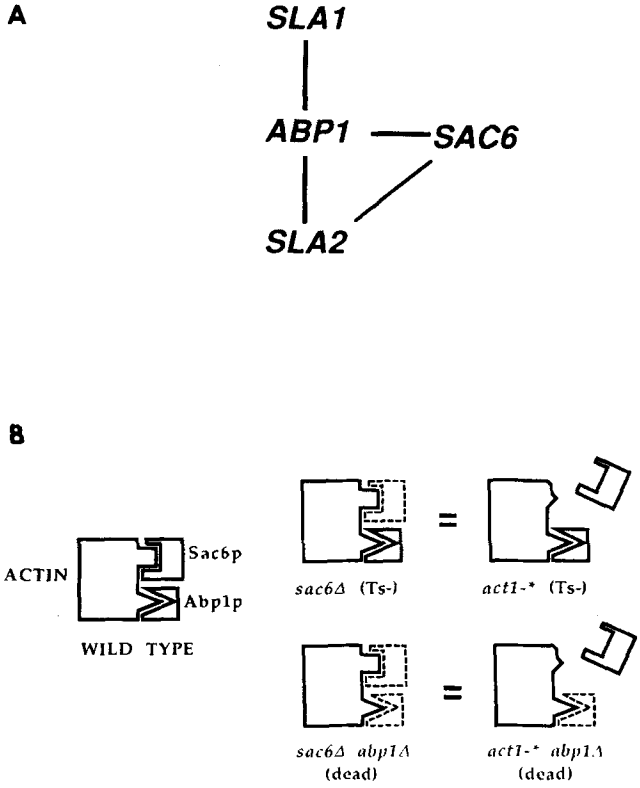


Figure 1. (*A*) Diagram showing synthetic-lethal interactions among components of the yeast cortical cytoskeleton. The lines connecting gene names signify synthetic–lethal interactions between null alleles of each gene. Each gene is nonessential for cell viability. When two genes are not connected by a line, no synthetic–lethal interaction occurs between null alleles of the two genes. (*B*) Schematic diagram illustrating the genetic principle for mapping protein–protein interactions on the actin filament. A null mutation in the *SAC6* gene (*sac6Δ*) results in temperature sensitivity for cell growth (Adams et al., 1991). Therefore, a mutation in the *ACT1* gene that disrupts the interaction between Sac6p and the surface of the actin filament is also predicted to result in temperature sensitivity. Moreover, if such a mutant actin allele is combined with a null allele of *ABP1* (*abp1Δ*) (or a null allele of *SLA2* that was not included in this cartoon for simplicity), a synthetic–lethal interaction between these mutations is predicted to result, despite the fact that the disruption of *ABP1* has no discernible effect in a wild-type actin background (Drubin et al., 1990; Adams et al., 1993; Holtzman et al., 1993). Therefore, by creating double mutants between mutant actin alleles and null alleles of *ABP1* and *SAC6*, and by looking for synthetic-lethal interactions, it should be possible to identify residues on the surface of actin that are important for the binding of Sac6p and/or Abplp.

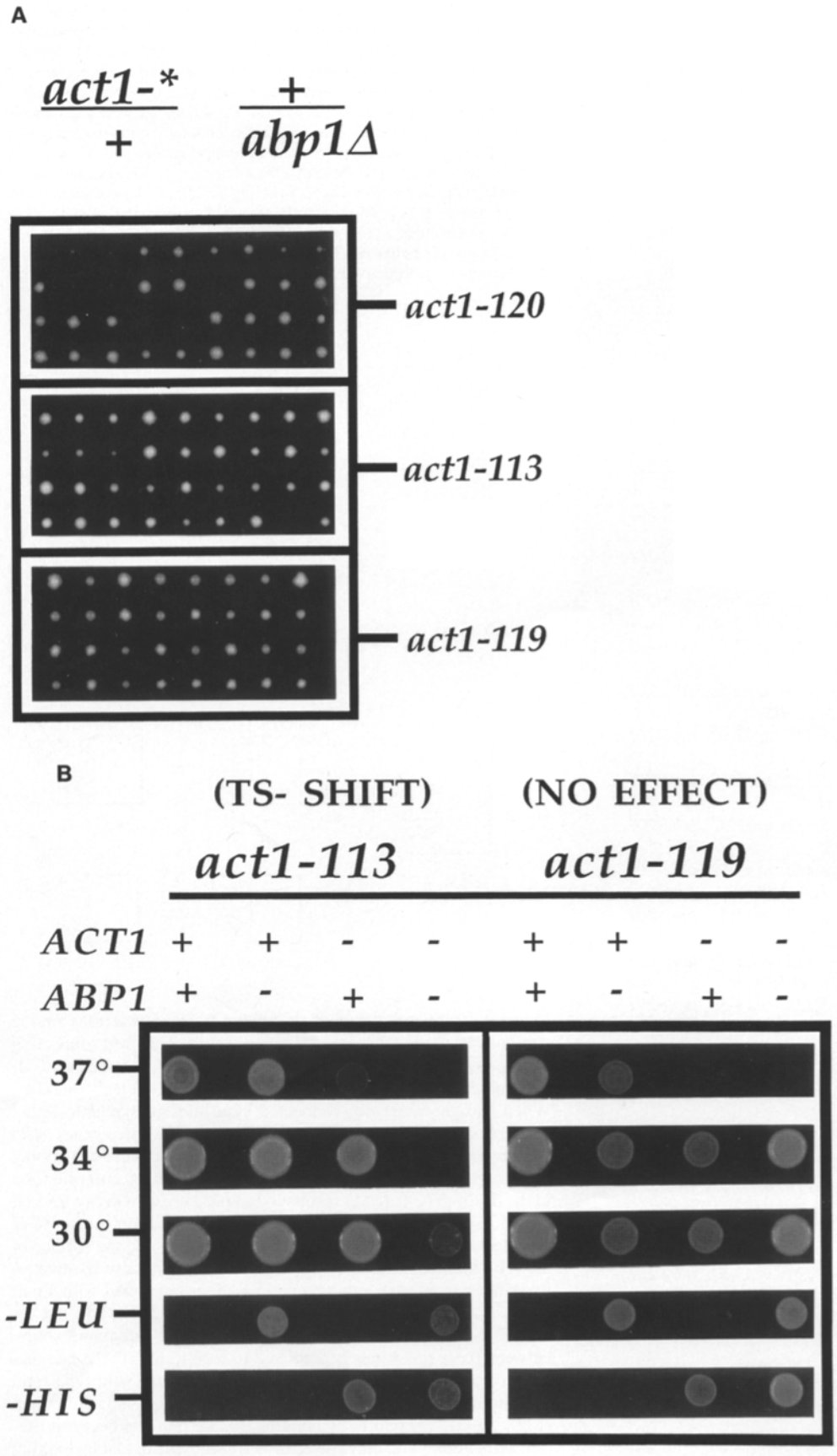


Figure 2. Growth properties of actin–actin cytoskeletal protein double mutants. (*A*) Spore colonies resulting from sporulation of *act1-*/+ abp1Δ/+* diploids were grown for 3 d at 25°C on rich medium. A synthetic-lethal defect results from combining the *act1-120* allele with the *abp1Δ* mutation. *act1-120-abp1Δ* double-mutant strains are either dead or extremely sick. In contrast, *act1-119* is unaffected by the *abp1Δ* mutation. The *act1-113-abp1Δ* interaction is an example of a modified permissive temperature range effect (see also *B*). (*B*) Example of a modified permissive temperature range effect (*MOD* in Table II). Replica plating of a tetratype tetrad from a dissection of a *act1-113/+ abp1Δ/+* strain shows that the permissive temperature range of the *act1-113* allele is narrowed by addition of the *abp1Δ* allele. Cells were replica plated using a multipronged replica-plating device, and the growth properties of viable spores were examined at a variety of temperatures. The segregation of mutant genes was followed by scoring the selectable marker genes linked to each mutant gene (i.e., *LEU2* for *sac6Δ* and *abp1-Δ1*; *URA3* for *sla1-Δ1* and *sla2-Δ2*; and *HIS3* for the *act1* alleles). This analysis was used to compare the permissive temperature range of each viable double mutant to the permissive temperature ranges of the single mutants (Table II). (*C*) Spore colonies from tetrad dissections of *act1-120/+ abp1Δ/+* strains transformed with centromere-based plasmids containing a dominant suppressor allele of *SAC6* (*sac6^{sup}*), the wild-type *SAC6* gene, or a control plasmid (YCp50). The control plasmid has no effect on the synthetic-lethal defect that results from combining the *act1-120* allele with the *abp1Δ* mutation. However, an allele of *sac6* that suppresses the temperature sensitivity of *act1-3* also suppresses the synthetic-lethal interaction between *act1-120* and *abp1Δ* (note that only 50% of the spore colonies are expected to inherit a plasmid in each cross). Increasing the copy number of the wild-type gene (*SAC6*) is able to enhance marginally the growth of the *act1-120-abp1Δ* double mutants that form colonies. Tetrads were dissected on rich media plates and were grown for 3 d at 25°C.

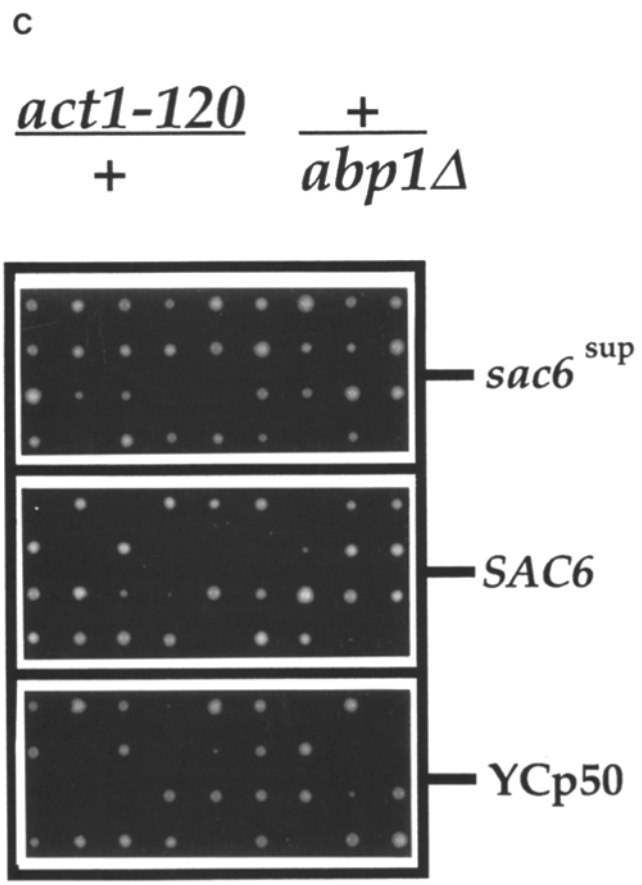


Figure 2. (continued)

equal volumes were loaded onto SDS-polyacrylamide gels as described (Laemmli, 1970).

Electron Microscopy

Electron microscopy of negatively stained actin filaments was performed by the procedure of Millonig et al. (1988), except that 1% aqueous uranyl acetate was used.

Results

Genetic Interactions

Our strategy to locate the binding sites of actin-binding proteins on the surface of actin is based on the expectation that mutations in the actin gene (*ACT1*) that impair binding to a specific actin-binding protein will cause a phenotype similar to that caused by a null mutation in the gene that encodes the actin-binding protein. For example, as shown in Fig. 1 *A*, null alleles of *SAC6* (the yeast fimbrin gene) are synthetically lethal with null alleles of *ABP1* (Adams et al., 1993; Holtzman et al., 1993) and *SLA2*, but not with null alleles of *SLA1* (Holtzman et al., 1993). Thus, actin mutants defective in Sac6p (fimbrin) binding are predicted to be synthetically lethal with *abp1* and *sla2* null alleles. This assumes that the phenotype of the actin-binding protein mutant results from loss of the interaction with actin. Further predictions for an actin mutant that is defective in Sac6p binding are that the mutant will not show synthetic lethality with *sla1* and *sac6* null alleles. This is because *sac6* null alleles do not show synthetic lethality with *sla1* null alleles, and because the phenotype of an *act1* mutant, whose phenotype results from a defective interaction with Sac6p, should not be made more severe by deletion of the *SAC6* gene. The cartoon shown in Fig. 1 *B* which, for simplicity, shows only Sac6p and Abp1p, illustrates the principle behind our mapping strategy. A mutation in *act1* that prevents Sac6p binding is predicted to cause a temperature-sensitive phenotype (because *sac6Δ* mutants are Ts^-) and to be lethal in combination with a null allele of *ABP1*. Accordingly, haploid strains containing mutations in *ACT1* (21 *act1* alleles were analyzed) were mated to null alleles of *ABP1*, *SAC6*, *SLA1*, and *SLA2*, and the double-heterozygotes were then sporulated and dissected.

Each double mutant was analyzed as shown in Fig. 2 *A*. For those crosses in which the double mutant combination produced viable spores, spore colonies from tetratype tetrads were replica plated and grown at several temperatures so that the growth of the wild-type strain, both single mutants and the double mutant, could be compared directly (Fig. 2 *B*). The consequence of combining *abp1Δ*, *sac6Δ*, *sla1Δ*, or *sla2Δ* mutations with *act1* alleles are classified in one of three ways in Table II: (*a*) no effect on the growth properties of the *act1* Mutant (NE); (*b*) a modification of the permissive temperature range (MOD); or (*c*) a synthetic-lethal effect (SL). An example of a modified permissive temperature range is shown in Fig. 2 *B*. While strains bearing the *act1-113* mutation grow well at 34°, the *act1-113-abp1Δ* double mutant fails to grow at 34°C and grows poorly at 30°C (Fig. 2 *B*). Thus, the defect in the *act1-113* strain is exacerbated by the *abp1Δ* mutation, even though the *abp1Δ* allele alone has no discernible phenotype. An example of synthetic lethality between an *act1* mutant allele and a null allele of a gene encoding a cytoskeletal protein is shown in Fig. 2 *A*. When an *act1-120/+ abp1Δ/+* strain was sporulated and analyzed by tetrad dissection, a high percentage of the spores were found to be inviable (see Fig. 2 *A*). In all cases, the dead spores could be deduced by linked marker segregation (see Fig. 2 *A* legend and Materials and Methods) to contain both the *act1-120* and *abp1Δ* mutations (Table II). Those double-mutant spores that did germinate were extremely sick (data not shown). Therefore, the *act1-120* mutation is synthetically lethal with *abp1Δ*.

The data summarized in Table II show that *abp1Δ*, *sac6Δ*, *sla1Δ*, and *sla2Δ* each show genetic interactions with a different subset of the 21 *act1* mutants analyzed. Interestingly, four out of seven pseudo–wild-type *act1* alleles (*act1-102*, *act1-115*, *act1-116*, and *act1-117*) show enhancement, or even synthetic lethality, when combined with certain null mutations in cytoskeletal protein genes. Significantly, two of the 21 *act1* mutations meet all four predictions for alleles that cause defects in the Sac6p-actin interaction. These alleles (*act1-3* and *act1-120*) are synthetically lethal with *sla2* and *abp1* null alleles, but not with *sla1* and *sac6* null alleles.

The only gene in addition to *SAC6* that was analyzed in this study and that is known to encode an actin-binding protein is *ABP1*. An *act1* mutation that compromises the interaction between actin and Abp1p is predicted to be unaffected by a null allele of *ABP1*. However, this same *act1* mutation is predicted to be synthetically lethal with null alleles of *SAC6*, *SLA1*, and *SLA2*, since a double mutant of *abp1Δ* and a null allele of any of these three genes is synthetically lethal.

Table II. Growth Properties of Double Mutants

act1 Allele*	Amino acid substitution(s)	Double-mutant phenotype: *act1* single mutant phenotype	*act1-* sac6Δ*	*act1-* abp1Δ*	*act1-* sla1Δ*	*act1-* sla2Δ*
act1-101	D363A,E364A	Ts-	MOD‡	MOD	NE	SL
act1-102	K359A,E361A	WT	MOD	NE	UC§	NE
act1-104	K315A,E316A	WT	NE	NE	NE	NE
act1-105	E311A,R312A	Cs-,Ts-	NE	NE	MOD	NE
act1-108	R256A,E259A	Cs-,Ts-	SL	MOD	SL	SL
act1-112	K213A,E214A,K215A	Cs-,Ts-	UC	SL	ND‖	UC
act1-113	R210A,D211A	Weak Ts-	SL	MOD	NE	NE
act1-115	E195A,R196A	WT	SL	NE	NE	SL
act1-116	D187A,K191A	WT	MOD	NE	NE	SL
act1-117	R183A,D184A	WT	MOD	NE	NE	NE
act1-119	R116A,E117A,K118A	Ts-	SL	NE	NE	UC
act1-120	E99A,E100A	Ts-	NE	SL	NE	SL
act1-121	E83A,K84A	Cs-,Ts-	SL	NE	ND	ND
act1-123	R68A,E72A	WT	NE	NE	NE	NE
act1-124	D56A,E57A	Ts-	SL	MOD	NE	SL
act1-125	K50A,D51A	Cs-,Ts-	SL	SL	NE	SL
act1-129	R177A,D179A	Ts-	SL	SL	NE	SL
act1-133	D24A,D25A	Cs-,Ts-	MOD	MOD	SL	SL
act1-135	E4A	WT	NE	NE	NE	NE
act1-136	D2A	Cs-,Ts-	SL	SL	UC	UC
act1-3	P32L	Ts-	NE	SL	NE	SL

Ts-, temperature sensitive; WT, wild type; Cs-, cold sensitive.
* For the location of each mutation on the atomic model of the actin monomer, and a complete description of the *act1* permissive temperature range, please see Wertman et al. (1992).
‡ MOD, (modifier) the double mutant has a permissive temperature range narrower than that of either single mutant; NE, no effect on the permissive temperature range; SL indicates that the double mutant is synthetically lethal.
§ UC, uncertain. Viability of spores was too low to confer significance to double-mutant phenotype.
‖ ND, not determined.

While one *act1* allele (*act1-115*) meets three out of four of these expectations, it fails to show the predicted synthetic lethality with the *sla1* null allele (Table 2).

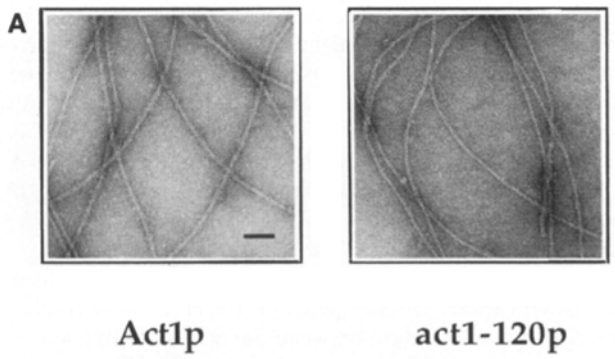
Suppression of act1-120-abp1Δ Synthetic Lethality

Adams and Botstein (1989) demonstrated that mutations in *SAC6* can be isolated as dominant suppressors of the *act1-3* allele, and their genetic analysis of the *act1-sac6* interaction strongly suggested that *act1-3* actin is defective in its interaction with wild-type Sac6p. More recently, biochemical studies demonstrated that *act1-3* actin filaments are indeed defective in the interaction with wild-type Sac6p (Honts et al., 1994). Recall that a *sac6Δ-abp1Δ* double mutant is synthetically lethal. If the lethality that results from combining the *act1-120* and *abp1Δ* mutations is caused by a defect in the interaction between *act1-120* filaments and Sac6p, then this is similar to having a cell that is *sac6Δ* and *abp1Δ*. Therefore, restoring the Sac6p interaction with actin should suppress the *act1-120-abp1Δ* synthetic lethality. Since *act1-3* and *act1-120* have the same synthetic lethal interactions (Table II), we reasoned that mutant forms of Sac6p encoded by alleles of *SAC6* (*sac6^{sup}*) that suppress *act1-3* might be able to interact functionally with *act1-120* actin. Therefore, we determined whether the synthetic lethality of the *act1-120-abp1Δ* double mutant could be suppressed by *sac6^{sup}* alleles.

As shown in Fig. 2 *C*, *act1-120-abp1Δ* double-mutant strains are rescued by a *sac6^{sup}* allele (*sac6-15*, Adams and Botstein [1989]) borne on a centromere plasmid (the increase of spore viability by 50%, rather than by 100%, is predicted based on the expected 2:2 segregation of centromere plasmids; compare *sac6^{sup}* with YCp50 tetrad dissection, Fig. 2 *C*, *top* and *bottom*, respectively). Moreover, these double-mutant strains grow in a *sac6^{sup}* plasmid-dependent manner (data not shown). Interestingly, wild-type Sac6p expressed from a centromere-based plasmid also affects the *act1-120-abp1Δ* double-mutant phenotype; those double-mutant spores that do germinate have a broader permissive temperature range (20–30°C) than spores that carry a control plasmid (20–25°C) (data not shown). Thus, changing either the nature or the dosage of *SAC6* can suppress the synthetic phenotype of the *act1-120-abp1Δ* double mutant. The same result was obtained for *act1-3-abp1Δ* double mutants (data not shown). These results strongly suggest that, like *act1-3* actin, *act1-120* actin is defective in the interaction with Sac6p, and that it is this defect (mimicking a *sac6Δ* mutation) that results in a synthetic-lethal interaction with *abp1Δ* mutations.

act1-120 Actin is Defective in the Interaction with Sac6p

To determine the physical basis of the synthetic-lethal interactions between *act1-120* and the *abp1Δ* and *sla2Δ* alleles, we purified actin from wild-type yeast and from a strain that bears the *act1-120* mutation. The mutant actin assembled into filaments similar to those formed by the wild-type actin as judged by electron microscopy (Fig. 3 *A*) and by velocity sedimentation assays (Fig. 3, *B* and *C*). However, while Sac6p associates with filaments assembled from wild-type yeast actin, the *act1-120* mutation significantly impairs this interaction (Fig. 3*B*). In five pelleting experiments, the amount of Sac6p in the *act1-120* actin pellet fraction varied



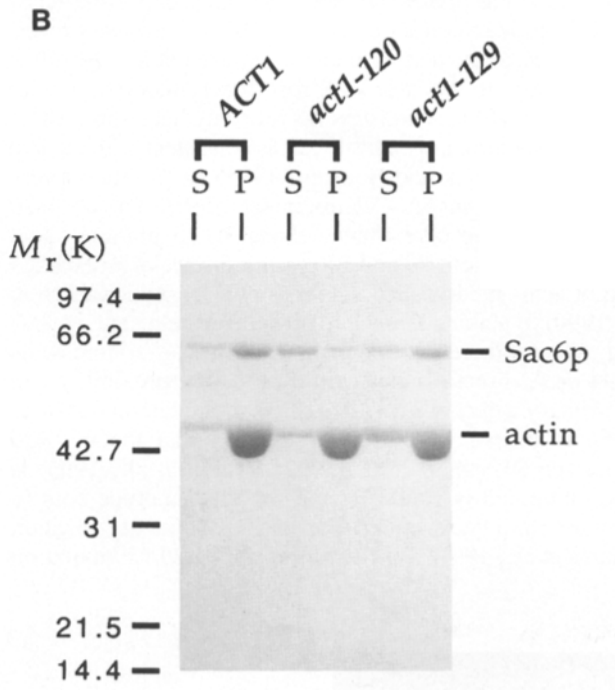
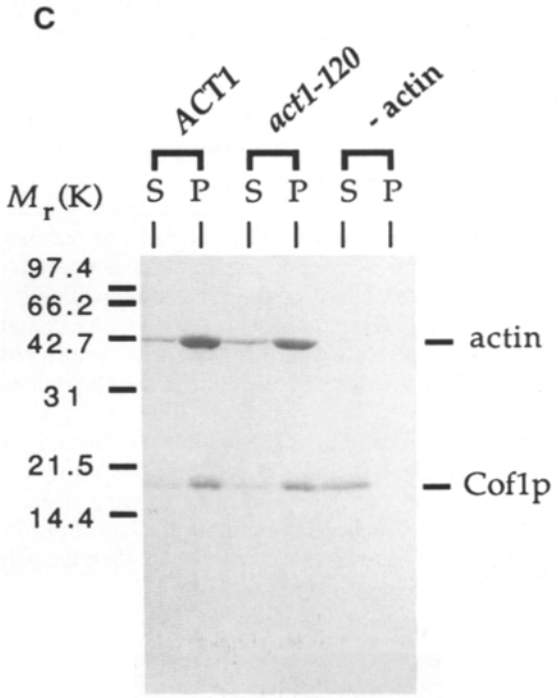


Figure 3. Biochemical analysis of filaments composed of *act1-120* actin. (*A*) Electron micrograph of negatively stained filaments assembled from *ACT1* and *act1-120* actin (bar, 50 nM). (*B*) While Sac6p pellets with actin filaments composed of actin purified from *ACT1* and *act1-129* strains, the pelleting activity is impaired when actin purified from the *act1-120* mutant strain is used. *S*, supernatant; *P*, pellet. Coomassie blue-stained SDS-polyacrylamide (8.5%) gel. (*C*) Actin filaments composed of actin purified from the *act1-120* strain show no defect in its association with the low molecular weight actin-binding protein Cof1p (yeast cofilin). Coomassie blue-stained SDS-polyacrylamide (13%) gel. See Materials and Methods for experimental details.



from being undetectable (not shown) to being noticeable but greatly reduced in amount compared to the amount in the wild-type actin pellet fraction (Fig. 3 *B*). For the experiments in which some Sac6p was detectable in the *act1-120* pellet fraction, a similar amount could be found in pellet fractions for control reactions that lacked any actin (not shown). Another temperature-sensitive mutation, *act1-129*, has no effect on the interaction with Sac6p (Fig. 3 *B*). The low molecular weight actin-binding protein Cof1p (Moon et al., 1993) copellets with both *ACT1* and *act1-120* filaments (Fig. 3 *C*), providing further evidence that the *act1-120* mutation (E99A,E100A) does not cause a gross defect in filament structure. Thus, the genetic interactions accurately predicted a defect in the physical association of Sac6p with yeast actin filaments where the glutamic acid residues at positions 99 and 100 have been replaced by alanine.

Discussion

To understand fully how actin-binding proteins regulate actin assembly and organize higher order structures such as filament bundles, it is necessary to define the molecular contacts that these proteins make with the surface of actin. Analysis of the data compiled in Table II leads to four important conclusions concerning the functions and interactions of actin and actin cytoskeleton proteins. The first point is that the *abp1*Δ, *sac6*Δ, *sla1*Δ, and *sla2*Δ mutations each show genetic interactions with a different subset of the 21 *act1* mutations analyzed. This demonstrates that the charged-to-alanine mutations in *ACT1* have diverse effects on actin. This applies even for those cases in which two *act1* alleles have similar effects on cell growth. For example, despite the fact that *act1-119* and *act1-120* mutants have similar permissive temperature ranges (Wertman et al., 1992), combining these mutations with *abp1*Δ and *sac6*Δ have opposite effects (Table II). The nonequivalence of the conditional *act1* alleles is not surprising because the mutations are distributed widely over the surface of the monomer and are therefore likely to affect different aspects of actin function.

The second important conclusion arises from the observation that while the *sla1*Δ allele only shows interactions with three *act1* alleles, the *abp1*Δ, *sac6*Δ, and *sla2*Δ alleles each affect 10–14 *act1* alleles, and each shows a distinct pattern of interactions with the *act1* mutations (Table II). The differences in the interactions with different *act1* alleles provide further evidence that although *SAC6*, *SLA1*, and *SLA2* are all

"genetically redundant" with *ABP1* (Fig. 1 *A*; Holtzman et al., 1993), their gene products play different roles in the regulation of the actin cytoskeleton.

The third point is that the double-mutant analysis shows that the amino acids changed in four actin mutants for which no phenotype had previously been identified (*act1-102*, *act1-115*, *act1-116*, and *act1-117*) do indeed contribute to actin function. Thus, *act1-115* appears to be wild type with respect to its permissive temperature range and sensitivity to osmotic stress (Wertman et al., 1992). However, when combined with a null mutation in *SAC6* or *SLA2*, *act1-115* mutants are inviable (Table II). One possibility is that the *act1-115* mutation impairs the ability of the mutant actin to bind to a protein whose interaction with actin is only important in the absence of Sac6p or Sla2p. Abp1p would be such a candidate since *abp1*Δ, like *act1-115*, is synthetically lethal with *sac6*Δ and *sla2*Δ. An observation that argues against this possibility is that the *act1-115* allele is not synthetically lethal with the *sla1*Δ mutation. This genetic interaction is expected because *abp1*Δ is synthetically lethal with *sla1*Δ. Despite this discrepancy, it is possible that the "genetic redundancy" observed between *ABP1* and *SLA1* is not manifested through the interaction of Abp1p with actin, but rather, through the interactions of the SH3 domains found on both Abp1p and Sla1p (Holtzman et al., 1993) with a common cellular ligand. Resolution of this issue will require biochemically assaying the interaction of purified Abp1p with *act1-115* actin.

The final important conclusion derived from the interactions summarized in Table II concerns the ability to genetically implicate two *act1* alleles as causing defects in the Sac6p-actin interaction. If a mutation in actin compromises the interaction between actin and Sac6p, then the deletion of *SAC6* should cause no further detriment to the growth properties of a strain bearing that mutation. Therefore, an *act1* mutation that causes a defect in Sac6p binding is predicted to show no further detriment in growth when combined with a *sac6*Δ allele. Furthermore, such an *act1* mutant is predicted to show synthetic lethal interactions with *abp1*Δ and *sla2*Δ, but not with *sla1*Δ (see Fig. 1 *A*). Of the 21 *act1* alleles analyzed, only *act1-3* (P32L) and *act1-120* (E99A, E100A) meet these criteria (Table II).

The double-mutant results (Table II) and the suppressor analysis with *sac6*^{sup} alleles (Fig. 2 *C*) accurately predicted a defect in the association between Sac6p and filaments assembled from *act1-120* (this study) and *act1-3* (Honts et al., 1994) actin. The *act1-3* mutation is a change of proline at position 32 to leucine (Shortle et al., 1984). Because proline 32 is buried in the actin monomer (Kabsch et al., 1990), it is not likely to be available for direct interaction with fimbrin, and alteration of this residue may have some effect on actin structure, a possibility that is consistent with the fact that *act1-3* (Novick and Botstein, 1985) has a more severe phenotype than *sac6*Δ (Adams et al., 1991). The *act1-120* mutation, on the other hand, changes two glutamic acid residues at positions 99 and 100 on the surface of an exposed loop of actin subdomain 1 (Holmes et al., 1990; Kabsch et al., 1990) to alanine (E99A, E100A) (Wertman et al., 1992) (Fig. 4), and therefore one or both of these glutamic acids might make direct contacts with Sac6p. The possibility that the primary defect of *act1-120* actin is in the interaction with Sac6p is further supported by the fact that the *act1-120* phenotype (Wertman et al., 1992; Drubin et al., 1993) is very subtle and is similar to the *sac6*Δ phenotype both in terms of the effects on growth and on actin organization (Adams et al., 1991). Furthermore, if the *act1-120* mutation

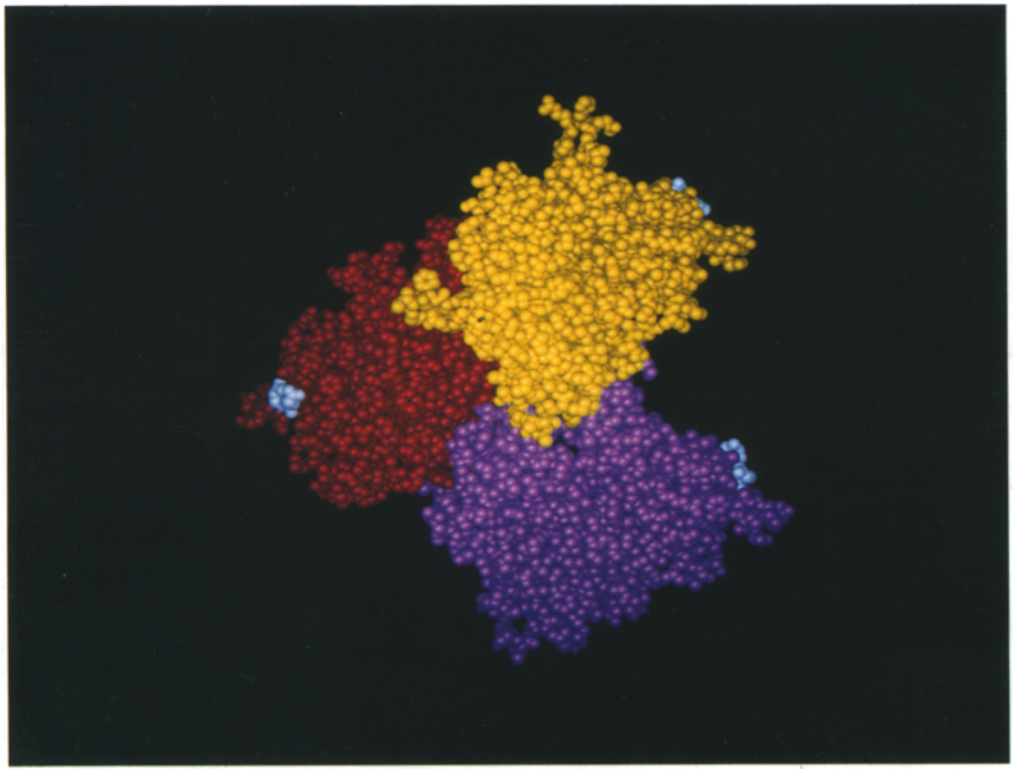


Figure 4. Location of the *act1-120* mutation in the three dimensional model of the actin filament (three monomers are shown). Glutamic acid residues 99 and 100 (*white*) are exposed on the surface of the filament in actin subdomain 1. The purple and yellow monomers are located in the same strand of the two-start helix, while the red monomer lies in the adjacent strand.

also caused defects in the interaction with either myosin, cofilin, profilin, or tropomyosin, or a combination of these, a more severe defect in cell growth would be expected, since mutations in the genes that encode these actin-binding proteins result in severe phenotypes or even death (reviewed by Welch et al., 1994). The observations that pure *actl-120* actin forms filaments that appear normal when analyzed by electron microscopy (Fig. 3 *A*) and when tested for the ability to interact with cofilin (Fig. 3 *C*) further suggests that the *actl-120* mutation only causes limited effects on the surface of actin.

Since Sac6p contains actin-binding domains homologous to domains of α-actinin, dystrophin, filamin, β-spectrin, and actin-gelation protein (see Adams et al. [1991] and the references therein), each of these proteins is likely to interact with a similar region of the actin filament. Interestingly, in vitro studies have shown that α-actinin can be cross-linked to a segment of actin (residues 87–119) (Mimura and Asano, 1987) that contains residues 99 and 100. In addition, antibodies raised against actin residues 95–113 interfere with the interaction between α-actinin and actin (Lebart et al., 1993). Recently, McGough et al. (1994) studied the structure of actin filaments decorated with the actin-binding domain of α-actinin using cryoelectron microscopy and image processing. Their results indicate that α-actinin interacts with actin subdomain 1, the subdomain in which the *actl-120* and *actl-3* mutations reside, and with actin subdomain 2. We note that the interaction between α-actinin and F-actin is postulated to be predominantly hydrophobic in nature (Kuhlman et al., 1992). Sac6p binding, however, is sensitive to salt concentration and thus is likely to have an electrostatic component (Drubin et al., 1988).

Actin residues 99 and 100 are likely to make electrostatic contacts with the S1 fragment of myosin (Schatz et al., 1986; Rayment et al., 1993; Schröder et al., 1993), and charge reversal of these residues in *Dictyostelium* actin interferes with the ATP-driven translocation of actin filaments by myosin (Johara et al., 1993). Since the *actl-120* mutation is not a charge reversal, but a change of the two glutamic acid residues to alanines, it is not possible to know without a direct test how this mutation will affect the interaction with myosin. However, as we point out above, an actin mutation that impairs the interaction of myosin with actin is predicted to have much more severe effects on cell growth than are observed for the *actl-120* mutation, suggesting that myosin can functionally interact with *actl-120* actin. While these considerations suggest that interactions with residues 99 and 100 are more important for the interaction between actin and fimbrin than between actin and myosin, the implied proximity of the binding sites for fimbrin and myosin on actin suggests that fimbrin and myosin might compete for binding sites on the actin filament. In this regard, it is relevant that filamin, a protein that, like fimbrin, contains a domain homologous to the actin-binding domain of α-actinin, appears to compete with myosin for an interaction site on actin (Davies et al., 1977; Dabrowska et al., 1985), while α-actinin does not (Malhotra et al., 1986). These and other studies suggest that α-actinin and filamin have overlapping but nonidentical footprints on the actin filament (Lebart et al., 1993). Competition binding assays could be used to test directly whether Sac6p binding inhibits the interaction of myosin, α-actinin, and/or filamin with actin filaments.

More than 60 *actl* alleles presently exist, and the number is growing steadily (e.g., Shortle et al., 1984; Johannes and Gallwitz, 1991; Cook et al., 1992, 1993; Wertman et al., 1992; Chen et al., 1993). Since these mutations cover many of the surfaces of actin and presumably interfere with many different protein–protein interactions, they can be used to create a functional map of the surface of actin. One way to do this is to use the genetic strategy described here. Moreover, since many synthetic lethal interactions have been observed between mutant cytoskeletal protein genes, the approach can be extended to a variety of actin-binding protein–actin interactions. Thus, capping protein (encoded by *CAP1* and *CAP2*) mutants show synthetic lethality with *sac6* (Adams et al., 1993), as well as with *slc1* and *slc2* (Karpova et al., 1993) mutants, and myosin (*myo2*) mutants show synthetic lethality with tropomyosin (*tpm1*) mutants (Liu and Bretscher, 1992).

By isolating suppressors of *sac6* mutations that map to actin subdomains 1 and 2, Honts et al. (1994) implicated a cluster of actin residues in the interaction with Sac6p (fimbrin). In addition, we (Wertman and Holtzman, unpublished observation) and Honts, et al. (1994) combined $sac6^{sup}$ alleles with the conditional-lethal charged-to-alanine *actl* alleles to test for genetic suppression. Significantly, *actl-120*, the allele shown here to encode an actin that is defective in the interaction with Sac6p, was suppressed by these suppressor alleles, adding further support to the conclusion that actin residues E99 and/or E100 are important for the interaction with fimbrin. Suppression was observed for one additional charged-to-alanine allele, *actl-125* (K50A,D51A; actin subdomain 2) (Honts et al., 1994). The *actl-125* allele was not implicated in the interaction with Sac6p by the synthetic lethal approach because this *actl* allele is synthetically lethal with *sac6*Δ (Table II). The synthetic lethality between *actl-125* and *sac6*Δ indicates that this *actl* allele affects more than the actin-Sac6p interaction. Otherwise, deletion of the *SAC6* gene should not make the *actl-125* phenotype more severe, a prediction that was met for the *actl-120* allele. Furthermore, a *sac6* null mutant is viable and grows well over a larger temperature range (Adams et al., 1991) than an *actl-125* mutant (Wertman et al., 1992). Presumably, the suppressor approach implicated *actl-125* as impairing the Sac6p-actin interaction because the interaction of the $sac6^{sup}$ protein with *actl-125* actin "repairs" or compensates for defects that may include, but certainly extend beyond, the Sac6p interaction.

In conclusion, the application of a combination of different genetic, biochemical, and structural approaches to problems of protein structure and function is necessary for gathering complementary information and for corroboration of conclusions derived from each approach.

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