Actin Mutations that Show Suppression with Fimbrin Mutations Identify a Likely Fimbrin-binding Site on Actin

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Abstract. Actin interacts with a large number of different proteins that modulate its assembly and mediate its functions. One such protein is the yeast actin-binding protein Sac6p, which is homologous to vertebrate fimbrin (Adams, A. E. M., D. Botstein, and D. G. Drubin. 1991. *Nature (Lond.).* 354:404–408.). Sac6p was originally identified both genetically (Adams, A. E. M., and D. Botstein. 1989. *Genetics.* 121:675–683.) by dominant, reciprocal suppression of a temperature-sensitive yeast actin mutation (*actl-1*), as well as biochemically (Drubin, D. G., K. G. Miller, and D. Botstein. 1988. *J. Cell Biol.* 107: 2551–2561.). To identify the region on actin that interacts with Sac6p, we have analyzed eight different *actl* mutations that show suppression with *sac6* mutant alleles, and have asked whether (*a*) these mutations occur in a small defined region on the crystal structure of actin; and (*b*) the mutant actins are defective in their interaction with Sac6p in vitro. Sequence analysis indicates that all of these mutations change residues that cluster in the small domain of the actin crystal structure, suggesting that this region is an important part of the Sac6p-binding domain. Biochemical analysis reveals defects in the ability of several of the mutant actins to bind Sac6p, and a reduction in Sac6p-induced cross-linking of mutant actin filaments. Together, these observations identify a likely site of interaction of fimbrin on actin.

THE actin cytoskeleton is a fundamental component of eukaryotic cells, with widely divergent roles within individual cells and among different cell types. For example, even within a single cell, actin may be important in cell locomotion, the intracellular movement of organelles, the structural integrity of the cell, and cytokinesis. For some of its functions, actin is found as a stable array of filaments, whereas for others, it is present in a highly dynamic state. Actin can exist in the cell as either monomers or filaments, and the filaments themselves can vary enormously in both length and degree of cross-linking. Such different assembly states and the changes between them must be very tightly controlled both temporally and spatially, but the mechanisms by which these processes are both mediated and regulated remain poorly understood.

A large number of proteins that can affect the assembly state of actin in vitro have been identified, and it is likely that many of these also affect the assembly state and distribution of actin in vivo. Many of these proteins have been well characterized biochemically, but in general, it has been difficult to determine which of the various biochemical activities have biological significance, what the roles of these proteins are in the cell, and how the various proteins function together in vivo. We and others have applied genetic methods to this problem. In particular, we have used yeast as a system to identify actin-binding proteins both genetically and biochemically, make mutations in the genes encoding these proteins, and then study the effects of these mutations both in vivo and in vitro.

One such actin-binding protein is the yeast homolog of the actin-filament cross-linking protein fimbrin (Adams et al., 1991), which is encoded by the *SAC6* gene. *SAC6* was originally identified both biochemically on actin affinity columns (Drubin et al., 1988; Adams et al., 1989), as well as genetically by dominant, allele-specific suppression of the temperature-sensitive (Ts)[1] *actl-3* mutation (Adams and Botstein, 1989). Further genetic analysis showed that suppression is reciprocal i.e., that not only do the *sac6* mutations suppress the *actl-3* mutation, but also *actl* mutations suppress temperature-sensitive *sac6* mutant alleles. The observation of allele-specific and reciprocal suppression between various *actl* and *sac6* mutations (Adams and Botstein, 1989) provides strong genetic evidence for a physical interaction between actin and the *SAC6* protein, Sac6p. Indeed, the physical association of Sac6p with actin has been demonstrated by its ability to bind to an F-actin affinity column (Drubin et al., 1988), its ability to bundle actin filaments in vitro (Adams et al., 1991), and by its colocalization with actin in vivo (Drubin et al., 1988). The deduced sequence of Sac6p, which indicates it is a member of the fimbrin family of actin-bundling proteins (Adams et al., 1991), provides further sup-

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1. *Abbreviation used in this paper*: Ts, temperature sensitive.

port for the notion that Sac6p is a bona fide actin-binding protein.

To fully understand the interaction between actin and Sac6p, it is necessary to identify the sites on actin that bind Sac6p. This analysis is greatly facilitated by the fact that the crystal structure of actin has been solved (Kabsch et al., 1990). We expected that mutations in actin that suppress (or are suppressed by) mutations in *SAC6* might define the Sac6p-binding site of actin, and in particular (*a*) would all change residues that lie in a discrete physical locus in the three-dimensional structure of actin; and (*b*) would interfere directly with Sac6p-actin binding, and hence Sac6p-induced cross-linking of actin filaments in vitro. We have therefore examined eight different *act1* mutations that show suppression with *sac6* mutations. We show that all of these mutations change residues that lie in a small region of the actin surface, and that some of these mutations result in defects in the binding of Sac6p to (and the subsequent Sac6p-induced cross-linking of) actin filaments. These results therefore define a likely binding site for Sac6p on actin, and have important implications for understanding the interaction between actin and Sac6p.

Materials and Methods

Yeast Strains and Media

The yeast strains used in this study are listed in Table I. Media for yeast growth were as described previously (Sherman et al., 1974), except that in the growth of yeast cultures for actin preparation, the media yeast extract-Bacto-peptide contained 4% dextrose.

Chemicals

DNase I from bovine pancreas (grade II) and ATP (crystallized disodium salt, special quality) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). DTT (molecular biology grade), formamide, and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). The formamide was deionized by use of a mixed bed resin (AG 501-X8; Bio Rad Laboratories, Richmond, CA) and stored in aliquots at −70°C.

Isolation and Sequencing of act1 Mutations

Four *act1* mutations (*act1-7, act1-8, act1-9,* and *act1-10*) that had been identified as suppressors of *sac6-6* or *sac6-7* (Adams and Botstein, 1989) were isolated from strains DBY5236, DBY5244, DBY5254, and DBY5241, respectively (Table I). Genomic DNA was isolated from these strains by a modification of the method of Struhl et al. (1979), the actin genes were amplified by PCR (Perkin-Elmer Cetus kit, Perkin-Elmer Cetus Instruments, Norwalk, CT), and the PCR products were then sequenced directly using a double-stranded DNA cycle sequencing system (catalogue no. 8196SA; GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions, except that γ-^{33}P-labeled ATP (catalogue no. NEG302H; New England Nuclear, Boston, MA) was used instead of γ-^{32}P-labeled ATP. For each mutation, the entire coding region of the actin gene was sequenced to ensure that the mutations identified were the only changes in the sequence and, thus, were responsible for the phenotypes.

Localizing the act1 Mutations on the Structural Model of Actin

The crystal structure of rabbit muscle actin in a 1:1 complex with DNAse I was described by Kabsch et al. (1990). Recently, a refined structure of the actin filament has been described by Lorenz et al. (1993), and the atomic coordinates of the F-actin monomer were kindly made available to us by M. Lorenz (Max-Planck Institut fur Medizinische Forschung Abteilung Biophysik, Heidelberg, Germany). These latter coordinates were used to display the actin models shown in Fig. 1. Images of the actin monomer were generated using an IRIS Indigo XZ 4000 workstation (Silicon Graphics Computer Systems, Mountain View, CA) using the Viewer module of the Insight II program (Biosym Technologies, San Diego, CA). Since the primary sequences of rabbit muscle and yeast actins are 87% identical and 92% similar overall, it is likely that the three-dimensional structures of these two proteins will prove to be nearly identical (Chothia and Lesk, 1986). All of the residues affected by the mutations described in this study are identical between these two molecules, and they lie in a region of the

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source/reference
AAY1021*	*MATa act1-3 tub2-201 sac6-2 ura3*	This study
AAY1111*	*MATα act1-3 sac6-5 his4-619*	This study
AAY1115*	*MATα act1-3 sac6-15 his4-619*	This study
AAY1643‡	*MATα act1-2 sac6-2 his4*	This study
AAY1644§	*MATa act1-7 ura3*	This study
AAY1645§	*MATa act1-8 leu2*	This study
AAY1646§	*MATa act1-9*	This study
BJ5628‖	*MATa/MATα ura3/ura3 trp1/+ his3/his3 pep4::HIS3/pep4::HIS3 prb1/prb1 can1/can1 leu2/+Gal*$^+$	Jones (1991)
DBY877	*MATα his4*	Adams and Botstein (1989)
DBY5236	*MATα sac6-6 act1-7 ura3-52*	Adams and Botstein (1989)
DBY5241	*MATα sac6-6 act1-10 ura3-52*	Adams and Botstein (1989)
DBY5244	*MATα sac6-6 act1-8 ura3-52*	Adams and Botstein (1989)
DBY5254	*MATa sac6-7 act1-9 ura3-52*	Adams and Botstein (1989)
KWY361¶	*MATa act1-120::HIS3 can1 ade4 his3 leu2 ura3 tub2-201 cry1?*	Ken Wertman
KWY376¶	*MATa act1-125::HIS3 can1 ade2 his3 leu2 ura3 tub2-201*	Ken Wertman

* These strains are segregants from the original diploid *act1-3/act1-3 sac6/+* revertants described in Adams and Botstein (1989).
‡ AAY1643 was a Ts+ (therefore *sac6-2)TUB2+* (therefore assumed to carry the tightly linked *act1-2* mutation) segregant obtained from a cross of AAY1021 to DBY1991 (*MATα act1-2 TUB2+ his4-619*; Adams and Botstein, 1989).
§ AAY1644, AAY1645, and AAY1646 are Ts segregants derived from crosses of DBY2059 or DBY2060 (Adams and Botstein, 1989) to DBY5236, DBY5244, and DBY5254 (Adams and Botstein, 1989), respectively.
‖ BJ5628 was obtained from the Yeast Genetics Stock Center (Donner Laboratory, University of California at Berkeley). These strains were obtained from K. Wertman (Selectide Corp., Oro Valley, AZ).
¶ The *act1* alleles in these strains are described in Wertman et al. (1992).

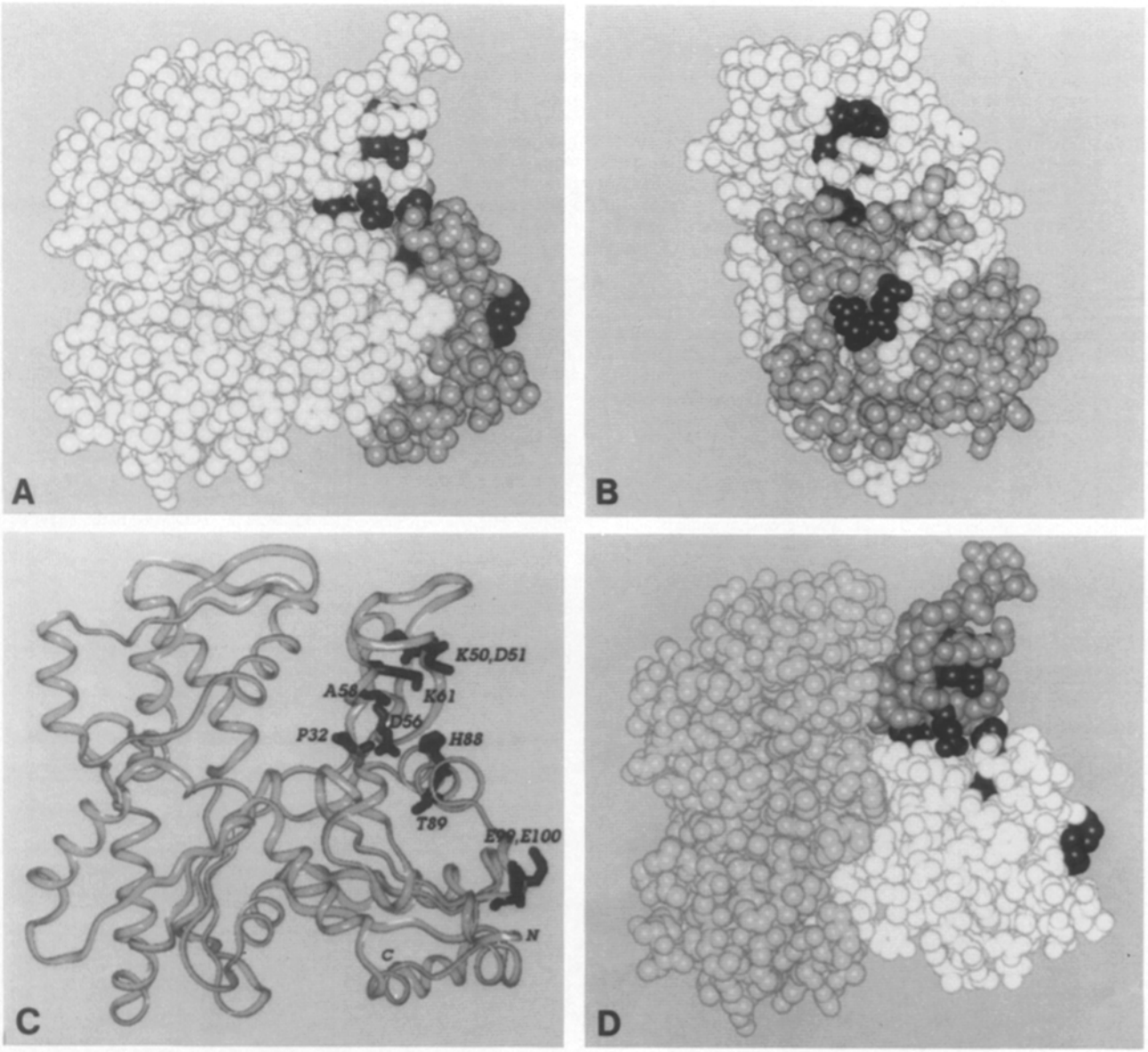

Figure 1. Location in a model of rabbit muscle actin of residues corresponding to those changed by the yeast *act1* suppressor mutations. A model of the rabbit muscle actin molecule was displayed from the atomic coordinates of the rabbit muscle actin as determined by Lorenz et al. (1993). (*A*) A space-filling model of the actin molecule (excluding the hydrogen atoms) is shown in approximately the same orientation as seen in Fig. 1 in the original actin crystal structure paper (Holmes et al., 1990). The residues corresponding to the mutations in yeast actin that suppress or are suppressed by *sac6* mutant alleles are indicated in black. The residues that correspond to peptides implicated by other studies (Mimura and Asano, 1987; Lebart et al., 1990, 1993; Levine et al., 1992; Méjean et al., 1992; Fabbrizio et al., 1993) as binding to the conserved actin binding domain referred to in the text (see Discussion) are shaded grey (actin residues 1–12, 83–125, and 350–375). (*B*) The actin molecule after a rotation of 90° from the orientation shown in Fig. 1 *A*. (*C*) A ribbon diagram of yeast actin in the same orientation as in Fig. 1 *A*. The amino acids affected by the suppressor mutations listed in Table II are represented as stick figures projecting from a ribbon describing the course of the polypeptide chain. The positions of the amino and carboxy termini of actin are also indicated (*N* and *C*, respectively). (*D*) Subdomains 1 and 2 (referred to in the text) of the actin monomer are shaded white and dark grey, respectively. These two subdomains constitute the "small domain" of actin (see Kabsch et al., 1985). The suppressor mutations are shaded black as in Fig. 1 *A*.

actin molecule that is highly conserved. The amino acid residues changed by the suppressor mutations are highlighted in black in Fig. 1.

Biochemical Purification of Yeast Actin

Yeast actin was purified from strains AAY1021, AAY1643, AAY1644, AAY1645, AAY1646, DBY877, DBY5241, KWY361, and KWY376 (Table I) by DNase I affinity chromatography essentially as described by Kron et al. (1992), but incorporating modifications described by Cook and Rubenstein (1992) and the additional changes noted below. Details of the purification will be provided by the authors upon request.

Subsequent to elution from the DNase I column, actin was eluted from a column (DE-52); Whatman Chemical Separation Inc., Clifton, NJ) as described by Cook and Rubenstein (1992), except that the elution used a step gradient of column buffer containing 50, 100, 200, and 300 mM KCl instead of a single 300-mM step. In most purifications, the majority of the actin

eluted with 200 mM KCl, and only this fraction was processed further. However, in the case of the *act1-120* strain (E99A, E100A), most of the actin eluted at 100 mM KCl.

The actin-enriched fractions from the DE-52 elution were dialyzed at room temperature for several hours against 2.0 liters of F-actin buffer (50 mM Hepes-K^+, pH 7.5, 50 mM KCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5 mM DTT) to reduce the concentration of KCl to ~50 mM. This step appeared to ensure the maximal recovery of the actin filaments, especially in the case of some of the mutant actins, which appeared to assemble poorly in elevated salt concentrations.

Finally, the F-actin was pelleted, depolymerized by dialysis (against 5 mM Hepes-K^+, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, plus protease inhibitors at final concentrations of 1 $\mu g\ ml^{-1}$ for chymostatin, pepstatin, leupeptin, antipain, and 1 mM for PMSF), and the resulting G-actin solution was clarified essentially as described previously. The supernatant was recovered, and a Bradford's dye binding assay (BioRad Laboratories) was used to estimate the protein concentration. If the protein concentration was $\geq$0.5 mg ml^{-1}, the clarified G-actin solution was rapidly frozen as small aliquots (30–50 μl) in liquid nitrogen and stored at −70°C until used in the binding and cross-linking assays described below. Clarified dialysates with protein concentrations <0.5 mg ml^{-1} usually indicated a failure to purify actin, and they were not processed further.

There were no obvious contaminating proteins or proteolytic fragments, as judged by SDS gel electrophoresis at the loadings (1.6 μg of actin) used for binding and cross-linking assays (Fig. 2). When greater amounts of actin were loaded (>10 μg/lane), several faint bands could be seen below the single major band at 43 kD. We presume, based on previous experiments, that these bands are products of proteolytic degradation of actin.

Biochemical Purification of Sac6p

Sac6p was purified from strain BJ5628 (Table I) containing a plasmid expressing Sac6p from the *GALI-10* promoter. The details of this purification will be presented in a separate publication (Sandrock, T., and A. Adams, manuscript in preparation). Briefly, the cell lysate was fractionated by DEAE chromatography, ammonium sulfate precipitation, and hydroxylapatite chromatography to yield fractions highly enriched in Sac6p. Elution from the hydroxylapatite column yielded two overlapping peaks, the first of which was used in all experiments described in this paper. Preliminary results suggest Sac6p from the second peak discriminates less well between wild-type and mutant actins in the binding and cross-linking assays. The nature of the biochemical difference between the protein in the two peaks is currently under active investigation.

Actin Filament Binding and Cross-linking Assays

The concentrations of purified actin and Sac6p were estimated by using the Bradford dye binding assay, with gamma globulin at a known concentration serving as the protein standard. Binding and cross-linking assays were set up as 100-μl reactions with actin at 3 μM and Sac6p at 0.3 μM, a molar ratio that had been shown by electron microscopy to produce actin bundles with wild-type yeast actin and Sac6p (Adams et al., 1991).

Immediately before use, frozen aliquots of G-actin were rapidly thawed and clarified by centrifugation at 70,000 rpm (190,000 *g*) for 30 min at 4°C in a TLA-100 rotor (Beckman Instruments, Palo Alto, CA) to remove insoluble aggregates. Purified Sac6p, which had been diluted in depolymerization buffer, was clarified in the same manner before use. The clarified protein solutions were kept on ice and were promptly used in the assays described below.

Binding assays were performed by adding actin to freshly made depolymerization buffer (with the protease inhibitors), followed by the addition of Sac6p. Finally, polymerization was initiated by the addition of 20× polymerization buffer (450 mM Hepes-K^+, pH 7.5, 20 mM EGTA, 500 mM KCl, and 80 mM $MgCl_2$), and the mixture was gently pipetted up and down. These mixtures were then incubated at 22°C for 90 min. After this time, a 25-μl sample was withdrawn from the mixture, and mixed with an

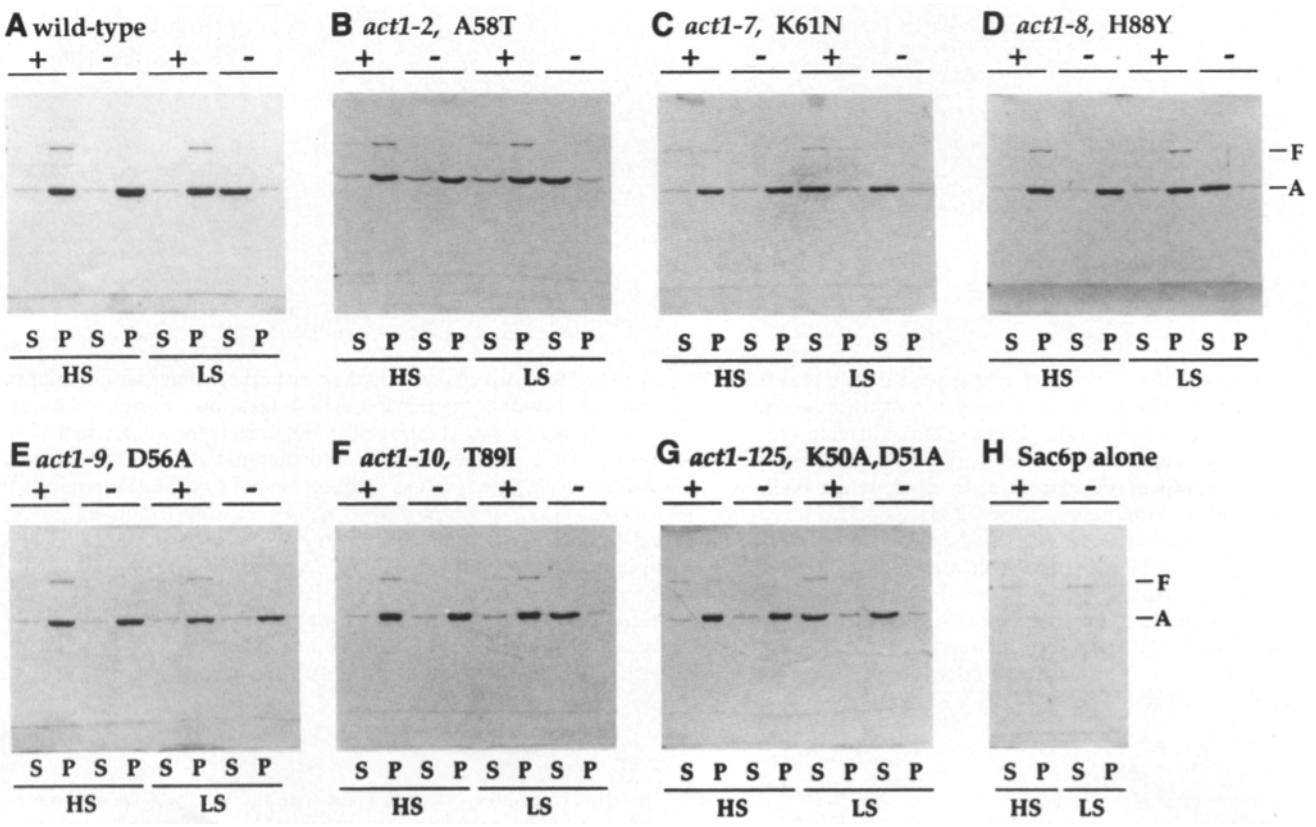

Figure 2. Actin-binding and cross-linking assays for wild-type and mutant actins paired with wild-type Sac6p. SDS-PAGE of supernatants (*S*) or pellets (*P*) obtained by high speed (*HS*) or low speed (*LS*) centrifugation of wild-type or mutant actins polymerized in the presence (+) or absence (−) of Sac6p, as described in Methods. The positions of yeast actin (*A*) and yeast fimbrin (*F*) protein bands in the gels are indicated. The proteins were resolved in a 10% SDS-polyacrylamide gel and stained with Coomassie blue R-250. The data are representative of at least two separate experiments.

equal volume of 2× gel sample buffer. The mixture (remaining 75 μl) was then centrifuged at 190,000 *g* in a TLA-100 rotor at 22°C for 30 min. Under these conditions, almost all wild-type actin sediments, whether or not Sac6p is present (Fig. 2). However, under nonpolymerizing conditions, essentially no actin sediments (not shown). After centrifugation, a 25-μl sample of the supernatant was withdrawn from the meniscus and mixed with an equal volume of 2× gel sample buffer. The remainder of the supernatant was carefully removed and discarded, and the remaining pellet (usually not visible) was solubilized with 150 μl of 1× gel sample buffer. Thus, the total mixture, the high speed supernatant, and the high speed pellet were all solubilized in gel sample buffer at the same relative concentration. The protein samples were boiled for 5 min, separated by SDS-PAGE (25 μl sample/lane), and then stained with Coomassie blue.

The cross-linking assays were performed in a similar manner, except that the mixtures were centrifuged in a TLA-100 rotor at 12,000 rpm (6,000 *g*) for 15 min at 22°C instead of 190,000 *g* for 30 min at 22°C. This degree of centrifugation was sufficient to pellet almost all wild-type actin and Sac6p, when the two were mixed in this assay, but it was insufficient to sediment most wild-type actin in the absence of Sac6p (Fig. 2). These assays do not distinguish between cross-linked filaments and nonspecific aggregates, but because the actin is found in the low speed pellet in the presence (but not the absence) of Sac6p (Fig. 2), we presume it is present as cross-linked filaments. In each experiment, control experiments without the addition of Sac6p were conducted in parallel to assess the self-aggregation ability of actin filaments.

These experiments were performed at 22°C, rather than at 37°C (the restrictive temperature of most of the *act1* mutants) because our preliminary observations indicated that even wild-type actin was poorly cross-linked by Sac6p at 37°C. The use of 22°C for these experiments is validated by the fact that some of the *act1* mutants grow poorly (and thus have defective actin) even at 22°C, and all of the *act1* mutant actins analyzed biochemically in this study can suppress the *sac6* temperature-sensitive defects (and thus show altered interactions with mutant Sac6p) at this temperature (Table II, legend).

Electron Microscopy

Samples prepared in the cross-linking assays described above were also used for electron microscopy. Aliquots were removed from the mixtures before centrifugation, and they were immediately negatively stained with 2% uranyl acetate. Using a JEOL 100cx or Hitachi 500 microscope, wild-type mutant filaments were scored by an observer unaware of their identity for ability to be bundled in the presence or absence of Sac6p.

Results

The goal of these experiments was to test the hypothesis that *act1* mutations that suppress or are suppressed by *sac6* mutations identify the Sac6p-binding domain on actin. Two predictions of this hypothesis were: (*a*) that the *act1* mutations would fall into a small region of the actin molecule, the likely Sac6p-binding site; and (*b*) that biochemical experiments with actin and Sac6p would reveal a direct defect in the interaction between these two proteins. Experiments were therefore conducted to test these predictions.

Identification of Additional act1 Mutations that Show Suppression with sac6 Mutants

We wished to analyze as large a collection as possible of *act1* mutations that suppress or can be suppressed by *sac6* mutant alleles. We already had at hand six *act1* alleles (*act1-2*, *act1-3*, *act1-7*, *act1-8*, *act1-9*, and *act1-10*; see Table II) that suppressed *sac6* mutations, but to further increase the pool, we examined a set of eight temperature-sensitive *act1* mutations generated by alanine-scanning mutagenesis (Wertman et al., 1992). These eight mutations were chosen from the larger collection of such mutants as those that showed the tightest temperature-sensitive growth defects at restrictive temperature, as well as reasonably good growth at permissive temperature (Wertman et al., 1992).

We first tested whether any of the eight *act1* mutations could be suppressed by any one of seven different *sac6* mutant alleles isolated previously as dominant suppressors of *act1-3*. Thus, temperature-sensitive *ura3* strains carrying *act1-105*, *act1-108*, *act1-112*, *act1-119*, *act1-120*, *act1-125*, *act1-132*, or *act1-133* (Wertman et al., 1992) were transformed with centromere-containing plasmid carrying the selectable marker *URA3* and either wild-type *SAC6* or one of the suppressor mutations *sac6-4*, *sac6-5*, *sac6-7*, *sac6-14*, *sac6-15*, *sac6-17*, or *sac6-19*. Ura$^+$ transformants were selected and tested for temperature sensitivity. Two *act1* alleles were found to be suppressed by at least some *sac6* alleles: *act1-120* was consistently and strongly suppressed by all seven *sac6* alleles tested, and *act1-125* was consistently (though less strongly) suppressed by plasmids carrying *sac6-4*, *sac6-5*, or *sac6-15*. Interestingly, even the centromere-containing plasmid carrying wild-type *SAC6* showed some suppression of *act1-125*, suggesting the defect in *act1-125* is caused by reduced affinity for Sac6p, and can also be suppressed by increased levels of wild-type Sac6 protein. None of the other *act1* mutants were consistently suppressed by any of the *sac6*

*Table II. Temperature Sensitivity and Sequence Changes of act1 Mutations**

act1 allele	*act1 SAC6*$^+$ Ts− or Ts+	Residue changed	Subdomain‡	Reference
act1-2	Ts−	A58T	2	Shortle et al. (1984)
act1-3	Ts−	P32L	1	Shortle et al. (1984)
act1-7	Ts−	K61N	2	Adams and Botstein (1989)
act1-8	Ts−	H88Y	1	Adams and Botstein (1989)
act1-9	Ts−	D56A§	2	Adams and Botstein (1989)
act1-10	Ts+	T89I	1	Adams and Botstein (1989)
act1-120	Ts−	E99A,E100A	1	Wertman et al. (1992)
act1-125	Ts−	K50A,D51A	2	Wertman et al. (1992)

The *act1* mutant alleles listed all suppress *sac6* mutations, and in all cases where the *act1* mutation is Ts in a *SAC6*+ genetic background (see table above), they are also suppressed by *sac6* mutant alleles.

* The temperature-sensitive phenotypes and sequences of *act1-2*, *act1-3*, *act1-120*, and *act1-125* have been reported previously (Shortle et al., 1984; Wertman et al., 1992). The phenotypes of the *act1-7*, *act1-8*, *act1-9*, and *act1-10* mutants were described previously (Adams and Botstein, 1989); the sequences of these latter four mutations were determined in this study (see text).

‡ Subdomains refer to the nomenclature of Kabsch et al. (1990).

§ The sequence of *act1-9* (D56A) reveals that this mutation is very similar to that of *act1-124* (D56A, E57A), described by Wertman et al. (1992). The suppressibility of *act1-124* by *sac6* mutations has not been tested.

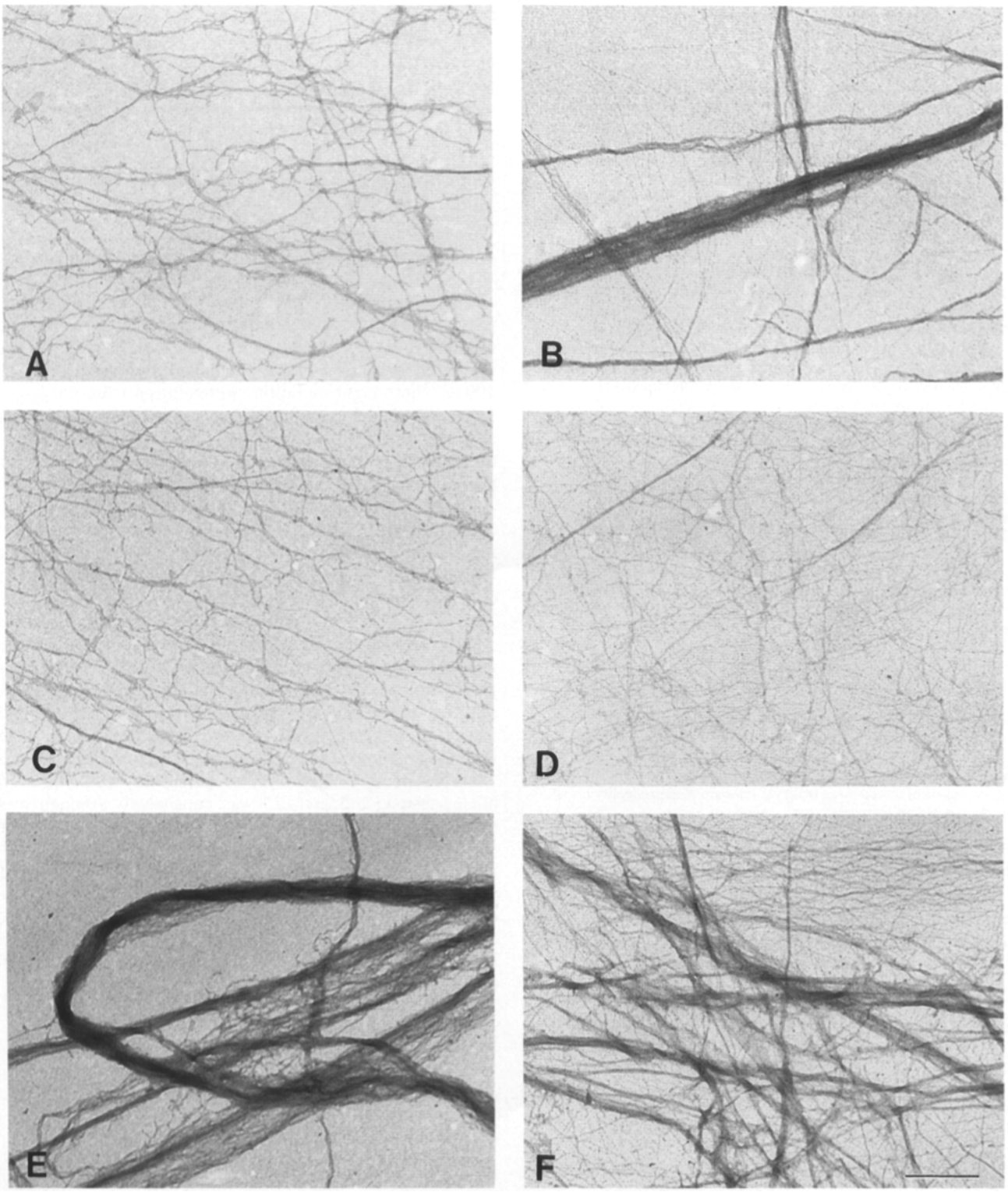

Figure 3. Electron microscopy of actin filaments in the absence and presence of Sac6p. Samples of the bundling assays were examined by negative staining using electron microscopy. The reaction conditions are described in Materials and Methods. (*A* and *B*) Wild-type actin filaments in the absence (*A*) and in the presence (*B*) of Sac6p. Most of the actin filaments appear to be found in bundles in the presence of Sac6p. (*C* and *D*) *act1-7* actin filaments in the absence (*C*) and presence (*D*) of Sac6p. *act1-7* filaments are clearly defective in the formation of bundles when compared to wild-type actin; instead, many single filaments are visible. (*E* and *F*) *act1-9* actin filaments in the absence (*E*) and presence (*F*) of Sac6p. Bundle-like aggregates of *act1-9* filaments are observed even in the absence of Sac6p, but the bundles seen in the presence of Sac6p appear more similar to wild type.

alleles tested, and they were not studied further. (It should be noted that if any of the *sac6* mutations were recessive suppressors of these *act1* alleles, however, they would not have shown suppression in this analysis.) This analysis therefore succeeded in identifying two *act1* alleles (*act1-120* and *act1-125*) that can be suppressed by *sac6* mutant alleles on plasmids.

Tetrad analysis was used to ensure that the observed suppression of *act1-120* and *act1-125* by *sac6* mutant alleles occurred not only when the *sac6* mutations were present on plasmids, but also when they were in the genome. Thus, in crosses between KWY361 (*act1-120 SAC6*$^+$) and AAY1115 (*act1-3 sac6-15*), and between KWY376 (*act1-125 SAC6*$^+$) and AAY1111 (*act1-3 sac6-5*), temperature sensitivity segregated 2:2, confirming that *act1-120* is suppressed by *sac6-15*, and *act1-125* is suppressed by *sac6-5* (data not shown). For the purposes of this study, we were interested in identifying *act1* mutations that could be suppressed by at least one *sac6* allele, rather than in identifying the spectrum of *sac6* mutations that could or could not suppress the various *act1* alleles. Lack of suppression is less meaningful than suppression itself because it is likely that suppression depends greatly on the nature of the mutational change and, therefore, that many possible amino acid substitutions at a particular residue will not cause suppression. Therefore, as *act1-120* and *act1-125* can both be suppressed by at least one *sac6* mutant allele, these mutations were added to the collection of mutants to be analyzed further, giving a total of eight (Table II).

Isolation and Sequencing of act1 Mutations

Four of the eight *act1* mutations that suppress or are suppressed by *sac6* mutant alleles (i.e., *act1-2*, *act1-3*, *act1-120*, and *act1-125*) had already been sequenced (Shortle et al., 1984; Wertman et al., 1992); the changes are listed in Table II. The remaining four mutations (*act1-7*, *act1-8*, *act1-9*, or *act1-10*) had not yet been sequenced, and were therefore isolated and analyzed. Genomic DNA was prepared from strains carrying these mutations (Materials and Methods), the mutant actin genes were amplified by PCR, and the *act1* mutations were identified by sequencing. In each of these four cases, the mutations identified (Table II) were shown to be the only changes in the entire *act1* sequence (see Materials and Methods), and thus were responsible for the suppression phenotype.

Location of Actin Mutations in a Model of Actin

The eight *act1* mutations that show suppression with *sac6* alleles are found widely distributed in the primary structure of actin, from residues 32–99/100 (Table II). If these *act1* mutations occur in the Sac6p-binding domain on actin, we predict that in the three-dimensional structure of actin the mutations might occur in a small region, rather than be scattered over the surface of the molecule. Because of the highly conserved nature of actin (see Materials and Methods), it is possible to identify the relative positions of the affected residues in the three-dimensional structure of either the rabbit or yeast actin molecules with a high degree of confidence. The molecular model of rabbit actin was used for Fig. 1. Strikingly, the eight actin mutations in Table II all change residues in one region of the "small domain" (Kabsch et al., 1985) of actin. According to the nomenclature of Kabsch et al. (1990), the small domain is comprised of subdomain 1 (residues 1–32, 70–144, and 338–375) and subdomain 2 (residues 33–69) (see Fig. 1 *D*). It can be seen in Fig. 1 that all of these mutations are relatively near to one another, and they localize either to subdomain 2 or to the part of subdomain 1 adjacent to subdomain 2 (Fig. 1 *D*). This clustering of mutant residues suggests that Sac6p binds to this region of the actin molecule; consistent with this idea, most mutations that are not suppressed by *sac6* mutations are located elsewhere in the molecule (not shown).

Biochemical Analysis of Wild-type and Mutant Actin and Sac6p

The finding that all the actin mutations that show suppression with *sac6* mutations alter residues in a relatively small region of the actin crystal structure suggested that this region of actin forms at least part of the Sac6p-binding domain. This led to the strong prediction that the mutant actins would be defective in their interactions with Sac6p, under conditions in which these were the only two proteins present. We therefore tested the ability of wild-type Sac6p to bind to and cross-link actin filaments in vitro, using purified Sac6p and actin.

Since both the Sac6p-binding and Sac6p-induced cross-linking assays depended on the ability of the mutant actins to polymerize, it was first necessary to show that polymerization was not grossly affected by the changes. Indeed, all of the mutant actins were able to undergo polymerization, as judged by increases in viscosity, ability to sediment during high speed centrifugation (Fig. 2), and electron microscopy of mutant filaments (e.g., Fig. 3). It was therefore possible to analyze all of the mutant actins in the Sac6p-binding and Sac6p-induced cross-linking experiments described below.

Binding of Sac6p to actin filaments was assessed by mixing actin and Sac6p, initiating the assembly of actin filaments, and recovering the filaments (either individual or cross-linked) by high speed centrifugation (see Materials and Methods). Samples of the mix before centrifugation (not shown), the supernatant after centrifugation, and the resulting pellet were solubilized for SDS-PAGE. The samples were adjusted to the same relative concentrations and separated in gels (Fig. 2). Defective binding of Sac6p to mutant actin would be expected to result in an increase in the amount of Sac6p remaining in the supernatant.

Cross-linking of actin filaments by Sac6p was tested similarly, except that cross-linked actin filaments, which sediment more rapidly than individual actin filaments, were recovered by low speed rather than high speed centrifugation. Under these conditions, the majority of individual filaments do not sediment (see below). Defective cross-linking of mutant actin by Sac6p would be expected to result in increased levels of both actin and Sac6p in the supernatant from the low speed centrifugation.

When wild-type actin was used in these experiments, almost all the actin and Sac6p was recovered in the high speed pellet (Fig. 2 *A*), indicating that actin was present as individual and/or cross-linked filaments to which Sac6p was bound. Since essentially all the actin and Sac6p was found in the low speed pellet also (Fig. 2 *A*), most of the actin in the assembly mix must be present as cross-linked structures, rather than individual filaments. This result is in contrast to

what is found in the absence of Sac6p, when essentially all the actin remains in the low speed supernatant.

When the various mutant actins were used in these assays, in each case, most of the actin was again found in the high speed pellet, and thus, like wild-type, was polymerization competent, as mentioned above. However, the amount of Sac6p that sedimented with the mutant actins varied. For example, in the case of *actl-125*, nearly all the Sac6p remained in the supernatant (Fig. 2 *G*), indicating that these filaments were drastically reduced in their ability to bind to Sac6p. In addition, most of these *actl-125* filaments were not sedimented by low speed centrifugation (Fig. 2 *G*), and thus were substantially less cross-linked than wild type. Similar defects in binding of Sac6p and ability to be cross-linked were observed for *actl-120* (Holtzman et al., 1994; our unpublished results). In the case of *actl-7*, about half of the Sac6p remained in the high speed supernatant (Fig. 2 *C*), indicating that these actin filaments did not bind Sac6p as well as did wild type. In addition, these *actl-7* filaments were not well sedimented by low speed centrifugation (Fig. 2 *C*), and thus were not as extensively cross-linked as wild type. Consistent with these data, relative to wild type, *actl-7* and *actl-125* actin filaments are defective in the formation of bundles as judged by electron microscopy (e.g., Fig. 3, *B* and *D*).

Less extreme defects in binding and crosslinking activities of Sac6p were observed in experiments with *actl-2*, *actl-8*, and *actl-10* filaments (Fig. 2, *B*, *D*, and *F*). For example, in the case of *actl-8* (Fig. 2 *D*), most of the Sac6p was found in the high speed pellet with the actin, indicating little or no defect in the binding of Sac6p to this mutant actin. Furthermore, most actin and Sac6p were also found in the low speed pellet, suggesting the mutant filaments were primarily cross-linked.

Interestingly, we could not assess the ability of *actl-9* actin filaments to be cross-linked because these mutant filaments were observed to undergo self-aggregation in the absence of Sac6p (Fig. 2 *E*), a result that was confirmed by electron microscopic observations of *actl-9* filaments in the absence of Sac6p (Fig. 3 *E*).

Unlike the mutant actins described above, *actl-3* actin showed variable degrees of defects in ability to be cross-linked by Sac6p in several different experiments (not shown), with the results ranging between those seen with *actl-2* and *actl-7* actins (Fig. 2, *B* and *C*, respectively).

Discussion

We report here the identification of a likely fimbrin-binding site on actin. This identification is based on two lines of evidence. First, eight out of eight actin mutations that show suppression with mutant alleles of yeast fimbrin (Sac6p) all cluster in one region of the "small domain" of actin (Fig. 1 *D*). Second, several of these *actl* mutations have a direct effect on the interaction of actin with Sac6p in vitro.

Location of actl Mutant Residues in the Actin Structure

The *actl* mutations that show suppression with *sac6* mutant alleles all lie between residues 32 and 100, and they cluster in the small domain of actin (Fig. 1 *D*). This concentration of mutant residues in the three-dimensional structure suggests that this region of actin is likely to be an important part of the Sac6p-binding site. Genetic studies of Holtzman et al. (1994) involving an analysis of synthetic-lethal interactions support this notion. Consistent with this possibility, this region is readily accessible in the filament model (Holmes et al., 1990; Milligan et al., 1990; Lorenz et al., 1993), and most of the *actl* mutations that fail to show suppression with *sac6* mutant alleles are located elsewhere in the molecule (not shown). It should be noted, however, that the *actl* suppressor mutations described in this study all show suppression with *sac6* alleles that were originally isolated as suppressors of *actl-3*. Our results therefore do not rule out the possibility that Sac6p also binds to other sites on actin. It might be possible, for example, to isolate a class of *sac6* mutations that show suppression with *actl* mutations that change residues elsewhere on the surface of actin. However, in the synthetic-lethal analysis of Holtzman et al. (1994), ~20 other *actl* mutations (that change residues all over the surface of actin) failed to show genetic interactions indicative of another Sac6p-binding site.

Defective Interactions of Mutant Actins with Sac6p

The hypothesis that the *actl* mutations analyzed in this study identify the Sac6p-binding domain on actin is also supported by the finding that several of the mutant actins examined are defective in their interactions with wild-type Sac6p in vitro. In particular, *actl-7*, *actl-120*, and *actl-125* actins show clear defects in their binding to (and subsequent cross-linking by) Sac6p, suggesting that the altered residues in the mutant proteins disrupt the normal interactions that occur between actin and Sac6p. For example, certain residues on actin and Sac6p may normally interact with each other, but can be prevented from doing so by mutations that directly change these particular residues. The actins most defective for binding to and cross-linking by Sac6p (*actl-7*, *actl-120*, and *actl-125*) all involve mutations that result in the substitution of polar or nonpolar residues for the charged residues of the wild-type form (Table II). This may imply that the actin-Sac6p interaction has an important electrostatic component.

Alternatively, the mutations may have more indirect effects, such as by causing local changes in the structure of the actin molecule in the region of the binding site. The *actl-2* mutation, for example, may have such an effect because it changes a residue that is believed to be buried in the actin molecule. The formal possibility that the mutations cause more global defects (e.g., in folding) in the whole actin monomer seems unlikely because the mutant actins are normal by several other criteria, including (*a*) ability to bind to other monomers in polymerization (as judged by the formation of a viscous solution upon addition of polymerization buffer and subsequent pelleting (Fig. 2), as well as by electron microscopy of mutant actin filaments (e.g., Fig. 3); (*b*) ability to bind to DNAse I in the initial stages of the purification (see Materials and Methods); and (*c*) at least in the case of *actl-120*, the mutant protein is still able to bind to cofilin (Holtzman et al., 1994). It therefore seems likely that the *actl* suppressor mutations identify a Sac6p-binding site on the surface of actin.

Comparison of Defects of Mutant Actin Filaments in Binding to (and Cross-linking by) Sac6p

Sac6p contains a tandem pair of imperfect repeats, both of which are homologous to the actin binding domains of sev-

eral other actin binding proteins (Matsudaira, 1991). Sac6p is presumably like other monomeric fimbrins (Bretscher, 1981; Glenney et al., 1981), which are assumed to use both actin-binding domains to cross-link adjacent actin filaments.

It is not known whether each of the actin-binding domains in fimbrin bind to the same or different sites on actin monomers in adjacent filaments. The homology between these domains suggests that they might interact with actin in a similar way. However, there are precedents for structurally homologous domains binding to different sites on actin. For example, the two homologous domains of the actin-bundling protein scruin are observed to bind to different subdomains of actin (Owen and DeRosier, 1993; Schmid et al., 1994).

Our data do not provide us with information as to whether the two actin-binding domains of Sac6p bind to the same or different sites on actin because we do not know how many Sac6p molecules are required to form an actin bundle, whether the interactions between actin and the two Sac6p domains are independent, or whether the two actin-binding domains are equivalent. For example, the finding that cross-linking is more defective that binding to Sac6p (e.g., for *actl-7*, and *actl-125*; see Fig. 2) could be explained by either model, i.e., that the two actin-binding domains bind to the same site or that they bind to different sites on actin.

Comparison of the Putative Binding Sites on Actin for Fimbrin and Related Actin-binding Proteins

Sac6p is a member of a group of actin cross-linking proteins including α-actinin, β-spectrin, filamin, dystrophin, fimbrin, and actin gelatin factor that share a homologous 27-kD actin-binding domain (Matsudaira, 1991). It might therefore be expected that these proteins would all bind to the same region on actin. The fimbrins (including yeast Sac6p) and closely related plastins possess a tandem repeat of this conserved actin-binding domain, but before this study, no information was available concerning the binding site for these proteins on the actin molecule. There have, however, been extensive studies of the binding site on actin for α-actinin, filamin, and dystrophin, each of which has a single conserved 27-kD actin-binding domain (Mimura and Asano, 1987; Lebart et al., 1990, 1993; Levine et al., 1992; Méjean et al., 1992; Fabbrizio et al., 1993). All of the peptide segments of actin implicated in binding to the conserved actin-binding domain in these studies are found within subdomain 1, and they are shown highlighted in gray in Fig. 1, *A* and *B*. These segments comprise residues 1–12 (NH_2-terminus of actin), residues 83–125, and residues 350–375 (COOH terminus of actin) (Mimura and Asano, 1987; Lebart et al., 1990, 1993; Levine et al., 1992; Méjean et al., 1992; Fabbrizio et al., 1993). However, there is little evidence for an interaction of the conserved actin-binding domain with actin in the regions encompassed by the suppressor mutations. In particular, only three (*actl-8*, *actl-10*, and *actl-120*, at residues 88, 89, and 99/100, respectively) of the eight suppressor mutations lie in any of the regions defined biochemically, the other five causing changes between residues 32 and 61 of actin.

It might seem from these comparisons that although α-actin, filamin, dystrophin, and fimbrin share a conserved actin-binding domain by sequence comparisons, they do not all bind to the same region on actin. As mentioned above, there are precedents for homologous domains not binding to the same sites on actin. However, it is also possible that these proteins all bind to the same region on actin, and that two fundamentally different approaches have identified different regions of actin that bind to the conserved actin-binding domain. In this case, it would be expected that biochemical experiments with actin and fimbrin, similar to those conducted for the other proteins and actin, would identify the same sites of interaction. It may be, for example, that many mutations in the region identified biochemically would be lethal, and thus not identified as suppressors. Or perhaps the residues identified by suppressor analysis interact more weakly with the conserved actin-binding domain than do those identified biochemically, so that in biochemical experiments, the weaker interaction is not detected. Consistent with this notion, a thrombic fragment of actin (residues 40–113), which contains most of the region identified genetically, has been found to be bound by filamin, albeit about two orders of magnitude more weakly than other peptides derived from subdomain 1 (Méjean et al., 1992). Of course, it may also be that the observed lower affinity of filamin for the actin thrombic peptide results from the inability of this peptide to refold after purification (Méjean et al., 1992), that it has a different structure, or that the weak binding is not physiologically relevant.

Evidence in favor of the notion that the conserved actin-binding domain binds to the sites on actin identified both genetically (this study and Holtzman et al., 1994) and biochemically (the previous studies described above) comes from the work of McGough et al. (1994). In that study, an image reconstruction of the actin-binding domain of α-actinin bound to filamentous actin is reported, and it presents evidence that the sites identified by both the biochemical and genetic approaches may both be relevant (McGough et al., 1994). Their analysis indicates that the actin-binding domain of α-actinin is centered around subdomain 2 and makes contacts with subdomain 1 of the same actin monomer, as well as subdomain 1 of the adjacent actin monomer along the long-pitch strand (McGough et al., 1994). Similar multidomain interactions have been observed for other actin-binding proteins. For example, the myosin subfragment 1 has been modeled as making contacts not only with subdomain 1, but also with subdomain 2 of actin (Rayment et al., 1993).

In summary, our results and those of Holtzman et al. (1994) identify a likely binding site on actin for yeast fimbrin. By comparison to the structural analysis of the actin-binding domain of α-actinin by McGough et al. (1994), it would seem reasonable to expect that fimbrin, α-actinin, and the other actin cross-linking proteins that share the conserved actin-binding domain may all bind to the same region of actin. However, even if these different proteins do share a common actin binding site, it remains to be seen whether there are additional contacts that serve to differentiate the interaction of these proteins with actin. In support of this notion, it has been noted that there are differences between α-actinin and filamin in the ionic strength dependence of their binding to actin, implying the existence of additional contacts that differentiate the two complexes (Lebart et al., 1993, 1994).

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