Mapping Actin Surfaces Required for Functional Interactions In Vivo

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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 (\textsc{sac6}), lethal in combination with null mutations in the \textsc{abp1} and \textsc{sla2} genes, and viable in combination with a null mutation in the \textsc{sla1} gene. Biochemical experiments with act\textsc{t}-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 \textsc{sac6}, \textsc{abp1}, \textsc{sla1}, and \textsc{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\textsubscript{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\textsubscript{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 scrin (Owen and DeRosier, 1993) on the surface of the actin filament have been modeled through cryoelectron microscopy of tropomyosin- or scrin-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
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 phallloidin (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 described 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* (HFB3), dpSalA (LEU2), *sac6a* (LEU2), *sla1a* (URA3), and *sla2a* (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 (OD600 ~8–10, 6 liters) of DDDY344 (ACT1) and DDDY547 (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 CaCl2/0.5 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 (12,000 rpm, 60 min, 4°C) followed by a high-speed spin (42,000 rpm in a rotor [45T; 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 CaCl2/0.5 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 NaCl 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 7.4/0.2 mM CaCl2/0.2 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-Sla6p/Coflp 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 MgCl2/5 mM ATP, pH 7.0) to a final volume of 100 μl. Copflp 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). Sla6p (yeast fimbrin), a generous gift of Tanya Sand-
### Table 1. Yeast Strains Used in this Study

| Name      | Genotype*                                      |
|-----------|------------------------------------------------|
| DDY318    | MATa his3-Δ200, leu2-3,112, lys2-801am, ura3-52 sac6Δ:LEU2 |
| DDY319    | MATa his3-Δ200, leu2-3,112, lys2-801am, ura3-52 sac6Δ:LEU2 |
| DDY321    | MATa his3-Δ200, leu2-3,112, ura3-52, act1-3, act1-3 |
| DDY322    | MATa his3-Δ200, leu2-3,112, ura3-52, act1-3, act1-3 |
| DDY332    | MATa his3-Δ200, ura3-52, slal-Δ1::URA3          |
| DDY333    | MATa his3-Δ200, ura3-52, slal-Δ1::URA3          |
| DDY345    | MATa his3-Δ200, leu2-3,112, ura3-52, slal-Δ1::URA3 |
| DDY346    | MATa his3-Δ200, leu2-3,112, lys2-801am, ura3-52, slal-Δ1::URA3 |
| DDY334    | MATa ade2-101, ura3-52, act1-4                 |
| DDY335    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY336    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY337    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY338    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY339    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY340    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY341    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY342    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY343    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY344    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY345    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY346    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY347    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY348    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY349    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY350    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY351    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY352    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY353    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY354    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY355    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY356    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY357    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |
| DDY654    | MATa his3-Δ200, leu2-3,112, ura3-52, tub2-201, act1-3 |

* All strains are derived from the S288C background.

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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 ACT7 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 Abp1p.
Figure 2. Growth properties of actin–actin cytoskeletal protein double mutants. (A) Spore colonies resulting from sporulation of actl-1+ + abp1Δ+ diploids were grown for 3 d at 25°C on rich medium. A synthetic-lethal defect results from combining the actl-120 allele with the abp1Δ mutation. actl-120-abp1Δ double-mutant strains are either dead or extremely sick. In contrast, actl-119 is unaffected by the abp1Δ mutation. The actl-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 an actl-113/ + abp1Δ/ + strain shows that the permissive temperature range of the actl-113 allele is narrowed by addition of the abp1Δ allele. Cells were replica plated using a multi-pronged 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 abp1Δ; URA3 for slalΔ and sla2-A2; and HIS3 for the actl 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 actl-120/ + abp1Δ/ + strains transformed with centromere-based plasmids containing a dominant suppressor allele of SAC6 (sac6ΔP), the wild-type SAC6 gene, or a control plasmid (YCpS0). The control plasmid has no effect on the synthetic-lethal defect that results from combining the actl-120 allele with the abp1Δ mutation. However, an allele of sac6 that suppresses the temperature sensitivity of actl-3 also suppresses the synthetic-lethal interaction between actl-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 actl-120-abp1Δ double mutants that form colonies. Tetrads were dissected on rich media plates and were grown for 3 d at 25°C.
Electron Microscopy
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 (*ACT7*) 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 *sac6*Δ 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 tetradtype 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 is 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°C, 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.
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 (sac6Δw) 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 sac6Δw alleles.

As shown in Fig. 2 C, act1-120-abp1Δ double-mutant strains are rescued by a sac6Δw 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 sac6Δw with YCp50 tetrad dissection, Fig. 2 C, top and bottom, respectively). Moreover, these double-mutant strains grow in a sac6Δw 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 (20–25°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 (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 (20–25°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.

### Table II. Growth Properties of Double Mutants

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

* 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 confine significance to double-mutant phenotype.
§ ND, not determined.
Figure 3. Biochemical analysis of filaments composed of actl-120 actin. (A) Electron micrograph of negatively stained filaments assembled from ACT1 and actl-120 actin (bar, 50 nM). (B) While Sac6p pellets with actin filaments composed of actin purified from ACT1 and actl-129 strains, the pelleting activity is impaired when actin purified from the actl-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 actl-120 strain show no defect in its association with the low molecular weight actin-binding protein Coflp (yeast coflin). 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 actl-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, actl-129, has no effect on the interaction with Sac6p (Fig. 3 B). The low molecular weight actin-binding protein Coflp (Moon et al., 1993) copellets with both ACT1 and actl-120 filaments (Fig. 3 C), providing further evidence that the actl-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 abplA, sac6A, slalA, and sla2A mutations each show genetic interactions with a different subset of the 21 actl 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 actl alleles have similar effects on cell growth. For example, despite the fact that actl-119 and actl-120 mutants have similar permissive temperature ranges (Wertman et al., 1992), combining these mutations with abplA and sac6A have opposite effects (Table II). The nonequivalence of the conditional actl 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 slalA allele only shows interactions with three actl alleles, the abplA, sac6A, and sla2A alleles each affect 10-14 actl alleles, and each shows a distinct pattern of interactions with the actl mutations (Table II). The differences in the interactions with different actl alleles provide further evidence that although SAC6, SLAI, and SLA2 are all
"genetically redundant" with ABP1 (Fig. 1A; 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 (actl-102, actl-115, actl-116, and actl-117) do indeed contribute to actin function. Thus, actl-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, actl-115 mutants are inviable (Table II). One possibility is that the actl-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. Abplp would be such a candidate since abplΔ, like actl-115, is synthetically lethal with sac6Δ and sla2Δ. An observation that argues against this possibility is that the actl-115 allele is not synthetically lethal with the slalΔ mutation. This genetic interaction is expected because abplΔ is synthetically lethal with slalΔ. Despite this discrepancy, it is possible that the "genetic redundancy" observed between ABP1 and SLA1 is not manifested through the interaction of Abplp with actin, but rather, through the interactions of the SH3 domains found on both Abplp and Slalp (Holtzman et al., 1993) with a common cellular ligand. Resolution of this issue will require biochemically assaying the interaction of purified Abplp with actl-115 actin.

The final important conclusion derived from the interactions summarized in Table II concerns the ability to genetically implicate two actl 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 actl mutation that causes a defect in Sac6p binding is predicted to show no further detriment in growth when combined with a sac6Δ allele. Furthermore, such an actl mutant is predicted to show synthetic lethal interactions with abplΔ and sla2Δ, but not with slalΔ (see Fig. 1A). Of the 21 actl alleles analyzed, only actl-3 (P32L) and actl-120 (E99A, E100A) meet these criteria (Table II).

The double-mutant results (Table II) and the suppressor analysis with sac6αw alleles (Fig. 2C) accurately predicted a defect in the association between Sac6p and filaments assembled from actl-120 (this study) and actl-3 (Honts et al., 1994) actin. The actl-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 actl-3 (Novick and Botstein, 1985) has a more severe phenotype than sac6Δ (Adams et al., 1991). The actl-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 actl-120 actin is in the interaction with Sac6p is further supported by the fact that the actl-120 phenotype (Wertman et al., 1992; Drubin et al., 1993) is very subtle and is similar to the sac6Δ phenotype both in terms of the effects on growth and on actin organization (Adams et al., 1991). Furthermore, if the actl-120 mutation

Figure 4. Location of the actl-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 act1-120 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 act1-120 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 cryo-electron microscopy and image processing. Their results indicate that α-actinin interacts with actin subdomain 1, the subdomain in which the act1-120 and act1-3 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 act1-120 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 act1-120 mutation, suggesting that myosin can functionally interact with act1-120 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 act1 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 s1c1 and s1c2 (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 sac6Δ alleles with the conditional-lethal charged-to-alanine act1 alleles to test for genetic suppression. Significantly, act1-120, 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 resides E99 and/or E100 are important for the interaction with fimbrin. Suppression was observed for one additional charged-to-alanine allele, act1-125 (K50A,D51A; actin subdomain 2) (Honts et al., 1994). The act1-125 allele was not implicated in the interaction with Sac6p by the synthetic lethal approach because this act1 allele is synthetically lethal with sac6Δ (Table II). The synthetic lethality between act1-125 and sac6Δ indicates that this act1 allele affects more than the actin-Sac6p interaction. Otherwise, deletion of the SAC6 gene should not make the act1-125 phenotype more severe, a prediction that was met for the act1-120 allele. Furthermore, a sac6 null mutant is viable and grows well over a larger temperature range (Adams et al., 1991) than an act1-125 mutant (Wertman et al., 1992). Presumably, the suppressor approach implicated act1-125 as impairing the Sac6p-actin interaction because the interaction of the sac6-null protein with act1-125 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.

We thank A. Adams and J. Honts for plasmids and helpful discussions; T. Sandrock and A. Moon for the generous gifts of yeast fimbrin and yeast cofilin, respectively; K. McDonald for performing the electron microscopy; H. Nelson for assistance with the molecular graphics; and K. Ayscough, G. Barnes, R. Li, and A. Moon for comments on the manuscript. We are grateful to A. McGough, M. Way, D. DeRosier, J. Honts, T. Sandrock, S. Brower, J. O'Dell, and A. Adams for sharing the results of their studies before publication.

This work was supported by grants from the National Institute of General
Medical Sciences (GM-42759) and the Searle Scholars/The Chicago Community Trust.

Received for publication 4 February 1994 and in revised form 29 March 1994.

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