In vitro analysis of the interaction between yeast fimbrin and actin: A model for a mechanism of allele-specific suppression

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IN VITRO ANALYSIS OF THE INTERACTION BETWEEN YEAST FIMBRIN AND ACTIN: A MODEL FOR A MECHANISM OF ALLELE-SPECIFIC SUPPRESSION

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by TANYA MARIE SANDROCK

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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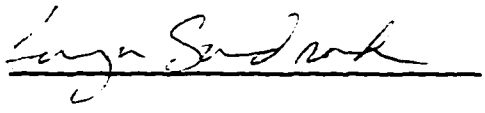
SIGNED: 

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DEDICATION

To Mom and Dad

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ABSTRACT

The actin cytoskeleton in yeast plays a role in many morphological events such as cell growth, secretion, polarity, bud emergence, and endocytosis (125). Some proteins have been identified that regulate the elongation, nucleation, and stability of the actin cytoskeleton (101). One of the many proteins controlling the state of the actin cytoskeleton is an actin bundling protein encoded by the gene *SAC6* (*s*uppressor of *ac*tin). In this study, I have examined some of the phenotypic consequences of overexpression of Sac6p and analyzed interactions between Sac6p and actin *in vitro*. In Chapter 3, I describe the purification of Sac6p and some initial characterization of the protein. The purification scheme described in Chapter 3 was used to analyze the potential biochemical mechanism of suppression seen between mutant *sac6* and *act1* alleles. This portion of the study is described in Chapter 4. I have also investigated the molecular basis of allele-specific suppression by analyzing the interaction between wild-type and mutant actin and wild-type and mutant Sac6p. The biochemical assays show *in vitro* suppression of binding and crosslinking activity between Sac6 suppressor protein and mutant actin protein. In addition, the Sac6 suppressor proteins tested have an increased affinity to wild-type actin. This analysis, as well as previous genetic data, is consistent with the idea that suppression results from an overall increase in affinity to actin rather than a strict "lock and key" mechanism previously hypothesized.

Overexpression of Sac6p under the control of a galactose inducible promoter results in growth inhibition, accumulation of multinucleated cells, and altered actin

cytoskeletal organization. To better understand why overexpression of Sac6p has detrimental effects, I devised a screen to isolate genomic mutations that can suppress the growth defects resulting from elevated levels of Sac6p. I have found that an *act1-3* strain is able to suppress Sac6p overexpression and one of the mutants isolated in the screen is also an *act1* mutant allele. In addition, the temperature sensitive and osmotic sensitive phenotypes are not complemented by an *act1-3* strain. These results suggest that the lethality is mediated through the interaction of Sac6p with actin. This study will be outlined in Chapter 5.

In the first chapter, I will first describe the yeast actin cytoskeleton, including some of the players identified that are needed to maintain the integrity of the cytoskeleton. In the second part of the introduction I will summarize a number of studies that employ allele-specific suppression and the models of suppression that have been hypothesized from these studies.

CHAPTER ONE: Introduction.

PART I: Introduction to the Cytoskeleton.

Actin.

There are two states of actin, a monomeric form referred to as G-actin (globular), and a filamentous form called F-actin [reviewed in (72, 101)]. Transition from G-actin to F-actin occurs in the presence of high mono- or divalent cations. During the formation of filamentous actin an ATP molecule bound to actin is hydrolyzed to ADP which remains firmly bound to the actin molecule. Polymerization of actin occurs by a rate-limiting nucleation step, the formation of a trimer, after which an elongation stage proceeds at a faster rate.

Since actin is a highly conserved protein common to all eukaryotes, the crystal structure for rabbit muscle actin has supplied biologists with a model to begin to decipher the location of binding sites on actin for actin binding proteins (see Figure 5.4 A, B), (43, 52, 67) for review see (72). The rabbit muscle actin structure is bi-lobed shaped, with the two sides being unequal in size. The small domain is composed of subdomain 1 (residues 1-32, 70-144, 338-375), and subdomain 2 (residues 33-69); the large domain is composed of subdomain 3 (residues 145-180, 270-337) and subdomain 4 (residues 181-269).

The major form of yeast actin is encoded by a single gene (*ACT1*) (32, 87) making replacement of wild type actin with mutated forms in yeast relatively straightforward (unless the mutation causes a dominant lethal phenotype). This then allows for the assessment of the phenotypes of these mutant actins *in vivo* possible. The high degree of

homology seen between yeast actin and that of higher eukaryotes, (for example yeast actin is 88% identical to rabbit muscle actin), suggests that the information learned about the yeast actin cytoskeleton may be applicable to other systems. A number of actin mutations have been generated (2, 49, 53, 83, 113, 128) and by far the largest collection was obtained by using "charged-to- alanine" mutagenesis (128).

Yeast actin, like other higher eukaryotic actins with the exception of *Tetrahymena* actin, binds DNase I (41, 58). The ability of actin to bind endonuclease DNase I in a 1:1 molar ratio, besides helping to crystallize actin, has provided a relatively easy purification scheme through the use of a DNase I affinity column (43, 52, 72, 97, 129). The capacity to purify both mutant and wild-type yeast actin has allowed for a number of biochemical studies (19, 44, 45, 53).

Actin Localization in Yeast.

In budding yeast, actin is normally distributed in cables and cortical patches which are close to, or at the surface of, the membrane. During the cell cycle the actin cytoskeleton undergoes rearrangement (54). Early in the cell cycle, actin is uniformly distributed in G1, after which a bud site is selected and actin becomes polarized which leads to the localization of actin in a ring prior to new bud growth. As the bud emerges from the boundaries of the ring, actin concentrates at the tip of the growing bud. Staining cells with FITC-ConA, which labels the cell wall, followed by a period of growth in the absence of FITC-ConA revealed that most new growth occurs at the tip of the bud (3). As the bud begins to equal the size of the mother the cortical patches become more uniform. At the end of the cell cycle the actin again concentrates to the neck region between the mother and daughter cell.

A ring of chitin is formed in the same area as the ring of actin, suggesting that actin may be needed for the deposition of the chitin ring. This ring remains as a bud scar and thus can be used to determine the age of the cell. Emanating from the cortical patches, cables are seen in various stages of the cell cycle. Electron microscopy of the cortical patches using gold-labeled actin antibodies revealed that at these sites the membrane invaginates, actin filaments wrap around the cytoplasmic side of the invaginated membrane and filaments extend into the cytoplasm possibly forming the cables seen by immunofluorescence (82).

Identification of Proteins that Genetically Interact with Actin.

The eukaryotic actin cytoskeleton is a dynamic structure composed of actin and other interacting proteins. *Saccharomyces cerevisiae* is a tractable system to study the actin cytoskeleton because both genetic and biochemical techniques are available that allow both *in vitro* and *in vivo* analysis. The actin mutations generated by a number of studies mentioned above (see section on actin) were used as a starting point for the isolation of a number of different proteins. For example, *SAC1* through *SAC6* (*s*uppressor of *ac*tin), *RAH1* through *RAH3* (*r*eversion of *a*ct1 *h*igh osmolarity sensitivity) were isolated as suppressors of the temperature-sensitive and osmotic-sensitive phenotypes, respectively of an *act1-1* mutation (2, 18, 89). *SAC7* was isolated as a suppressor of an *act1-4* mutation (25). *ANC*1 through *ANC*4 (*a*ctin *n*on-*c*omplementing) were isolated as mutations that failed to complement *act1* mutations (126).

Yeast Fimbrin, Sac6p.

SAC6 was identified as a dominant suppressor of an *act1-1* mutant (2). Certain *sac6* and *act1* mutations showed reciprocal suppression, for example, *sac6-2* and *act1-1* are able to suppress each others' growth defects, indicating that Sac6p may bind actin directly (2, 5). This hypothesis was confirmed as Sac6p was independently identified as a protein that binds to an F-actin affinity column (24). Furthermore, Sac6p can bundle actin *in vitro* and localizes to cortical spots and actin filaments *in vivo* (4, 24). At the amino acid level, Sac6p has 40% identity to human plastin and chicken fimbrin (4). Similar to the higher eukaryotic homologs, Sac6p has actin crosslinking activity. Chicken fimbrin has also been shown to bundle actin *in vitro* (33) and localizes to the actin bundles in the microvilli (15).

Structurally, fimbrins are composed of a head piece region, with sequence similarity to the calcium binding site of calmodulin, and a core domain that contains two putative actin binding domains that are thought to promote bundling of actin filaments (22). All three share homology to a putative actin binding domain found in other important actin binding proteins such as α-actinin, dystrophin, and ß-spectrin (4). The core domain of fimbrin contains four similar regions designated A, B (first actin binding domain), A' and B' (second actin binding domain) [see Chapter 3, Figure 3.1, (22)]. Sac6p, as well as a number of other members of the spectrin family, have a highly conserved 27 amino acid region in subdomain A and A', (4, 13, 14, 62). This region has been implicated as an important region for binding to actin (13, 14). Since chicken fimbrin behaves as a monomeric protein as assayed by gel filtration, it has been hypothesized that the two actin-

binding domains function to bundle actin filaments with a spacing of fimbrin-actin bundles of 11 nm (15, 74).

A *sac6* null mutation imparts temperature-sensitivity at 37° C, yet these cells can still grow at 30° C. This may indicate that the function of Sac6p is not needed at 30° C or that there are additional protein(s) that have overlapping function with Sac6p (4). Mutations in genes encoding two proteins, Abp1p, originally identified via retention on an actin affinity column, and *CAP1* and *CAP2*, two genes that encode proteins homologous to α and ß subunits of capping proteins, are synthetically lethal with *SAC6*; however, null mutations of *ABP1* and *CAP2* are not synthetically lethal with each other (6, 7). One can propose two or more models for this result; one in which Sac6p has overlapping functions with both capping protein and Abp1p, or another in which the levels of actin stability dictate life or lethality. For example, there could be a critical ratio of the concentration of actin monomers to filaments within the cell. Changing the levels of an actin binding protein may alter the ratio possibly resulting in inviability.

Previous binding assays revealed that some mutant actin proteins [*act1-125* (K50A, D51A), *act1-3* (P32L), *act1-7* (K61N), *act1-120* (E99A, E100A), *act1-108* (R256A,R254A), *act1-101* (D363A,E364A), *act1-113* (R210A,D211A), and *act1-133* (D24A,D25A)], have a reduced affinity for Sac6p, suggesting that these residues may be close to residues that contact Sac6p or are involved in direct contacts with Sac6p (44, 45, 53). Interestingly, these residues are primarily located in subdomain 1 and subdomain 2 in the actin molecule, making it likely that this area is a binding site for Sac6p (44, 45, 53). Evidence in favor of the notion that this area defines a Sac6p-binding domain comes from the work of McGough *et al.* (77). Image reconstruction of the actin-binding domain of α-

actinin bound to filamentous actin reveals that the actin-binding domain 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 (77). In addition, crosslinking studies using α-actinin revealed that residues 86-112 in actin may be a binding site (37, 61). As α-actinin and fimbrin share homology in the actin binding domain and the same region on actin has been implicated as being important for binding of both proteins provides support that a binding site on actin for Sac6p has been identified.

Brower *et al.* 1995 reported the sequence of the *sac6* mutations that suppress the defects of various actin alleles and found that they cluster in three areas within the first actin binding domain (16). The first two regions have been implicated in other studies to be important for the interacteration of other proteins that have a homologous actin binding domain; however, the third cluster has not been implicated before. Mutations in *SAC6* and *ACT1* suppress each other's defects (2, 5), consistent with the lock-and-key model. One of the predictions of the lock-and-key model is that there be great specificity: any one mutant allele should only be suppressible by a mutation that alters its counterpart in the interacting protein. Interestingly, in the case of the actin-Sac6p interaction, mutations that change any one of eight residues in the likely Sac6p-binding domain of actin (but not elsewhere in actin) can all be suppressed by any one of several different *sac6* alleles; thus, each suppressor appears not to restore a specific lost interaction, but rather to restore any one of a number of defective interactions (16, 45). The nature of the suppression is the focus of this research and is discussed in Chapter 4.

PART II: Allele-Specific Suppression.

A suppressor is a mutation that can correct the phenotypic defect of the primary mutation. Why do a suppressor screen? Suppressor screens are often done for a number of reasons: to identify new proteins that may interact with your protein of interest; to yield insight into how and where two known proteins interact with each other; to learn what role a protein has in the cell; and to discover if there is an independent pathway that can bypass the requirement of the original protein. Hunts for suppressors of a specific mutant phenotype often yield a plethora of secondary mutations that can revert the phenotypic defect of the original mutant. These may be involved in a general cellular process that is not the specific focus of what you are studying. Hence, how does one decide on which mutations to focus upon? Allele-specific suppressors are frequently chosen since this phenomenon is often, but not exclusively, observed between genes that encode interacting proteins. Allele-specific suppressors can be extragenic or intragenic. Intragenic suppression occurs when the second-site suppressor is in the same gene as the original mutation. Extragenic suppression occurs when a mutation in one gene compensates for a mutation in a second gene. In the case of extragenic allele-specific suppression, the suppressor mutation in gene "A" compensates for only a subset of mutant alleles in a second gene "B".

Molecular Mechanisms of Allele-Specific Suppression.

Extragenic suppression can occur through compensating changes in two interacting proteins. Jarvik and Botstein postulated this type of "*compensatory alterations*" for suppressors of phage P22 mutations (48). The discovery that a suppressor

is allele-specific suggests that the suppressor does not simply bypass the requirement of the wild-type gene product (2). The specificity may reflect direct contacts between residues in different proteins. This rationale led to the classic "lock and key" model, shown in Figure 1.1, as a way of explaining the genetic interactions. This model has several predictions that are testable by measuring the affinities between mutant and wild-type protein combinations. First, the primary mutation should alter protein A such that it does not interact with wild-type protein B as well as wild-type protein A interacts with wild-type protein B (see Figure 1.1 A). Second, the suppressor protein B should restore the interaction with the mutant protein A. Third, the modified mutant B protein should interact worse with wild-type A than wild-type B does with wild -type A. Notably, in extreme cases this model predicts that the suppressor mutation can sometimes yield a mutant phenotype in the presence of a wild-type copy of the original gene.

"Lock and key" type interactions have been observed in protein-DNA complexes. For instance, in one study, all the possible changes in two amino acid positions were created in the *trp* repressor (99). Interestingly, 12 of the mutant trp repressors could bind 1 or more mutant operators better than could the wild-type *trp* repressor. Eleven of the 12 mutant trp repressors retained the ability to bind to the wild-type operator. Thus, some of these mutants may be described as "master keys" if the mutant protein retains the same affinity to the wild-type operator as the wild-type repressor has to the wild-type operator (see Figure 1.1 B). However, one mutant trp repressor had a clear classic "altered specificity"; thus, the mutant trp repressor binds a specific mutant operator, but not other mutant or wild-type operators tested (see Figure 1.1 A). In other words, this mutant trp repressor does not bind to the wild-type trp operator as well as does

the wild-type repressor, but has a higher affinity to a mutant trp operator than does the wild-type repressor.

Mutational analyses of proteins that form dimers provide examples of strict specificity in protein-protein interactions. The interhelical electrostatic contacts between leucine zippers contribute to this specificity (91, 122). Vinson *et al.* (122) created "self-complementary" (homodimerization preferred) and "mutually complementary" (heterodimeriztion preferred) derivatives of C/EBP (CCAAT/*e*nhancing *b*inding *p*rotein) to test specificity of interactions. A set of mutations were generated in the C/EBP leucine zipper. These mutants were each mixed with a shorter molecule that still contained the wild-type C/EBP leucine zipper such that homodimerization verses heterodimerization could be assayed. The attractive forces generated in the same molecule, "self-complementary", resulted in a preference for homodimerization. Electrostatic attraction created between molecules "mutually complementary", yielded molecules that prefer to heterodimerize. The net repulsion or attraction due to electrostatic forces dictated the relative amount of homodimers or heterodimes formed when the mutant and wild-type proteins were mixed.

In a different study, some mutants generated in human growth hormone, hGH, had a higher affinity for hGH receptor than did wild-type hGH. Interestingly, a single mutation in hGH displayed increased affinity to hGH receptor yet reduced affinity to hPRL, prolactin receptor compared to wild-type hGH (68). As a single mutation can alter the specificity of interactions between proteins suggests that altering the key by a single mutation can switch which lock the key fits.

The next models need a broader interpretation of the definition of allele-specific suppression. In the strictest sense, allele-specific suppression denotes suppression of only

one mutant allele of a gene by a single allele in another gene. In a more liberal definition, if a suppressor fails to suppress just one mutant allele out of a large number within a gene, it is still an allele-specific suppressor. Categorically, the apparent degree of allele-specificity depends on the repertoire of mutations that are available.

Although the examples described above provide precedent for the "lock and key" model, the "increased affinity model", (see Figure 1.1 B) may be a more common mechanism of allele-specific suppression. This model also has certain biochemical predictions. The "increased affinity model" has some similarity to the "lock and key" model in that one of the two proteins, in this case mutant A, still has a reduced affinity for wild-type B and the combination of the two mutant proteins restores a functional interaction. However, contrary to the first model, the suppressor protein B has an overall increase in affinity to the protein A by the addition of new contact sites rather than restoration of a previously existing site. Also, contrary to the first model, the second model suggests that an independent phenotype of a suppressor may result from a gain in affinity that leads to a protein complex that is too stable, rather than a loss of affinity.

Mutations leading to increases in protein-protein or protein-DNA affinities are not uncommon. For example, a second site reversion mutation that suppresses a loss of function mutation in λ repressor increases the affinity of the λ repressor protein for DNA (10, 85, 86). Similarly, mutations in the LexA repressor of *E. coli* were isolated that enhance their binding affinity to DNA (92) and mutations in the Lac repressor ("super-repressors") (11, 12) and hGH(human growth hormone) (21) show increases in their affinity toward their respective substrates.

In *Escherichia coli*, allele-specific suppression was observed between *malE* mutant alleles, encoding maltose binding protein (MBP), and *malF* and *malG* mutant

alleles, encoding integral transmembrane proteins important for transport of maltose (23, 46, 120, 121). The mechanism of suppression is thought to result from an overall balance in affinities between the two sets of interacting mutant proteins (MalF or MalG) and MalE (see Figure 1.1 C). In brief, mutations in *malG* and *malF* were isolated that could not function in the presence of a wild-type copy of *malE*. Suppressors of the transport defect of *malG* and *malF* mutations were isolated in *malE* (46, 120). *In vitro* assays suggest that the MalF and MalG mutant proteins bound too tightly to wild-type MBP and that the *malE* suppressor mutations reduced the affinity of MalE for MalG or MalF (23). The *in vitro* analysis suggests that the observation that mutant MalF and MalG proteins are defective in the presence of wild-type MBP is most likely attributed to an interaction that is too strong. Thus, restoration of a functional balance in affinity seen between pairs of allele-specific suppressors is a plausible molecular mechanism of suppression.

Two other models in which suppression is not a compensatory change reflecting a direct interaction between the original mutant gene product and the suppressor gene product include "factor X". These models suggest that the suppression is not a direct interaction but is mediated by a third protein. One could imagine if a protein had multiple functions, or was involved in a number of biological processes, that certain mutations would disrupt one process, for example secretion, but not another, like endocytosis. Therefore, a suppressor may be a "bypass" suppressor for the requirement of a protein in one biological process, but an allele-specific suppressor of another process because the original protein has two essential functions. Alternatively, the two interacting proteins could also each interact with a third component. Thus, if all three interact with each other in a complex, a mutation in one protein could reduce the affinity for the third protein. This

would be suppressable by an increase in affinity of the second protein with the third protein to stablize the complex. This model is diagramed in Figure 1.1 C.

Allele-Specific High Copy Suppression.

If simply increasing the affinity for a certain protein results in suppression, one may predict that allele-specific high copy suppressors can be isolated. Indeed, there are numerous examples of allele-specific high copy suppressors in yeast. For example, *TSD4*, encoding a RNA polymerase III transcription factor, is an allele-specific high copy suppressor of mutations in TATA-binding protein (17). Similarly, overexpression of *SWP1*, suppresses an *wbp1* mutation (39). *SWP1* and *WBP1* are both essential components of the *N*-oligosaccaryl transferase complex (39). The observation that allele-specific suppressors can be isolated by simply increasing the copy number of a gene suggests that increasing the cellular concentration of a protein is enough to promote a functional protein-protein complex.

Destabilization may also be a mode of allele-specific high copy suppression. For example, overexpression of *JSN1* (*j*ust *s*ay *n*o) suppresses the tubulin mutants *tub2-150* and *tub2-404*. This suppression is allele-specific as other tubulin mutants are not suppressed (70). These tubulin mutants are defective in spindle elongation and appear to increase the stability of microtubules as judged by growth of cells in the presence of a microtubule destabilizing drug, benomyl (70). The viability of *tub2-150* is higher with the addition of benomyl than in the absence of benomyl. The reverse is true for a wild-type strain. Thus, overexpression of *JSN1* may decrease microtubule stability to levels that support the dynamic instability of the spindle that is necessary for growth. In agreement

with this idea, wild-type cells are more sensitive to benomyl if the levels of *JSN1* are elevated. Whether this reflects a direct interaction between Jsn1p and tubulin has yet to be determined.

Allele-Specific Reciprocal Suppression.

Reciprocal suppression is a phenomenon whereby two intra- or intergenic mutations, which by themselves impart either different or similar mutant phenotypes, confer a wild-type phenotype upon the organism when both are present. Disruption of components in large multi-protein complexes often results in pleiotropic phenotypes. Allele-specific reciprocal suppression provides an additional level of information that is often indicative of protein-protein interaction. The cytoskeleton and the transcriptional machinery are examples of elaborate complexes whose analyses have benefited from the use of reciprocal suppression.

Suppression of the temperature-sensitive *act1-1* mutation, led to isolation of *sac6* (*s*uppressor of *ac*tin) mutations that could allow growth of *act1-1* at high temperature (2). The suppression was allele-specific, as the *sac6* mutations failed to suppress the *act1-4* mutation. Interestingly, some of these *sac6* mutations had an independent phenotype in a wild-type background under the same conditions that the *act1 sac6* double mutant could grow. This indicated that not only did *sac6* mutations suppress *act1* defects, but mutations in actin could suppress *sac6* mutant alleles. Thus, the *sac6* and *act1* mutant alleles reciprocally suppressed each other's defects. The discovery that *sac6* and *act1* mutations can suppress each other's defects strongly indicated that the Sac6p interacted with actin (2). Indeed, Sac6p was also concurrently isolated *via* an actin affinity column (24).

Mutations in either *spt3* or *spt15* (TFIID) cause defects in transcription (26); however, if specific *spt3* and *spt15* mutant alleles are combined they suppress each other's defect. Sequencing of suppressor alleles in TFIID and SPT3 reveal that the mutations cluster, possibly defining a region important for interaction. Furthermore, Spt3p coimmunoprecipitates with TFIID, suggesting that Spt3p and TFIID may interact *in vivo*.

Although allele-specificity is often indicative of direct interactions, one could imagine cases of reciprocal suppression that are not due to direct interactions. For example, elevated levels of activity of a kinase may lead to a phenotypic defect that could be corrected by increasing the levels of a phosphatase, such that there is an intermediate level of phosphorylation activity necessary for growth.

Cycling-Suppressor Screens.

Cycling-suppressor isolation, based on reciprocal suppression, is a useful tool for increasing the number of alleles of a particular gene. A cycling-suppressor screen is a method used to isolate a suppressor in a second gene and subsequently use that suppressor to isolate a mutation in the original gene. This process can be repeated over and over until the number of alleles is saturated. As described above, suppression of the temperature-sensitive *act1-1* mutation led to the isolation of *sac6* mutations that could allow growth at high temperatures. Some of these *sac6* mutations had an independent phenotype in a wild-type actin backround. The isolation of suppressors of the *sac6* mutant phenotype led to the identification of additional actin alleles (2).

This type of screen was also used to study the maltose transport system (see section on molecular mechanisms of allele-specific suppression). This cycling-

suppression method was similarly used in *E. coli* to explore the interactions between CheW, a cytoplasmic component, and Tsr, a membrane bound chemoreceptor, during chemoreceptor signaling (65) and interactions between *fliG* and *cheY* (106).

Defining Domains.

The increased number of mutant alleles obtained by cycling suppression selection are invaluable in determining an area in a protein structure that is important for protein-protein interaction. DNA sequencing of the mutations in actin alleles suppressible by *sac6* mutations showed that these mutations cluster in subdomains 1 and 2 in the three dimensional structure of actin (45). The locations of the genetically interacting residues between (*malF* or *malG)* and *malE* mapped onto the three-dimensional structure of MBP cluster in two areas (46, 120), one area on MBP for mutations that suppress or are suppressed by *malF* mutations and one area for mutations that suppress or are suppressed by *malG* mutations. These regions possibly define the MalF and MalG binding sites.

The location of interacting residues also gives insight into the mechanism of suppression. In transcription, for example, mutations in the second largest subunit of RNA polymerase II in *Drosophila* were isolated as dominant allele-specific suppressors of a mutation in the largest subunit of RNA polymerase II (80). To examine the hypothesis that allele-specific suppressors define domains within proteins that interact, the genetically interacting mutations were later modeled into the crystal structure of DNA polymerase I from *E.coli.* Interestingly, the residues resided in the same domain defining the DNA binding cleft (55); however, it is unclear whether the suppression occurs through an alteration of the DNA binding affinity or by an alteration of subunit interactions.

The genetic interaction of the two largest subunits of yeast RNA polymerase II was also examined through extragenic suppressor analysis. Suppressors of an allele in the largest subunit were isolated by mutagenizing a plasmid containing the second largest subunit. The suppressor mutations clustered in one region of the second largest subunit and were allele-specific, since they did not suppress six other *rbp1* mutations that are spread out over the *RBP1* gene (73). In addition, one of the suppressors was dominant and the others were slightly or semi-dominant.

Dominant vs. Recessive Suppressors.

Allele-specific suppression is not limited to the isolation of dominant suppressors. Interestingly, both dominant and recessive allele-specific suppressors of a mutation in the *FLA10* locus of *Chlamydomonas reinhardtii* were isolated that mapped to a single locus. Lux *et al.* (69), suggest that dominance or recessiveness of a suppressor results from a competition between the suppressor protein and the wild-type protein for the original mutant protein. Thus, if the suppressor is recessive, in the diploid cell the wild-type protein has a higher affinity for the original mutant protein and the complex formed is inactive. However, if the suppressor is dominant, the suppressor protein has a higher affinity for the mutant protein. Suppression in a haploid cell containing either a dominant or recessive suppressor could be phenotypically equivalent since the wild-type protein is not around to compete for binding.

Null Allele-Specific Suppressors.

Although there are examples in which allele-specific interactions have been suggested to indicate restoration of a protein-protein interaction, there are other examples which do not support this hypothesis. For example, mutations in *SAC2*, and *SAC7* are allele-specific suppressors of certain actin mutations. However, null mutations in these proteins can suppress an *act1-1* mutation (25, 57).

Allele-Specific Intergenic Noncomplementation.

A recessive suppressor cannot suppress the phenotype of the original mutation in the presence of another copy of the wild-type genome. However, intergenic noncomplementation of a suppressor can occur, if recessive suppressors in two different genes crossed to each other yields a diploid in which the mutations can suppress the phenotype of the original mutation. If only certain combinations of alleles in each of the genes results in suppression then the intergenic noncomplementation is allele-specific. This type of interaction has been observed in *Caenorhabditis elegans* for some alleles of *sog-1* [suppressors *of glp-1*(germ *l*ine *p*roliferation defect)] (71). Maine and Kimble (71) also define the interaction between these genes as "dominant enhancers of each other" and suggested that this may be indicative of proteins that interact with each other.

Intragenic Suppressors.

If a mutation affects the folding, or protein stability, of a protein under restrictive conditions, a second mutation in the same gene may compensate for the initial defect in the relative stability of the protein. For example, some double mutant combinations in *Staphylococcal* nuclease (*nuc*) have been shown to have compensatory effects on protein stability (34). Intragenic suppressors of *nuc* mutations were able to suppress the nuclease-minus phenotype of multiple mutant alleles (111, 112).

Although many intragenic suppressors are thought to affect general stability, intragenic suppressors can act globally, suppressing multiple mutations, by increasing the activity of a protein. This mechanism has been suggested for intragenic suppressors of mutations in the *E.coli trp* repressor protein (56).

The original mutation that is chosen for a suppressor screen can make a large difference in the proportion of intragenic or extragenic suppressor mutations isolated. For example, in the isolation of suppressors of *Chlamydomonas reinhardtii fla10* mutant alleles, one original mutation (*fla10-1*) resulted in 0/68 extragenic suppressors/total suppressors, while a different mutation (*fla10-14*) resulted in isolation of 47/69 extragenic suppressors/total suppressors (69).

Other Examples.

There are many examples of allele-specific extragenic suppression at the level of nucleic acid-nucleic acid, nucleic acid-protein, and protein-protein interactions. For example, informational suppressors that allow translation of nonsense codons, UAA, UAG, and UGA, display allele-specificity, although, they do not exhibit locus specificity [reviewed in (79)]. Compensatory base pair changes in U2-U6 snRNA structure yielded allele-specific suppressors of respective base pair mutations. This type of analysis has offered insight into the base pairing in the U2-U6 snRNA structure necessary for mRNA splicing (117).

Other Techniques.

Two useful techniques to assay allele-specific suppressors include allele-shuffling and two-hybrid system analysis (29, 114). However, the use of the two-hybrid system in determining relative affinities between proteins warrants caution (27). Monovalent phage display (68) may aid in studing protein-protein interactions. Many different genetic approaches are used to identify genes that interact with each other including extragenic suppression, reciprocal suppression, synthetic lethality, unlinked noncomplementation, and suppression by overexpression [see for review, (35, 36, 100)]. However, relatively little is known about how the mutation that is isolated works at the molecular level to cause the resulting phenotype.

Concluding Remarks.

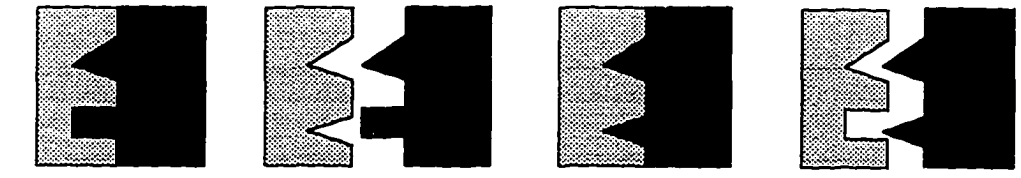
How do we define allele-specificity? There are numerous examples of allele-specific suppression. However, the degree of allele-specificity differs depending on the mutation. If a suppressor can suppress a number of alleles, yet not suppress only one, does this meet the criterion for the definition of allele-specific? Ironically, expanding the number of mutations may decrease the degree of allele-specificity, yet fine-tune the location of important residues involved in protein-protein recognition.

Allele-specific suppression in many cases signifies compensatory changes in two interacting proteins, nevertheless, as with most genetic analysis many possible explanations are conceivable. Therefore, models suggested for the observed genetic interactions between genes encoding protein components of a large complex requires carefully scrutiny. Hence, coupling the *in vivo* genetics with *in vitro* biochemical assays will help elucidate the molecular mechanisms of suppression.

FIGURE 1.1. Molecular mechanisms of allele-specific suppression.

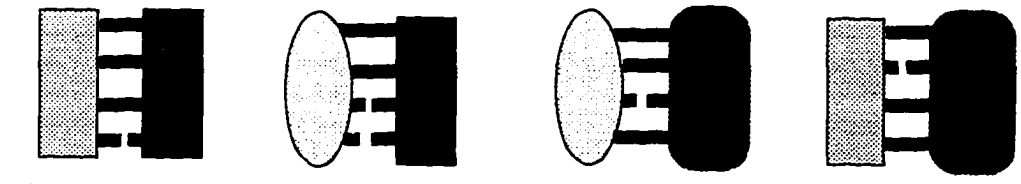
See text for details.

A. Lock and key



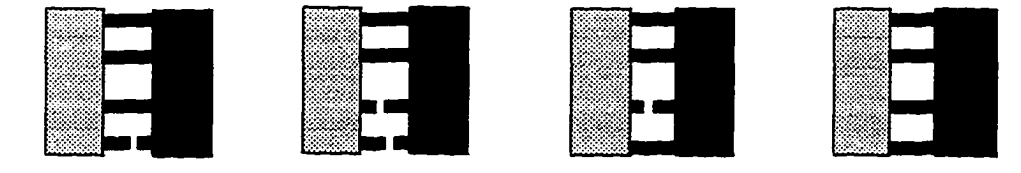
Wild-type A Wild-type B | Mutant A Wild-type B | Mutant A Mutant B | Wild-type A Mutant B

B. Master key



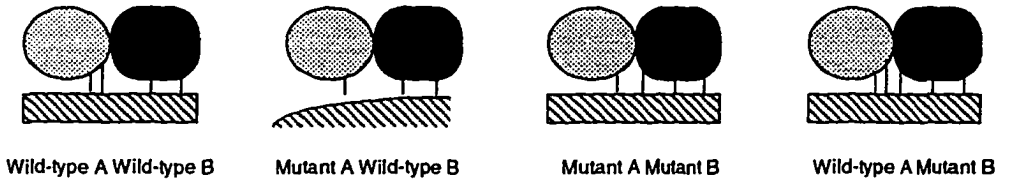
Wild-type A Wild-type B | Mutant A Wild-type B | Mutant A Mutant B | Wild-type A Mutant B

C. Increased affinity



Wild-type A Wild-type B | Mutant A Wild-type B | Mutant A Mutant B | Wild-type A Mutant B

D. Indirect restoration of affinity to factor X



Wild-type A Wild-type B | Mutant A Wild-type B | Mutant A Mutant B | Wild-type A Mutant B

CHAPTER TWO: Materials and Methods.

Sac6p Purification.

Yeast fimbrin was purified with some modifications similar to the purification protocol for l-plastin of Shibaya, M. *et al.* (110). A protease deficient yeast strain, AAY1918 *MAT a sac6::LEU2 ura3 trp1 leu2 his3 pep4::HIS3 prb1 can1 GAL*, which contains a 2μ *GAL1-SAC6* plasmid was grown to mid log in -ura media (selecting for retention of the plasmid) supplemented with 2% raffinose. Galactose was added at a final concentration of 2% to induce expression of *SAC6* from the *GAL1* promoter and the culture was grown for an additional 4-6 hours. Cells were spun down (Beckman J6B 3,500 rpm), washed once with cold wash buffer (10 mM Tris, pH 8.0, 5 mM NaCl, 1 mM EGTA, 1 mM EDTA) and resuspended in approximately a 1:1 ratio of cells to wash buffer, after which an equal volume of 50% glycerol was added. The cells were quickly frozen in liquid nitrogen and stored at -80° C. Frozen cells were thawed the day of purification, spun down and resuspended in lysis buffer (10 mM Tris, pH 8.0, 0.2% NP40, 5 mM NaCl, 1 mM EGTA). Cells were transferred to a 75 ml bead beater chamber (BioSpec Products) containing 25 ml of glass beads, protease inhibitors were added to a final concentration of 1 mM PMSF, 1 μg/ml (chymostatin, pepstatin, leupeptin, and antipain) and additional lysis buffer was added to fill the chamber. Cells were homogenized for 30 min. with a 20 sec on, 80 sec off cycle. Cell lysis was checked under the microscope, and additional cycles were added if cell lysis was incomplete. The lysed cells were first spun down at low speed for 10 min. at 10,000 rpm in a SS-34 rotor, then at

high speed for 30 min. at 45,000 rpm in a Ti80 rotor. The high speed supernatant was passed through a 0.2μ filter and applied to a DEAE Sepharose CL-6B (Sigma) column equilibrated with 10 mM Tris HCl, pH 8.0, 5 mM NaCl. The anion exchange resin was washed with 10 column volumes of 20 mM Bis-Tris, pH6.5 50 mM NaCl. Protein was eluted using a linear NaCl gradient (50 to 400 mM) in 20 mM Bis-Tris pH 6.5. Column fractions were monitored by dot blot and SDS PAGE. Sac6p-containing fractions were pooled, adjusted to 1 mM DTT, 0.2 mM EGTA, 0.5 mM EDTA and dry ammonium sulfate was added to a final saturation of 65%. The precipitated proteins were collected by centrifugation (15 min. at 10,000 rpm in SS-34 rotor). Concentrated protein was resuspended in 3 ml of 5 mM $NaPO_4$, pH 7.0 and passed through an Econo-Pac 10DG desalting column (BioRad cat. no. 732-2010) equilibrated with same buffer (following manufacture's instructions). Protein was then applied to a Hydroxylapatite Bio-Gel HT column (BioRad cat. no. 130-0150) and eluted with a 5 to 300 mM $NaPO_4$ buffer, pH 7.0. Sac6p eluted as two separate peaks in the first third of the gradient and were designated "peak 1" and "peak 2".

Protein Sequence Analysis.

Protein sequencing was performed at the University of Arizona Biotechnology Core Facility using an Applied Biosystems 477A Protein/Peptide Sequencer (Edman chemistry) interfaced with a 120A HPLC (C-18 PTH, reverse-phase chromatography) Analyzer to determine phenylthiohydantoin (PTH) amino acids.

CNBr Cleavage for Sequencing.

Cyanogen bromide (CNBr) cleavage on PVDF (Immobilon P, Millipore) blotted protein was performed as described by (75) with modifications. Briefly, 200 µg of peak 2-protein was run out on a 10% polyacrylamide gel and electroblotted to PVDF membrane. The PVDF membrane was stained with Ponseau S (Sigma cat. no. P7767) and full length peak 2-protein was excised and cut into 2-3 mm^2 fragments and placed into an eppendorf tube. A volume of 795 µl formic acid and 205 µl of H_20 and 2 crystals of CNBr was added to the tube. The tube was incubated overnight in the dark at room temperature on a rotator. The liquid was removed with a pipetman. The excess liquid was removed by vacuum centrifugation in a speedvac. After the PVDF paper was dry, 50 µl of H_20 was added, the tube was vortexed and allowed to redry using the speedvac, this was repeated twice. Seventy microliters of elution buffer (2% SDS, 1% Triton X-100 in 50 mM Tris (pH 9.2-9.5) was added and the mixture was incubated at room temperature for 90 min. Glycerol and bromophenol blue (Sigma NO. B-6131) was added to a final concentration of 6.25% and 0.01%, respectively. The CNBr mixture, including the membrane fragments, was loaded and run into a 12% polyacrylamide gel. The protein was transferred to PVDF for 30 min. at 50V at 4° C. The electroblotting buffer was 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer in 10% methanol. One hundred millimolar (10X) stock CAPS buffer, which was stored at 4° C was made by dissolving 22.13g CAPS in 900 mL of double distilled water, titrating the mixture with 2N NaOH (20 ml) to pH 11, and adjusting the final volume to 1L with H_20. Again the PVDF was stained with Ponseau S and a 38 kDa fragment was isolated corresponding to the N-terminal region of Sac6p was use for N-terminal sequence analysis. Sequence analysis

was performed on the first 15 amino acids and indicated that the N-terminus of Sac6p was intact.

Trypsin Digestion.

A mixture containing 20 µl of Sac6p (10 mg/ml), 33.25 µl H_2O, 1.25 µl of 0.045 M DTT, 5.0 µl of 1M NH_4HCO_3, 0.5 µl of 10 mg/ml trypsin suspended in 10 mM HCL (Sigma 8642, lot 22H7110,12,600 units/mg TPCK treated) was incubated overnight at 37° C.

Binding and Bundling Assays for Calcium Experiment.

Bundling and binding experiments were performed as described in (45) with the following modifications. For Ca^{2+} studies, actin and Sac6p (used in crystal growth experiments) were kindly provided by Jerry Honts. Mixtures were centrifuged in a TLA-100 rotor at 18,000 rpm for 15 min. at 22° C unless otherwise indicated. Bovine Serum Albumin was used as a standard in Bradford assays to determine the concentration of Sac6p and actin. The assays were set up in a 25 µl total volume. After incubation for 90 min. 10 µl was used for the high speed spin assay and 10 µl was used for the low speed assay. Supernatants were resuspended in an equal volume of 2X sample buffer, and the resulting pellets were resuspended in 20 µl of 1X sample buffer. Conditions: actin concentration: 10 µM; Sac6p concentration: 5 µM; T-fimbrin concentration: 5 µM; 20X buffer concentration: 450 mM HEPES - KOH pH7.5, 1 M NaCl, 20 mM $MgCl_2$, (2 mM

EGTA or 20 mM $CaCl_2$); actin contribution 32 μM $CaCl_2$ (should be eliminated by the EGTA in the buffer)

Anti-Sac6p Antibody Purification.

Antibodies were isolated as described in (119) with additional modifications. Briefly, protein (200 μg) was run out on a polyacrylamide gel using the mini gel apparatus (Mighty Small II SE250, Hoefer Scientific instruments) and transferred to nitrocellulose. The protein was stained using Ponceau S (38). The desired band was cut out of the nitrocellulose and destained using water. The nitrocellulose strip was blocked using 1X TBS and 5% dry milk (1L of 10 XTBS, for example, 90g NaCl, 12.1g Tris base, pH 7.4 with conc. HCl, diluted to 1L) for 1 hour. The strip was washed 3 times in 1X TBS. A piece of parafilm was placed on the bottom of a petri dish and a wet kimwipe was attached with tape to the top of the petri dish. The nitrocellulose strip was placed on the parafilm, protein-side up, 200 μl (enough to cover the strip), of crude antibody was carefully placed along the top of the strip and incubated for 2-3 hours (shaking slowly). The antibody was removed and saved for future use. The strip was washed 3X with 10 ml of 1X TBS. The antibody was eluted from the membrane by incubating 200 μl of cold 0.2 M glycine/1 mM EGTA pH 2.7 on the strip for 30 min. The antibody solution was removed and neutralized with 4-4.5 μl of 3 N NaOH to about pH 7.0 as determined using pH paper.

Gel Filtration.

Purified Sac6p from the peak 2 fraction, approximately 200 µg, was applied to a Superose 12 column (Pharmacia) using a Walters HPLC system (Millipore, Bedford, NJ) and eluted at 0.4 ml/min. Eluted protein was monitored at 280 nm absorbance. BSA possessing a molecular weight of 66 kDa (Sigma A 8531) and β-amylase sweet potato (200 kDa; Sigma A 8781) were used as standards.

Western Blot for Antibody Specificity.

Protein was run into a 10% polyacrylamide gel and transferred onto nitrocellulose using a Hoefer transfer apparatus. Transfer buffer (121.1g Tris Base, 56.3g Glycine, 1.0 L MeOH, 4.0 L H_20). Protein was transferred overnight at 35V in the cold room with stir bar. The blot was stained with Ponuau S and destained in H_20, washed 2X in H_20 and incubated in 1XTBS (10X TBS, 90 g NaCl, 12.1 g Tris base, pH to 7.4 with concentrated HCl, dilute to 1 L with H_20), 5% dry milk for 1 hr. Blocking buffer was removed and antibody 1:1000 dilution (1X TBS, 5% dry milk, 0.2% NaAzide) was added and incubated for 1 hour. The antibody was removed (the antibody can be reused for several months) and the blot was washed 5 times with 1X TBS. The filter was probed with antirabbit IgG horseradish peroxidase 1:10,000 dilution in 1X TBS, 5% dry milk. The secondary antibody was discarded. The blot was washed 5 times in 1X TBS. The filter was then incubated with10 ml of Solution 1, non radioactive ECL® chemiluminesence detection system (Amersham, Arelington heights, IL) and 10 ml of Solution 2 for 1 min. The excess liquid was removed and the filter was placed in saran wrap. In the dark room, a piece of HCL film was placed onto the filter and exposed for about 30 seconds.

Two-Dimensional Gel Analysis.

Two-dimensional gel analysis was performed as described in (90) modified by Tim Getzoff with additional modifications for analysis of Sac6p. Solution K (sample overlay mix) was composed of 0.54g urea, 5.0 µl ampholines pH 3.5-10, 20 µl ampholines pH 5-6 and 600 ul of H_20. Solution A (IEF sample buffer) was composed of 0.57g urea, 200 µl 5 % chaps, 100 µl 10 % DTT, 10 µl ampholine pH 3.5 to 10, 40 µl ampholine pH 5-6 and 220 µl of H_20. Buffer O (IEF gel equilabration and agarose preparation) was composed of 10% glycerol, 0.25% DTE, 2.3% SDS and Trizma base pH 6.8 0.625M. The lower running buffer was 0.3% citric acid and the upper running buffer was 0.5% ethanolamine. The negative electrode was connected to the upper reservoir and the positive electrode was connect to the lower reservoir. The second dimension was run out into a 10% SDS polyacrylamide gel.

Chemicals.

X-Gal and benomyl were obtained from the Genetics Society of America. Galactose was purchased from USB (cat. no. 15996).

Yeast Strains and Methods.

Yeast strains are listed in Appendix A. Transformation was performed by the lithium acetate method as described in (109). Recipes for media are described in (108). Synthetic media containing glucose (SD) or galactose (SG). Methods for sporulation and tetrad dissection are described in (108).

Plasmid Construction.

A *GAL1-SAC6* overexpression vector pAAB157 was constructed in two steps. The first step placed a portion of the 5' end of the *SAC6* gene behind a *GAL1* promoter in pAAB156. The vector pRP23 (same as pAAB121), a centromere-containing plasmid carrying the yeast *URA3* gene and *GAL1-10* promoter (obtained from Roy Parker), was prepared by cutting with *Bam HI*, filling the ends then cutting with *XbaI*. A fragment containing the 5' end of the *SAC6* gene, (from nucleotide position 694 of *SAC6* to 952, described in Adams *et al.* Nature 1991) was obtained from a cDNA construct described previously (4), by digestion at the 5' end (in the polylinker) with *Eco*RI followed by conversion to blunt ends, and at the 3' end (in *SAC6* coding sequences) with *Xba*I. This fragment was ligated into the blunt and *Xba*I ends, respectively, of pRP23 (above) to create pAAB156. The *BamHI* site was destroyed in the cloning process. The following step involved insertion the rest of the 3' portion of *SAC6* into pAAB156 to create a vector containing the entire *SAC6* coding region pAAB157. Thus, pAAB156 was cut with *SphI* (downstream of the *Xba*I site, in the polylinker), the ends were made blunt, and the plasmid was cut with *XbaI*. The 3' end of *SAC6* (from a wild-type genomic clone H-119-14-1, described in (4), from the *XbaI* site in the coding sequence to the *Bam*HI site (blunt-ended, and 960 bp 3' of the stop codon), was inserted into the *Xba*I-blunt *Sph*I sites of pAAB156 (above), to generate pAAB157. The cloning junctions at the 5' end of the gene and the *XbaI* site were sequenced. The first part of the construct was derived from a cDNA clone and thus does not contain the *SAC6* intron.

The *GAL1-SAC6* fragment, flanked by *Eco*RI (5' of the *GAL1* promoter) and *Bam*HI (3' of *SAC6*) sites, from pAAB157 was inserted into the *Eco*RI-*Bam*HI sites of

various vectors with different selectable markers *i.e.*, pRS305 (*LEU2*) (115) and pRS317 (LYS2) to create pAAB328 and pAAB329, respectively. *EcoRI-BamHI* fragment containing a *GAL1-SAC6* fragment from pAAB157 was ligated the *EcoRI-BamHI* site of the 2μ vector pRS426 (115) containing a *URA3* marker generating pAAB415, this plasmid was used to purify wild-type Sac6p. pRP127, (*GAL1-LacZ URA3 CEN*), was obtained from Roy Parker.

Isolation of Suppressors.

Spontaneous suppressors of the growth inhibition due to *SAC6* over-expression were isolated as follows. A *SAC6*$^+$ strain (AAY1942 or AAY1943) carrying a *SAC6* over-expression plasmid pAAB329 (*GAL1-SAC6 LYS2 CEN*) and reporter plasmid pRP127 (*GAL1-LacZ URA3 CEN*), was plated onto synthetic-glucose media lacking uracil and lysine (to select for maintenance of both plasmids) at approximately 100-200 cells per plate and grown for 7-10 days. Unless otherwise indicated, cells were grown at 30° C. Individual colonies were resuspended in sterile water and spread onto about 146 synthetic-galactose plates, lacking uracil and lysine, such that one colony from the glucose plate was put on one entire galactose plate. Cells were grown on galactose plates for 5-7 days. Isolates were struck for single colonies on synthetic-galactose medium lacking uracil and lysine. To help insure isolation of independent suppressors, only two to three colonies from each galactose plate were struck to single colonies, and subsequently, only one isolate from any one plate was retained, unless it was clear that additional isolates were genotypically or phenotypically distinct. A total of approximately 368 colonies were analyzed.

X-GAL Overlays.

X-Gal overlays procedure was obtained from Roy Parker and was slightly modified. Briefly, a 1% solution of agarose/H_2O was prepared and mixed 1:1 with 1M $NaPO_4$ pH7.0. Two hundred microliters of 2% X-GAL instead of 10% X-GAL was added per 10 mls of agar $NaPO_4$ mix. 2 ml of DMF was added per 100 ml total. The SDS and DMF are needed to break open the cells. Strains containing the *GAL1-LacZ* construct were struck out onto galactose plates selecting for maintenance of the plasmid and grown for 3-4 days at 30° C. Molten X-GAL mix was poured directly on top of the patches of cells on the plate. Color development was assayed after incubation for 1 - 2 hrs at 37° C.

Fluorescence Microscopy.

Cells were stained with antibodies for indirect immunofluorescence microscopy, with rhodamine-labelled phalloidin, or with DAPI as described previously (103) and were viewed with a Zeiss Axioscop.

Anti-actin and anti-tubulin staining was preformed as described in (54). Briefly, 5 ml of cells were placed into a 10 ml glass tube containing 0.67 ml of formaldehyde (use a fresh bottle if available). The tube was vortexed briefly and allowed to sit at room temperature for 1 hr. The cells were then spun down and washed twice with 2ml of 1.2 M sorbitol/0.1 M KPO_4 pH 7.5 (Pi/sorb), resuspended in 0.5 ml of phosphate/sorbitol solution + 1µl of βME + 10 µl or 10 µg 3mg/ml 20 T zymolyase and incubated for 40 min at 30° C. 2 ml of the phosphate/sorbitol solution was then added, the cells were spun down for 2 min and washed once with Pi/Sorb solution and gently resuspended in 1 ml of the same solution. 15 µl of cells were carefully placed onto a polylysine coaded slide.

After 60 min. the solution was aspirated off and the slide was allowed to air dry. At this point the slide was viewed under the microscope to see if the cells were distributed evenly and not clumped. For actin staining the slide was immersed in MeOH at -20° C for exactly 6 min and then immediately placed in acetone -20° C for exactly 30 seconds. The slide was then allowed to air dry completely. A 50 µl of a 1:9 dilution of fetal calf serum in solution F containing add 0.01 g $KHPO_4$ to 90 ml H_2O and titrated to pH 7.4 with 0.1 N KOH, diluted to 100 mls, 0.85 g of NaCl, 0.1 g bovine serum albumin and 0.1 g of NaN_3) was placed in each well. The slide was incubated for 45 min, after which the solution was aspirated off and the slide was washed 1 time in solution F and again the solution was aspirated off. 15 µl of primary anti-actin (1/50 dilution) or 30 µl of anti-tubulin (1:200) was added to a well. The primary antibody was incubated with the cells for at least 1 hr. The primary antibody was aspirated off and the wells were each washed 12 times with a drop of solution F. For the last wash, slightly more liquid was aspirated off, but not enough to completely dry the slide, then 30µl of secondary anti-body (anti-rat for tubulin, and anti-rabbit for actin or anti-Sac6p, 1:500 dilution) was added to the well. The secondary antibody was incubated with the cells for 1 hr, after which the wells were washed as before after the primary antibody. A drop of mounting media was placed on the slide (1 drop per 4 wells) and covered with a coverslip. The slides were stored at -20° C in the dark when not being viewed. For each of the incubation steps the slide was put under a petri dish lid (wrapped in foil) that had a wet kimwipe attached to the top.

Polylysine slides were prepared by adding 15 µl of 0.1% polylysine (size 400,000) per slide [Flow Laboratories, U.K. catalogue No. 60-408-05] after which the solution was aspirated off after 5-10 sec, making sure polylysine does not bead, and then the slide was allowed to air dry completely. Each well was washed 3 times with a drop of water that is

removed by aspiration or put in 50 ml Falcon tube and shaken for a couple of minutes. The slide was allowed to air dry completely before use.

For immunofluorescence of cells overexpressing Sac6p, see TABLE 5.1, the cells were grown in 2% sucrose to midlog then washed once and shifted to 2% galactose for 12hrs. The number of nuclei was determined by DAPI staining of asynchronous culture at 30° C. The percentage of DAPI stained cells is an average of 3 different experiments. Starting cultures were between ~1.52 X10^6 to ~4 X10^6 cells/ml, and after 12 hrs the number of cells containing a *GAL1-SAC6* plasmid is 6.67 X10^6 to 9.12 X10^6. Controls, grown in galactose, containing pPRP23 increased from 8.8 X10^5 to1.56 X10^6 to 8.76 X10^6 to 3.8 X 10^7, respectively. At 5 hrs, cells overexpressing Sac6p stop growing and viability drops as determined by placing cells back onto glucose plates after various times of induction and counting the number of cells that are able to form colonies compared to cells at time 0 and the control (pPRP23) (not shown). Actin and tubulin structures were determined by immunofluoresence using anti-actin antibodies and anti-tubulin antibodies, respectively. Wild-type strain AAY1016 with a control plasmid (pAAB121) was compared to AAY1016 with a plasmid containing *GAL1-SAC6* (pAAB157). Greater than 300 cells from 3 experiments were counted for DAPI staining and more than 200 cells, 100 cells from two separate experiments, was counted for anti-actin and anti-tubulin staining. For the three sets of experiments listed in TABLE 5.1 the percentages attained in each experiment did not vary by more than 7% at the 12 hr. time point. Note that these percentages may change depending on the mode of induction *ie.* from raffinose to galactose versus sucrose to galactose or the time after induction chosen to count the cells; however the end phenotypic consequences may be the same. For example at 6 hrs after

induction, the number of cells that are multinucleated is much lower, only 8 out of 312 cells counted were multinucleated.

Sequencing of the *sup25b* Mutation.

The mutant *act1* gene in a strain *sup25b* AAY1944 was cycle-sequenced as described previously (45). The entire coding region was sequenced on one strand, and the segment containing the mutation was sequenced on both strands.

Molecular Cloning of the SUP56c Gene.

The wild-type gene that carries a mutation in strain 56c was cloned by complementation of the Cs phenotype of *sup56c* (see RESULTS). Thus, the YCp50-based genomic library (obtained from Ted Weinert) was used to transform AAY1945 and AAY1946, and approximately 12,600 transformants were double selected for complementation of the cold-sensitive phenotype and *URA3* marker. Three colonies that were able to grow at 11° C on plates lacking uracil were struck to single colonies, and plasmid DNA was isolated, and amplified in *E. coli*. Restriction mapping showed that all 3 plasmids were identical, with an approximately 7 kB insert. A subclone lacking an internal *Sal*I fragment failed to complement the cold-sensitive, Ts, and osmotic phenotypes of *sup56c*, and sequencing of it revealed that the *Sal*I fragment was internal to the coding region of *SPT5* (118). Moreover, plasmids pMS18 (AAB272), pMS19 (AAB273), pMS37 (AAB274), and pMS4, containing various deletions of *SPT5* (118), "see RESULTS, Chapter 5", also failed to complement the Cs, Ts, and osmotic phenotypes of

sup56c where the complete *SPT5* gene was able to complement these phenotypes, consistent with *sup56c* having a mutation in *SPT5*.

CHAPTER 3: Purification and Characterization of Sac6p.

INTRODUCTION

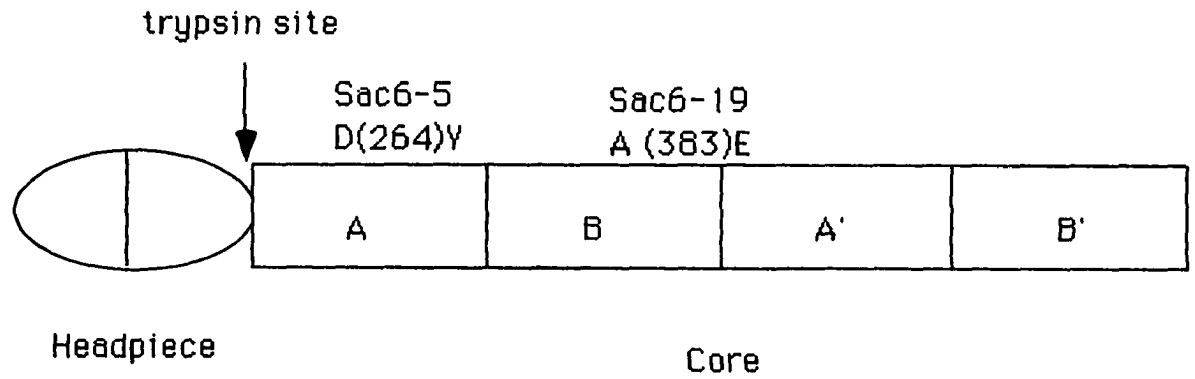
Sac6p belongs to the spectrin superfamily of proteins which include spectrin, α-actinin, dystrophin, ABP280/filamin, ABP120/*Dictyostelium* gelation protein, adducin and fimbrin/plastin. All contain an actin-binding domain that shares sequence similarity (37). Sac6p, yeast fimbrin, is most homologous to the fimbrin/plastin subfamily (4). Fimbrin was first identified in brush border microvilli as an actin bundling protein (15). Human fimbrin (plastin) was first identified as a protein that was increased during tumorigenic transformation of human cells and subsequently shown to belong to a family containing three isoforms T-fimbrin, L-fimbrin and I-fimbrin (15, 60, 63).

Structurally, fimbrins are composed of a head piece region, with sequence similarity to the calcium binding site of calmodulin, and a core domain that contains two putative actin binding domains that are thought to promote bundling of actin filaments (22). This N-terminal head piece region contains two predicted EF-hand motifs that are hypothesized to be important for Ca^{2+} binding. Although chicken fimbrin has been found to bind one calcium ion, conflicting results regarding the effect of Ca^{2+} on binding and bundling activities exist (15, 33). Yeast fimbrin, Sac6p, lacks crucial residues important for coordinating Ca^{2+} ions, which makes it unlikely that the head piece region binds Ca^{2+} (4). Conversely, mammalian L-fimbrin/plastin binding to actin is inhibited by micromolar amounts of Ca^{2+} (84).

The core domain of fimbrin contains four similar regions designated A, B (first actin binding domain), A' and B' (second actin binding domain) (22) (see Figure 3.1). Sac6p, as well as a number of other members of the spectrin family have a highly conserved 27 amino acid region in subdomain A and A' (4, 13, 14). This region has been implicated as an important region for binding to actin (13, 14). Since chicken fimbrin behaves as a monomeric protein as assayed by gel filtration, it has been hypothesized that the two actin-binding domains function to bundle actin filaments with a spacing of fimbrin-actin bundles of 11 nm (15, 74).

The present work describes the purification and partial characterization of yeast fimbrin, Sac6p. Interestingly, I have found similar properties between yeast fimbrin and higher eukaryotic homologues. First, Sac6p behaves as a monomeric protein as judged by gel filtration, similar to chicken fimbrin (15). Second, Sac6p contains a protease sensitive site that separates the head piece from the core domain which has also been defined for plastin (95). Third, purification methods similar to those used for L-plastin yielded two peaks after HA chromatography (110). As reported for L-plastin (110), the protein in each of these peaks yielded multiple spots by 2-D gel analysis. The multiple species in the case of L-plastin were a result of phosphorylation (110). However, we do not yet know if Sac6p is phosphorylated. Lastly, analogous to chicken fimbrin (15), we do not see Ca^{2+} inhibition of binding of Sac6p to actin.

FIGURE. 3.1. Domain organization of Sac6p based on (22). The head piece region has sequence similarity to the calcium binding site of calmodulin, and a core domain that contains two putative actin binding domains. The core domain of fimbrin contains four similar regions designated A, B, A' and B'. As described in (22), the 4 regions were determined by matrix comparisons of fimbrin and plastin polypeptide sequences using the program compare (UW,GCG) with stringency set at 25. Note a trypsin sensitive site is located between the headpiece region and the core domain in Sac6p giving the following N-terminal sequence I-I-V-A-G-S-Q-T-G-T-T-H-T (starting at amino acid position 124). The locations of *sac6-5* and *sac6-19* are shown and will be discussed in Chapter 3.


trypsin site
Sac6-5
D(264)Y
Sac6-19
A (383)E
A
B
A'
B'
Headpiece
Core

RESULTS

Purification of Sac6p.

Sac6p was purified as described in Materials and Methods. Briefly, to generate large amounts of protein, Sac6p was overexpressed in a protease deficient strain harboring a 2μ *GAL1-SAC6* (see Materials and Methods for construction of plasmid). I originally had trouble expressing *SAC6* in a protease deficient strain BJ2168. This may have been due to the fact that disruption of the *TRP1* locus in this strain affects the expression of the nearby *GAL3* locus, critical for galactose induction (51). Therefore, a strain BJ5465 that did not contain the *trp1* deletion was obtained from the Yeast Genetic Stock Center (51). Cells were grown in raffinose to midlog (see Figure 3.2, lane 1) and induced with 2% galactose. Cells overexpressing Sac6p (see Figure 3.2, lane 2) were lysed in a buffer containing10 mM Tris, pH 8.0, 0.2% NP40, 5 mM NaCl, 1 mM EGTA plus protease inhibitors, using a bead beater chamber. The lysed cells were first centrifuged at low speed for 10 min. at 10,000 rpm, then at high speed for 30 min. at 45,000 rpm. The high speed supernatant, (see Figure 3.2, lane3) was applied to a DEAE Sepharose CL-6B (Sigma) column equilibrated with 10 mM Tris, pH 8.0, 5 mM NaCl. The anion exchange resin was washed with 10 column volumes of 20 mM Bis-Tris, pH6.5 50 mM NaCl. Protein was eluted in a linear NaCl gradient (50 to 400 mM) in 20 mM Bis-Tris pH 6.5. Low pH reduces the amount of a higher molecular weight protein from coeluting with Sac6p in the HA column. The column fractions were monitored by dot blots and SDS PAGE. Sac6p-containing fractions were pooled (see Figure 3.2, lane 4), adjusted to 1 mM DTT, 0.2 mM EGTA, 0.5 mM EDTA and dry ammonium sulfate was added to a final saturation of

65%. The precipitated proteins were collected by centrifugation. Concentrated protein was resuspended in 3 ml of 5 mM $NaPO_4$, pH 7.0 and passed through a desalting column equilibrated with the same buffer (see Figure 3.2, lane 5). Protein was then applied to a Hydroxylapatite Bio-Gel HT column and eluted with a 5 to 300 mM $NaPO_4$ buffer, pH 7.0. Sac6p eluted as two separate peaks (see Figure 3.2, lanes 6,7) in the first 1/3 of the gradient.

This procedure resulted in highly enriched fractions of Sac6p; however, Sac6p eluted in two peaks from the final hydroxyapatite column. In uninduced cells, Sac6p constitutes 0.01% of the total cellular protein (24); after the purification steps, the highly enriched Sac6p peak fractions comprise 2% of the total soluble protein (protein in the high speed supernatant), which indicates Sac6p is induced over 200 fold (see Figure 3.2, lanes 1,2,6,7). The Sac6p in the second peak migrated approximately 1 kDa slower than Sac6p in the first peak. This difference could be due to translational modification, such as phosphorylation or degradation during purification. Variations in gel mobility have been reported in the purification of chicken fimbrin (15), and dual peaks of human plastin eluting from a hydroxyapatite column have also been noted (110). The biological relevance of the two Sac6p species, if any, has yet to be determined.

Differences Between the Two Hydroxylapatite Sac6p Peaks.

To determine if the difference in the two peaks could be due to degradation, protein in both peaks was subjected to N-terminal amino acid analysis as described in Materials and Methods. Protein in the first peak gave the following sequence, P-I-L-T-Q,

indicating that at least a portion of Sac6p in peak 1 was missing the first ten amino acids. The protein in the second peak failed to give any sequence; therefore, peak 2 protein was cleaved with CNBr to unblock the N-terminus. Since most proteins begin with the amino acid methionine, peak two protein was treated with CNBr which cuts on the carboxy side of methionine residues in proteins. From analysis of the primary sequence data using GCG program PEPTIDESORT, cleavage of denatured Sac6p should yield 8 fragments with the following molecular weights: 0.1492 kDa (a.a. 1-1), 0.4565 kDa (a.a. 639-643), 0.9902 kDa (a.a. 518-525), 1.0812 kDa (a.a. 526-535), 7.0861 kDa (a.a 452-517), 11.4309 kDa (a.a. 536-638), 11.9504 kDa (a.a. 347-451), 38.7356 kDa (a.a. 2-346). The cleaved protein was run out on a SDS polyacrylamide gel, transferred to nitrocellulose and a 38 kDa band was isolated (as described in Materials and Methods). The 38 kDa fragment of Sac6p should correspond to the first 346 amino acids with the exception of the first methionine. Sequence analysis of the 38 kDa band gave the following amino acids identical to the predicted translational amino acid sequence from amino acid 2-16, N-I-V-K-L-Q-R-K-F-P-I-L-T-Q-E, indicating Sac6p in the second peak contained an intact N-terminus. For future reference, small amino acid peptide inhibitors from Sigma that contain amino acids phenylalanine-proline (F and P) are available, and may be of use for future purifications.

To try to determine whether peak 1 had any biological significance, I attempted to isolate specific antibodies to the protein in each of the two peaks. The purpose of isolating specific antibodies would be to use them for immunofluorescence to determine if each of the Sac6p species had differences in localization patterns, for example, one to the

cortical patches and one to cables. Antibodies were affinity purified as described in the Materials and Methods. Briefly, Sac6p peak 1 and Sac6p peak 2 were transferred to nitrocellulose, crude antiserum (able to recognize Sac6p in both peaks) was incubated with peak 2 protein, bound antibody was eluted and then bound to peak 1 protein. The antibody that was not absorbed onto Sac6p peak 1 nitrocellulose should be specific for Sac6p peak 2 whereas the antibody eluted from what bound to Sac6p peak 1 nitrocellulose should be able to recognize both Sac6p species. Specificity was first determined by dot blot analysis and then by Western blot analysis (see Figure 3.3). The same procedure was repeated in reverse to purify Sac6p peak 1-specific antibodies. Antibodies specific to peak 2 protein were obtained (see Figure 3.3, compare lane 1 and 2 between blot A and B); however, peak 1-specific antibodies were not recovered.

Peak 2-specific antibodies were used for immunofluorescence; however, the antibodies did not recognize any structures *in vivo*. The inability to recognize cellular structures may be due to a number of possibilities. For example, the epitope that the peak 2-specific antibody recognizes may be hidden in the native structure *in vivo*, or altered by formaldehyde fixation. Alternatively, Sac6p could be modified by a postranslational modification, thus the active form does not contain the peak 2-specific epitope.

To better determine what epitope the peak 2-specific antibodies are binding to and to confirm that the protein in the two peaks was identical, peak 1 and peak 2 protein was cleaved with trypsin under native conditions as described in Materials and Methods.

Cleavage with trypsin resulted in a fragment between the 67 kDa and 45 kDa markers. Both peak 1 and peak 2 trypsin fragments were subjected to N-terminal sequence analysis. Peak 1 and Peak 2 trypsin fragments gave identical amino acid sequence I-I-V-A-G-S-Q-T-G-T-T-H-T (starting at amino acid position 124). Peak 2-specific antibodies failed to recognize this size of band by Western blot analysis, but did recognize the 38 kDa CNBr cleaved N-terminus indicating that these antibodies recognize something at the N-terminus, prior to the actin binding domain; however, it cannot be ruled out that the antibodies could also recognize some amino acids very close to the C-terminus that was clipped during the digestion procedures.

Phosphorylation.

Phosphorylation can also affect the mobility of proteins on a SDS polyacrylamide gel. Therefore, potential phosphorylation sites in Sac6p were looked for using Mac Pattern Program (see Table 1). There are a number of potential phosphorylation sites, including those within the head piece region of Sac6p. Interestingly, some *sac6* suppressor mutations mapped near potential phosphorylation sites. These include *sac6-17, sac6-5, sac6-14, sac6-6, sac6-7.* These mutations are near CK2 sites. Casein kinase II is a serine/theonine kinase. The following is a summary of the results using the Mac Pattern program:

1. the consensus pattern is [ST]- X(2)-[DE], where X is any amino acid.

2. serine is a better substrate than threonine, an acidic residue (either an aspartic acid or a flutamic acid) must be three residues to the C-terminal of the phosphate acceptor site.

3. more acidic residues at positions +1, +2, +4, and +5 improve the rate of phosphorylation.

4. aspartic acid is favored to glutamic acid as the acidic determinants.

5. a basic residue on the N-terminal side of the acceptor site reduces the rate of phosphorylation, but an acidic one will do the opposite.

If serine (261) is a casein kinase II phosphorylation site, the *sac6-5* mutation D(264)Y would reduce the phosphorylation rate, since as mentioned above, an acidic residue must be 3 amino acids to the C-terminal side of the phosphate acceptor site.

Since Sac6p had these potential phosphorylation sites, Sac6p was sent to Dr. Clover's lab (University of Georgia) to determine if Sac6p in peak 1 or peak 2 could be phosphorylated by casein kinase II (CKII). If the difference between the two peaks was indeed due to phosphorylation then one of the two peaks may be phosphorylated and the other one may not be phosphorylated. Interestingly, Ashok Bidwai, in Dr. Clover's lab, (personal communication) was able to phosphorylate peak 1 with CKII; however, peak 2 could not be phosphorylated. Protein was also sent to a CKI lab, and against the second peak could not be phosphorylated whereas the first could be phosphorylated (Namrita Dillian, Icos corporation). Interestingly, in a preliminary experiment, multiple spots are observed by two-dimensional gel analysis (Figure 3.4). However, whether these spots are due to phosphorylation or some other modification has yet to be determined.

As an added note, a 1:5000 dilution of CKI antibodies (anti-HRR25p antibodies, a gift from Namrita Dillian, Icos corporation) can recognize protein in the second peak overlapping the position of Sac6p; however, these antibodies are not able to recognize

protein in the first peak. The CKI gene homologues from *S. cerevisiae* -- HRR25 (57kD), YCK1 (61.7kD), YCK2, (62.1 kD)-- are all smaller in size than Sac6p making it unlikely that the protein in the band migrating at about 67 kD recognized by anti-HRR25 is one of these CKI proteins (42, 105). There is a faint band in total cellular protein that migrates close to Sac6p that is recognized by anti-HRR25. In addition, in total cellular protein taken from cells overexpressing Sac6p a band close to or overlapping the position of Sac6p increases in intensity compared to uninduced cells. This result suggests that CKI and Sac6p peak 2 may share some common epitope. CKI antibodies were made as a *TRP1* fusion construct (Namrita Dillian, personal communication). To test if the epitope is specific to an antigen other than that similar to TRP1, CBP1-TRP1 antibodies (a gift from Carol Dieckmann) were tested; however, the CBP1-TRP1 antibodies failed to detect peak 1 or peak 2 protein.

Effect of Ca^{2+} on Bundling and Binding activity of Sac6p.

Unlike human T-fimbrin (Wenyan Chen, Navin Pokala, Whitehead Institute for Biomedical Research & M.I.T. Dept. of Biology, personal communication) and L-fimbrin (84), whose bundling and binding activites have been shown to be inhibited in the presence of calcium, Sac6p does not appear to be inhibited by calcium under the conditions tested. Binding of Sac6p to actin filaments was assessed by mixing actin and Sac6p, initiating the assembly of actin filaments, and recovering the filaments by high-speed centrifugation. Under these conditions, polymerized actin with bound Sac6p is found in the pellet, whereas G-actin and unbound Sac6p remain in the supernatant. Bundling was assayed by low-speed centrifugation; conditions under which bundles but not individual filaments of actin

will sediment. The conditions used to assay calcium sensitivity were similar to those reported for I-fimbrin (63). With the addition of Ca^{2+}, T-fimbrin is defective in bundling yeast actin, yet yeast fimbrin is still proficient at bundling and binding (see Figure 3.5). These experiments do not exclude the possibility that calcium is affecting the actin structure and therefore not allowing T-fimbrin to bind. In addition, T-fimbrin bound to yeast actin much worse than yeast fimbrin (see Figure 3.5). Although no apparent effect on binding and bundling activity was identified under these conditions, it does not preclude the possibility that Sac6p does bind to Ca^{2+}, but that the divalent cation is required for some as yet unidentified function Sac6p. Chicken fimbrin has been found to bind either one calcium or one magnesium ion; however, conflicting results of the effect of Ca^{2+} on binding and bundling activities exist (15, 33). One caveat to these experiments is that the conditions for Sac6p binding may be such that differences in bundling in the presence or absence of calcium may not be observed. These experiments should be repeated using 100-200 mM NaCl and in the absence of magnesium. Addition of NaCl loosens the binding of Sac6p to actin and may accentuate differences that may be seen with the addition of various substrates like calcium, magnesium and ATP.

Sac6p Behaves as a Monomeric Protein.

The discovery of a putative leucine zipper, a dimerization domain, found in Sac6p using the MacPattern program, above, hinted at the possibility that Sac6p may function as a dimer (see Table 1). However, the proline located in this sequence makes it unlikely that a leucine zipper will form (47). Sac6p suppressor protein restores bundling with mutant actin (see Chapt. 4, Figure4.3) even though the suppressor mutation can be obtained in

only one of the two actin binding domains. We envisioned that this latter observation could be obtained if Sac6p dimerizes and thus, an increase in affinity of one actin binding domain to actin could restore bundling. To test if Sac6p behaves as a dimer in solution, Sac6p was applied to a Superose 12 gel filtration column in a solution similar to that used in bundling assays. The Sac6p (71 kDa) elution profile essentially overlapped with B.S.A. (66 kDa) indicating that under the conditions used, Sac6p exists in solution as a monomeric protein (see Figure 3.6). However, we cannot rule out that binding to actin is required for dimerization.

DISCUSSION

In summary, a number of interesting characteristics of Sac6p, including a trypsin sensitive site that may be useful for future crystal studies, have been identified. The N-terminus is sensitive to degradation, this may be one factor leading to the isolation of two Sac6p species eluting from a hydroxylapatite column. Sac6p in the second peak migrated approximately 1 kDa slower than Sac6p in the first peak. This difference could be due to translational modification, such as phosphorylation or degradation during purification. Slight variations in gel motility have been reported in the purification of chicken fimbrin and L-plastin (15, 31), and dual hydroxylapatite peaks containing human plastin have also been noted (110). For the case of plastin, the difference could possibly be due to allelic charged varients; however, the nature of the differences between the two species has not been determined in any of these cases. The biological relevance of the two Sac6p species, if any, has yet to be determined. I was successful in isolating antibodies that are specific to the second peak. These antibodies may be useful for competition experiments if a protein is found that binds to the N-terminal region of Sac6p, or for future Western blot analysis if an *in vivo* role for Sac6p peak 1 is discovered. The peak2 antibodies appear to recognize some epitope specific to the head piece of Sac6p, as suggested by the inability to recognize a trypsin fragment from amino acid 124 and the ability to recognize a CNBr fragment from amino acid 2-346. Another way to test this would be to determine if only the first or second actin binding domain is recognized by peak2-specific antibody. If the antibody recognizes something in the head piece, I would predict that the antibodies should not recognize protein extracted from a *sac6* null strain expressing just the first or second

actin-binding domain. Interestingly, if these antibodies do recognize only the head piece, then proteolysis of Sac6p most likely proceeds from the C-terminus to the N-terminus since the major degradation products recognized by antibodies against both peaks are also recognized by peak 2 specific antibodies (see Figure 3.3).

The observation that Sac6p yields multiple spots by 2-D gel analysis forms precedent to determine if Sac6p is phosphorylated *in vivo* and may yield insight into how Sac6p is regulated. There is precedent for phosphorylation of fimbrin homologs. Specifically, L-plastin is phosphorylated on Ser residues in the head piece region (31, 78, 110, 130). What role phosphorylation has in L-plastin function remains obscure. The discovery that Sac6p behaves as a monomeric protein and Sac6p binding to actin is insensitive to calcium, suggests that Sac6p behaves similarly to some forms of fimbrin in higher eukaryotes.

TABLE 3.1: Motifs in Sac6p from "Mac Pattern" program

amino acid position	motif	sequence	near sac6 mutation
14	CKII phospho site	TQED	
20	CKII phospho site	STIE	
47	TYR phospho site	KDGDATY	
52	CKII phospho site	TYDE	
59	PKC phospho site	TLK	
64	myristyl	GTTHTI	
68	PKC phospho site	SGR	
128	myristyl	GSQTGT	
132	myristyl	GTTHTI	
136	CKII phoso site	TI**N**E	sac6-17
179	myristyl	GLVLSK	
187	ASN glycosylation	NDSV	
189	CKII phospho site	SVPD	
223	PKC phospho site	SAK	
260	Leucine zipper	L**L**SKI**D**IKL**H**PELY RLLEDDETL	sac6-14,6-5,6-6,6-7
261	CKII phoso site	SKI**D**	sac 6-5
308	CAMP phospho site	RRVT	
312	ASN glycosylation	NFSK	
315	TYR phospho site	KDVSDGENY	
318	CKII phospho site	SDGE	
322	ASN glycosylation site	NYTI	
432	CKII phospho site	SLFD	
466	CKII phospho site	SGAE	
467	myristyl	GAEISR	
474	TYR phopho site	KALENTNY	
495	myristyl	GIEGSD	
520	ASN glycosylation	NISI	
524	PKC phospho site	TMK	
531	CKII phospho site	SGRD	
531	PKC phospho site	SGR	
551	myristyl	GGKNST	
554	ASN glycosylation	NSTI	
556	PKC phospho site	TIR	
559	PKC phospho site	SFK	
577	myristyl	GIAPGY	
593	CKII phospho site	TEEE	

* Positions of sac6 suppressor mutation are underlined and highlighted in bold.

FIGURE. 3.2. SDS-PAGE of steps in purification of Sac6p from strain AAY1918 overexpressing Sac6p. Lane (1), total cellular protein, protein from uninduced cells was extracted when the cells were at a density of ~ 8-9 X 10^6 cells/ml (klett 35); (2), total cellular protein, protein was extracted from cells after induction, the cells were at a density of ~3.15 X 10^7 cells/ml (klett 86); (3), high speed supernatant, 30 μg; (4), ion exchange group (see text), 10 μg; (5), 65% ammonium sulfate precipitation, 10 μg; (6), hydroxylapatite peak 1 fraction, 5 μg; (7), hydroxylapatite peak 2 fraction, 5 μg. Details of the purification are provided in the materials and methods. Protein concentration was estimated by the Bradfords assay using γ–globulin as a standard. The gel was stained with Coomassie Blue R-250. Klett readings were determined using a Klett-Summerson photoelectric colorimeter, MFG. CO., New York. The arrow denotes the position of Sac6p. The amount of sample loaded in lane 1 and lane 2 was adjusted such that the same amount of protein was loaded in each lane based on Klett readings.

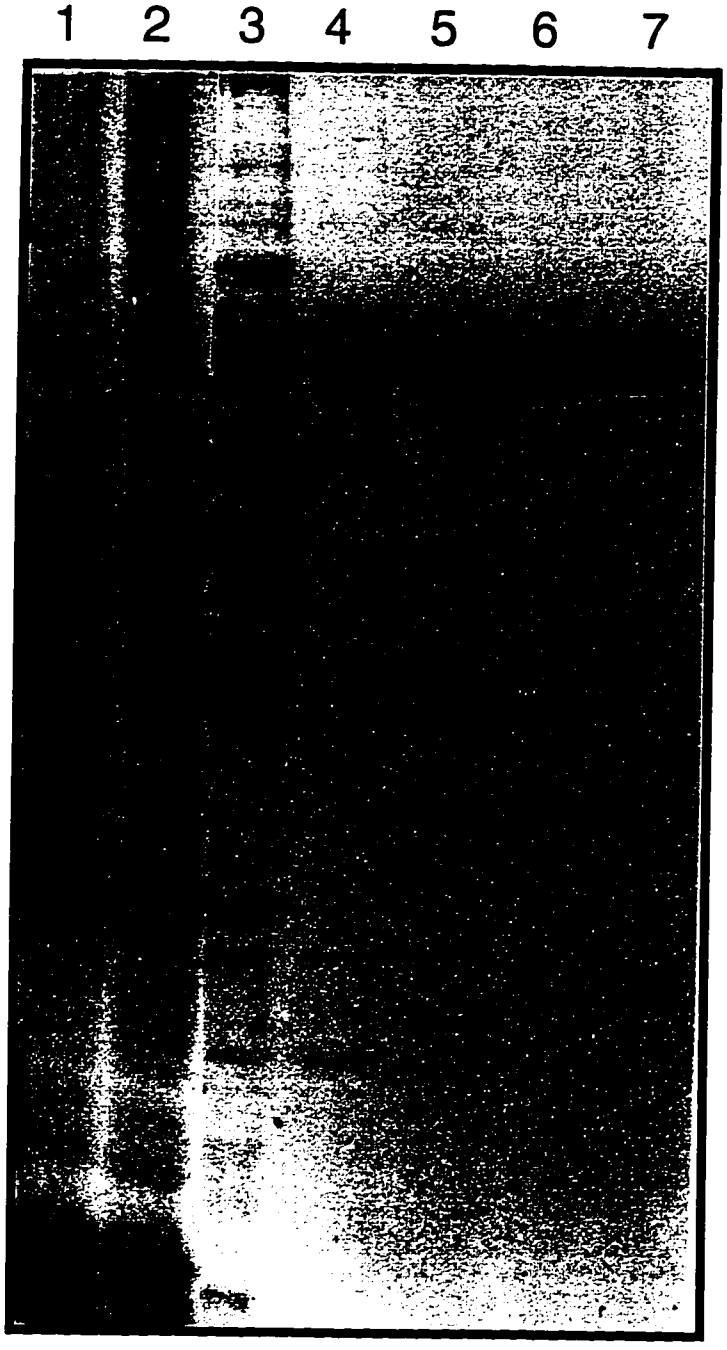
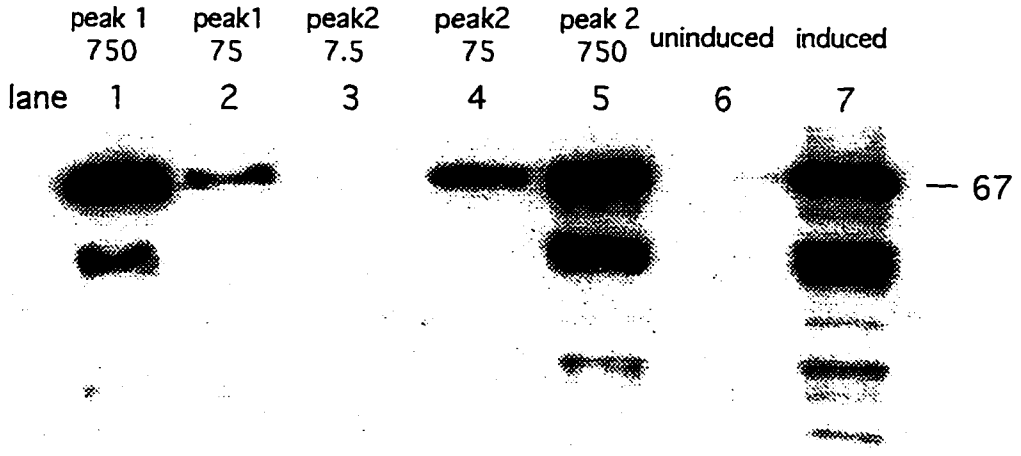

1
2
3
4
5
6
7

FIGURE. 3.3. Western blot showing specificity of peak 2-specific antibodies. The same samples were loaded on gel (A) and gel (B), protein was transferred to nitrocellulose as judged by Ponseau S staining. Lane (1), 750 ng of peak 1 Sac6p; (2), 75 ng of peak 1 Sac6p; (3), 7.5 ng of peak 2 Sac6p; (4), 75 ng of peak 2 Sac6p; (5), 750 ng of peak 2 Sac6p; (6), uninduced TSY 195; (7), induced TSY195. Blot A was probed with antibodies that were isolated against both peak 1 and peak 2 protein. Blot B was probed with Sac6p peak 2-specific antibodies. Note that peak 2-specific antibodies fail to recognize protein in lanes 1 and 2 indicating that the antibodies are specific for an epitope found only in protein located in peak 2. Similar results were obtained in four other experiments. Also, note that the major degradation fragments seen in A, lane 7; are also recognized by peak 2-specific antibodies B, lane 7; suggesting that degradation proceeds from the C-terminus.

A. Anti-peak1 + peak2



B. Anti-peak2

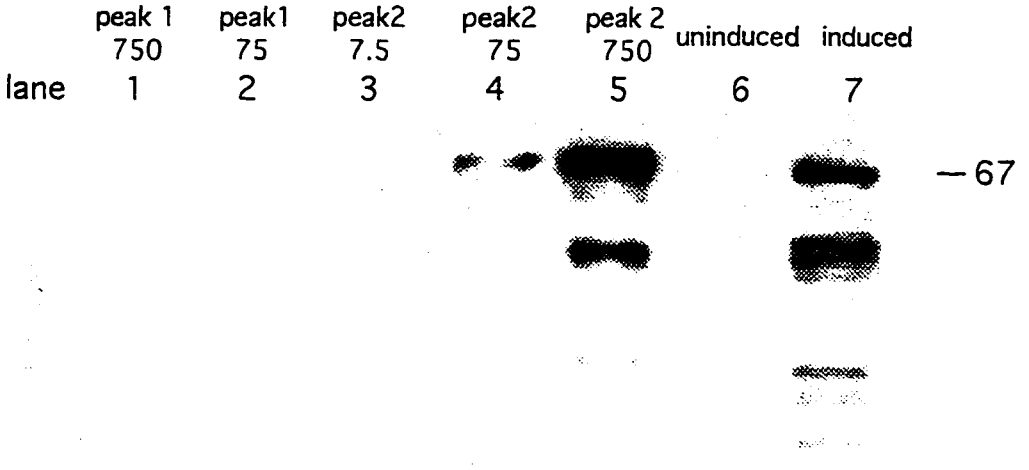


FIGURE. 3.4. Preliminary 2-D gel analysis of protein in HA Sac6p peak 1, (A); and Sac6p peak 2, (B). Note the presence of multiple spots in both Sac6p obtained from peak1 and Sac6p obtained from peak 2. 3μg of protein was loaded onto the 2-D gel. This experiment was only performed once. The orientation of the gels were difficult to determine; thus, the pH gradient is not shown.

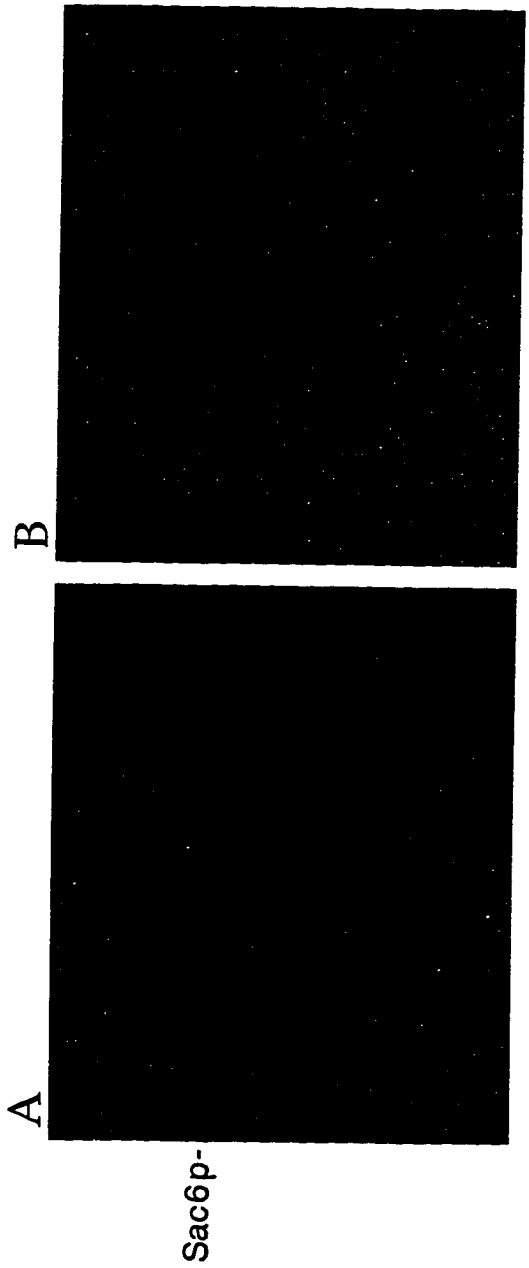

B
A
Sac6p-

FIGURE. 3.5. Effect of Ca^{2+} on binding and bundling activity. Binding of Sac6p to actin filaments was assessed by mixing actin and Sac6p, initiating the assembly of actin filaments, and recovering the filaments by high-speed centrifugation. Under these conditions, polymerized actin with bound Sac6p sediments (HSP, high speed pellet), whereas G-actin and unbound Sac6p remain in the supernatant (HSS, high speed supernatant). Bundling was analyzed using low-speed centrifugation - conditions under which bundles (LSP, low speed pellet), but not individual filaments (LSS, low speed supernatant), of actin will sediment (45). Shown are SDS-PAGE analysis of supernatants (s) and pellets (p) obtained from (+) 1 mM calcium, (A); or (-) calcium, (B). Bundling activity of T-fimbrin has been previously shown to be inhibited by calcium; therefore, it was used as a control.

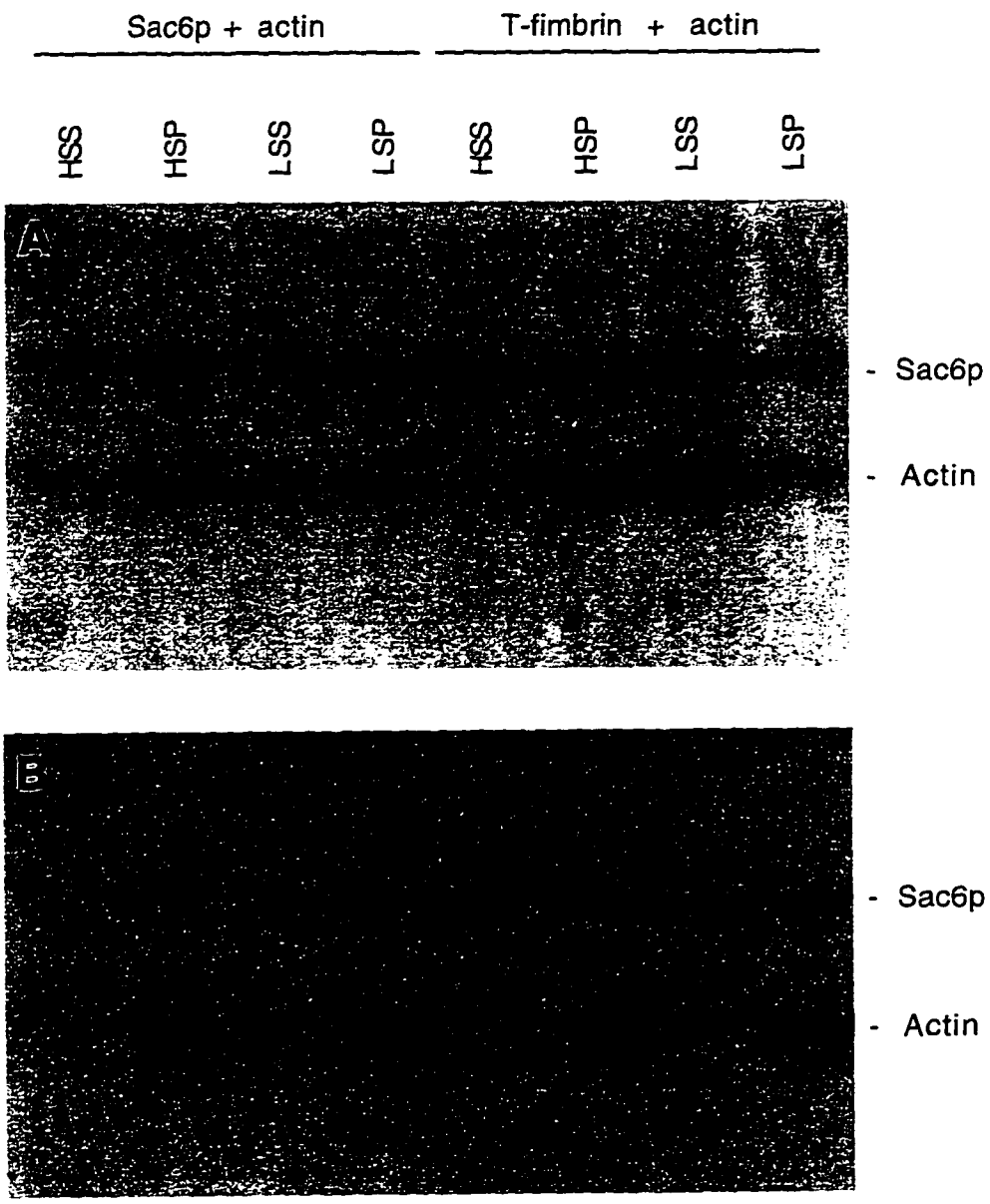
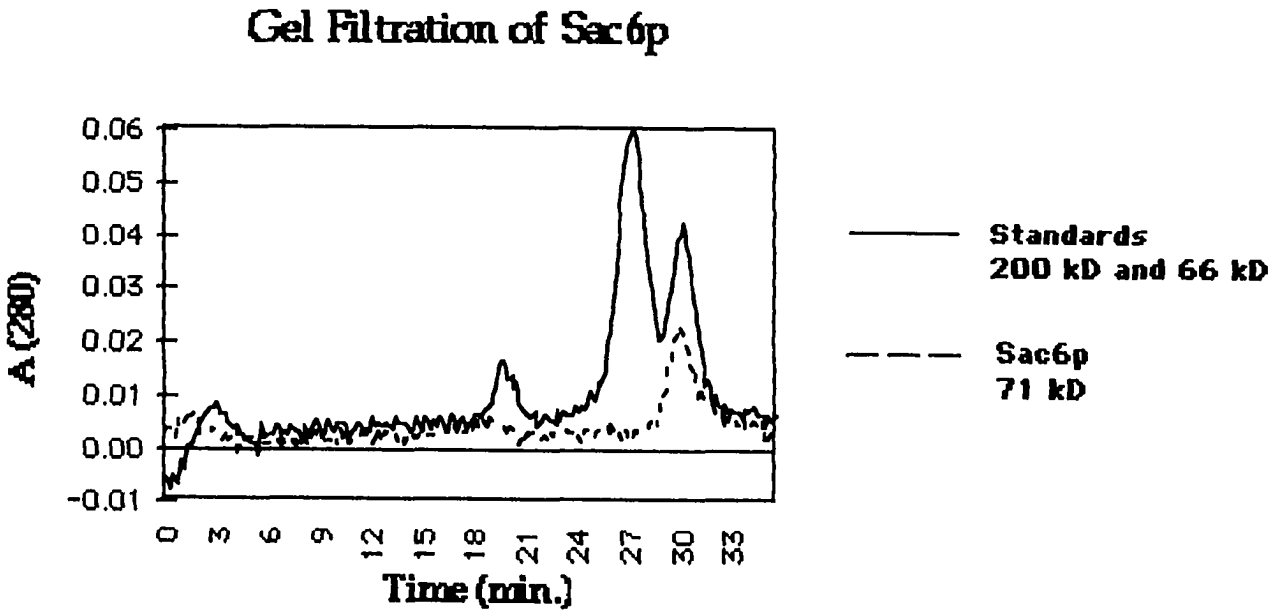

Sac6p + actin
T-fimbrin + actin
HSS
HSP
LSS
LSP
HSS
HSP
LSS
LSP
A
- Sac6p
- Actin
B
- Sac6p
- Actin

FIGURE. 3.6. Elution profile of purified Sac6p from the peak 2 fraction, approximately 200 μg, applied to a Superose 12 column (Pharmacia) using a Walters HPLC system (Millipore, Bedford, NJ) and eluted at 0.4 ml/min. Eluted protein was monitored at 280 absorbance. BSA (66 kDa) and β-amylase sweet potato (200 kDa) were used as standards.


Gel Filtration of Sac6p
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
A (280)
0
3
6
9
12
15
18
21
24
27
30
33
Time (min.)
Standards
200 kD and 66 kD
Sac6p
71 kD

CHAPTER 4: Molecular Basis of Allele-Specific Suppression Through Analysis of the Yeast Actin-Fimbrin Interaction.

INTRODUCTION

We have investigated the molecular basis of allele-specific suppression by analyzing the interaction between yeast actin and Sac6p, the yeast homolog of the actin-bundling protein fimbrin (4). Mutations in *SAC6* and *ACT1* suppress each other's defects (2, 5), consistent with the lock-and-key model. One of the predictions of the lock-and-key model is that there be great specificity: any one mutant allele should only be suppressible by a mutation that alters its counterpart in the interacting protein. Previously, Brower *et al.* (16) showed that in the case of the actin-Sac6p interaction, mutations that change any one of eight residues in the likely Sac6p-binding domain of actin (but not elsewhere in actin) can all be suppressed by any one of several different *sac6* alleles; thus, each suppressor appears not to restore a specific lost interaction, but rather to restore any one of a number of defective interactions (16, 45). This finding led to the suggestion that the *sac6* suppressor mutations might each increase the overall affinity between Sac6p and actin, thereby overcoming weakened interactions caused by any one of a number of *act1* alleles [(16); see Figure 4.4]. This idea further led to the predictions that (i) the mutant *sac6* alleles might also increase the interaction with wild-type actin, and (ii) those *sac6* mutant alleles (*e.g.*, *sac6-19*) that show defects in an *ACT1*$^+$ background (2), may give rise to interactions with actin that are too strong (16). We have tested these predictions biochemically, as described below.

RESULTS

In the present study, the basis of the suppression of an actin mutation (*act1-120*) by two different *sac6* alleles (*sac6-5* and *sac6-19*) is analyzed. These two *sac6* alleles were chosen, as (i) they both suppress the Ts defect of *act1-120* (*i.e., act1-120 sac6-5* and *act1-120 sac6-19* strains are Ts^+), (see Figure 4.1); and (ii) they have different effects in a wild-type actin background. Thus, *sac6-19 ACT1*$^+$ strains are Ts^- *i.e., sac6-19* is reciprocally suppressed by the *act1 -120* mutant allele and *sac6-5 ACT1*$^+$ strains are Ts^+.

The interactions between various combinations of wild-type and mutant actin and Sac6p proteins were tested through binding and bundling assays, as described previously (45). Thus, binding of Sac6p to actin filaments was assessed by mixing actin and Sac6p (purified as described in Figure 4.2), initiating the assembly of actin filaments, and recovering the filaments by high-speed centrifugation. Under these conditions, polymerized actin with bound Sac6p sediments, whereas G-actin and unbound Sac6p remain in the supernatant. Previously, this assay was used to show that several mutant actins whose *in vivo* defects are suppressible by *sac6* mutant alleles are defective in their interactions with wild-type Sac6p (44, 45, 53). For example, in the presence of wild-type actin and 0.125 M NaCl, most wild-type $Sac6^+$ protein is found in the pellet (Figure 4.2 A), whereas in the presence of Act1-120 mutant actin and the same concentration of salt, most $Sac6^+$ protein is found in the supernatant (Figure 4.2 E).

To test whether the *sac6* mutations suppress the *act1* mutant alleles by restoring the interaction between mutant actin and Sac6p, we examined the interactions between Act1-

120 and Sac6-5 or Sac6-19 mutant proteins. As shown in Figure 4.1 F,G at 0.125 M salt, most mutant Sac6 protein and Act1-120p is found in the pellet, indicating the mutant Sac6 proteins interact more strongly with Act1-120 actin than does wild-type Sac6p (compare with Figure 4.2 E). This level of interaction is similar to that seen between wild-type actin and Sac6 proteins (Figure 4.2 A), and reflects the *in vivo* suppression seen between these mutant alleles (2).

If the suppressors act by increasing the overall affinity of Sac6p for actin, the Sac6-5 and Sac6-19 mutant proteins should bind more tightly to wild-type actin than does wild-type Sac6p. This prediction is in contrast to that made by the "lock-and-key" model in which mutant Sac6 protein would be predicted to have a decreased affinity for wild-type actin. To test this prediction, it was first necessary to identify *in vitro* conditions under which binding of wild-type Sac6p to actin was weakened so that interactions even stronger than the wild-type interaction could be detected. Increased concentrations of NaCl, from 0.125 M to 1.0 M, resulted in increased levels of Sac6$^+$p in the supernatant (Figure 4.2 A), and thus diminished the wild-type actin-Sac6p interaction. This finding enabled us to test whether the mutant Sac6-5 and Sac6-19 proteins bind more tightly to wild-type actin than does wild-type Sac6p. At a given salt concentration (0.5 M or 1.0 M NaCl), more mutant than wild-type Sac6p is found in the pellet (Figure 4.2 B,C compared with Figure 4.2 A), indicating that, as predicted, mutant Sac6-5 and Sac6-19 proteins interact more strongly with wild-type actin than does wild-type Sac6p.

The finding that Sac6-5 and Sac6-19 mutant proteins have a higher affinity for wild-type actin than does wild-type Sac6p, suggested that the *in vivo* defect seen in *ACT1*$^+$ *sac6-19* might be due to an interaction between Sac6-19p and wild-type actin that is too

strong, rather than one that is too weak. As *ACT1*$^+$ *sac6-5* strains are not similarly defective *in vivo*, we predicted that Sac6-19p might bind more tightly to wild-type actin than does Sac6-5p. To test this hypothesesis, we examined the relative strengths of the interactions of these mutant Sac6 proteins with wild-type actin. As shown in Figure 4.2 B,C, at 1.0 M salt, most Sac6-19, but not Sac6-5, protein remains bound to wild-type actin, indicating that the former has a greater affinity for actin, as predicted. [As expected, even less wild-type Sac6p binds to wild-type actin under these conditions (Figure 4.2 A)]. This very strong interaction between Sac6-19p and actin is presumably responsible for the poor growth phenotype of *sac6-19 ACT1*$^+$ cells seen *in vivo* (2), as interactions that are too strong are probably as detrimental as interactions that are too weak. It is likely, for example, that improper interactions that arise during bundle formation (123) may not easily be corrected if individual interactions are too strong. Moreover, the dynamic nature of the cytoskeleton, which is necessary for reorganization of the cytoskeleton during the cell cycle, makes it likely that the formation of actin-filament bundle formation must be reversible. Thus, the optimal interaction between actin and Sac6p might not necessarily be the strongest.

As Sac6p is an actin-filament bundling protein, we expected that increased binding of Sac6p to actin filaments would result in increased bundling activity, and thus that the Sac6 suppressor proteins would restore bundling of, as well as binding to, actin filaments. To test this prediction, the abilities of wild-type and mutant Sac6 proteins to bundle wild-type and mutant actins was assayed. Two methods were used: (i) SDS-PAGE analysis of supernatants and pellets obtained from low-speed centrifugation - conditions under which bundles, but not individual filaments, of actin will sediment (45);

and (ii) electron microscopy of the mixtures prior to centrifugation. Electron microscopy was performed by Johanna O' Dell. For these experiments, 0.125 M NaCl was used. When wild-type or Act1-120 mutant actin was used in the absence of Sac6p, almost all the actin remained in the low-speed supernatant (Figure 4.3 D,H). This result indicated that most actin was present as single filaments, and electron microscopy of the mixtures prior to centrifugation confirmed this conclusion (Figure 4.3 D,H). In the presence of wild-type Sac6$^+$p, almost all the wild-type actin and Sac6$^+$p was recovered in the low speed pellet, indicating that most Act1$^+$ actin was present as cross-linked filaments to which Sac6p was bound (Figure 4.3 A). Electron microscopy confirmed that the majority of this actin was present as bundles, rather than individual filaments (Figure 4.3 A). In contrast, in the presence of wild-type Sac6p, most (but not all) of the Act1-120 mutant actin and Sac6$^+$p was found in the supernatant, suggesting that the majority of this actin was present as individual filaments, and only a minority was present as bundles. Electron microscopy again confirmed this conclusion (Figure 4.3 E). However, in the presence of Sac6-5p or Sac6-19p, almost all the Act1-120p was found in the low-speed pellet, indicating that the majority of the filaments were bundled; as before, electron microscopy confirmed this conclusion (Figure 4.3 F,G). Comparisons between samples containing Act1-120 and Sac6$^+$p, Sac6-5p, or Sac6-19p, by either electron microscopy or SDS-PAGE (Figure 4.3 E,F,G), revealed that the ratio of bundles to individual filaments in each case reflects the relative binding affinities of these various Sac6 proteins for actin (Figure 4.2). Thus, ability to bind actin and bundle filaments is greatest for Sac6-19p, less for Sac6-5p, and least for Sac6$^+$p. Together, these results demonstrate that the increased affinities of the mutant Sac6 proteins for mutant actin result in increased actin-filament bundle formation, and indicate

that the *in vivo* suppression seen between *act1* and *sac6* alleles is caused by restoration of actin filament bundle formation.

DISCUSSION

"Lock and key" type interactions have been reported previously. For instance, in one study, all the possible changes in two amino acid positions were created in the trp repressor (99). One mutant trp repressor had a clear classic "altered specificity"; thus, the mutant trp repressor binds a specific mutant operator, but not other mutant operators tested, better than it binds to the wild-type operator. Furthermore, the trp repressor bound the wild-type operator worse than wild-type repressor bound wild-type operator. In contrast to the mutant trp repressor mentioned above, the Sac6 suppressor proteins tested in this study do not behave in a typical "lock and key" fashion, but display an overall increase in affinity to actin. The interactions between *sac6* suppressors and *act1* alleles are more similar to allele-specific suppression observed between *malE* mutant alleles, encoding maltose binding protein (MBP), and *malF* or *malG* mutant alleles, encoding integral transmembrane proteins important for transport of maltose. In this case, the mechanism of suppression is thought to result from an overall balance in affinities between the two interacting mutant proteins (MalE and MalF or MalG). Interestingly, *in vitro* studies suggest that the MalF and MalG mutant proteins may bind too tightly to wild-type MBP (23). However, it should be noted that the assay used was an indirect one rather than a direct assay of affinities between the components since transport with various concentrations of protein is being measured rather than direct binding affinities based on gel shift assays. Mutations in malE, which reduced the affinity to MalF and MalG mutant proteins, suppressed the transport defects caused by these mutations. As with the case of the *sac6-2* mutant allele with wild-type actin, the observation that *malF* or *malG* mutations

are defective in the presence of a wild-type MBP is most likely attributed to an interaction that is too strong. Thus, restoration of a functional balance in affinity seen between pairs of allele-specific suppressors is a plausible molecular mechanism of suppression.

A number of reciprocal suppressors of *sac6* mutants were isolated and found linked to *SAC6* (2). One may predict from the second model that these suppressors should reduce the affinity of Sac6p for actin. If model one was plausible, that the *sac6* suppressors with an independent phenotype had a reduced affinity for wild-type actin, one may predict that the suppressors of the *sac6* mutations may increase the affinity of Sac6p for actin. Although we have shown that Sac6p mutant protein binds better to Act1p than wild-type Sac6p we do not know whether these effects are due to an increase in modification that results in increases in activity, or due to a global conformational change or a direct additional contact between Sac6p and Act1p. Interestingly, a number of suppressors of *sac6-2* do not map to *ACT1* (2) thus, it would be interesting to determine the exact mechanism of how this mutation increases the affinity of Sac6p for Act1p.

FIGURE 4.1. Suppression of *act1-120* by *sac6-5* and *sac6-19*. Shown are segregants of a tetratype from the diploid AAY1912 *act1-120/act1-3 SAC6*$^{+}$/*sac6-19*, (A); and AAY1937 *act1-120/act1-3 SAC6*$^{+}$/*sac6-5*, (B). *act1-120* is linked to *tub2-201* which confers benomyl resistance. In total, 18 tetrads derived from AAY1912 were examined. For 10 tetrads, all four spores in each tetrad were viable and segregated 2:2 Ts^{+} osm^{r} : Ts^{-} osm^{s}. Seven tetrads had three viable spores, four segregated 1:2:1 Ts^{+} osm^{r} : Ts^{-} osm^{s} : dead and three segregated 2:1:1 Ts^{+} osm^{r} : Ts^{-} osm^{s} : dead and one tetrad had two viable spores 1:1:2 Ts^{+} osm^{r} : Ts^{-} osm^{s} : dead. resistance. 17/33 benomyl resistant spores were Ts^{+}. Six tetrads derived from AAY 1937 were examined. All six tetrads had four viable spores which segregated all 2:2 Ts^{+} , osm^{r} : Ts^{-} ,osm^{s}. 5/12 benomyl resistant spores were Ts^{+}. The tetrad data indicates that both *act1-120* and *act1-3* are suppressed by both *sac6-5* and *sac6-19*. Cells were grown on YEPD plates at the following temperatures 30° C, 37° C, and 38° C and on YEPD plus 900 mM NaCl plates at 30° C to test osmotic sensitivity (osm^{s}) or osmotic resistance (osm^{r}). Note at 38° C *act1-3* is suppressed by *sac6-19* and *sac6-5* better than *act1-120* is by *sac6-19* or *sac6-5*. Both actin mutations are suppressed by *sac6-19* and *sac6-5* at 37° C and on 900 mM NaCl. *sac6-2* and *sac6-19* have identical nucleotide and amino acid changes (16).

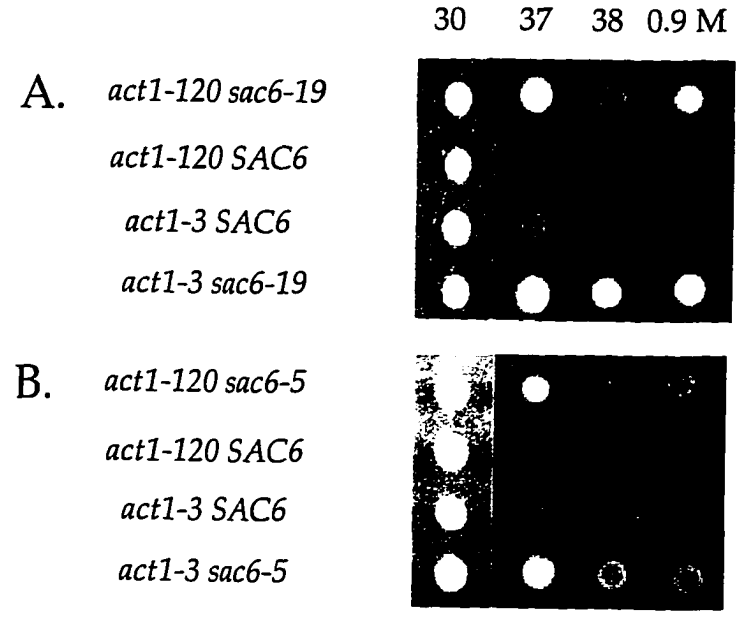

30
37
38
0.9 M
A.
act1-120 sac6-19
act1-120 SAC6
act1-3 SAC6
act1-3 sac6-19
B.
act1-120 sac6-5
act1-120 SAC6
act1-3 SAC6
act1-3 sac6-5

FIGURE 4.2. Binding assays of various combinations of wild-type and mutant actin and Sac6p, at 0.125, 0.5, or 1.0 M NaCl. 10% SDS-PAGE analysis of supernatant (**s**) and pellet (**p**) from high-speed centrifugation (45), following polymerization of actin (Act1p) in the presence or absence of Sac6p. The positions of Sac6p (hollow arrowheads) and Act1p (solid arrowheads) are indicated. Wild-type and mutant actins were purified as described previously (45), except DEAE Sepharose CL-6B (Sigma) column was used instead of the DE52 column. Wild-type and mutant Sac6p was purified as described in Figure 1. Mutant Sac6-5p and Sac6-19p was purified from strains AAY1916 and AAY1917, respectively, which are identical to AAY1918 except the plasmids contained in these strains carry *sac6-5* and *sac6-19*, respectively, instead of wild-type $SAC6^{+}$ (plasmids containing the mutants were constructed by Jerry Honts). Binding experiments were performed as described previously (45) with the following modifications: (i) to improve solubility of Sac6p following thawing, HA2 fractions were pooled and passed through a 2μ filter. Using Centricon 30 tubes (Amicon, product #4208), the fractions were then concentrated and buffer exchanged into 5 mM Hepes pH 7.5, 5 mM β-mercaptoethanol, 100 mM NaCl, 1 mM EGTA, 0.01% Na Azide, 0.1 mM PMSF; and (ii) 0.4 μM Sac6p and 3 μM actin were used.

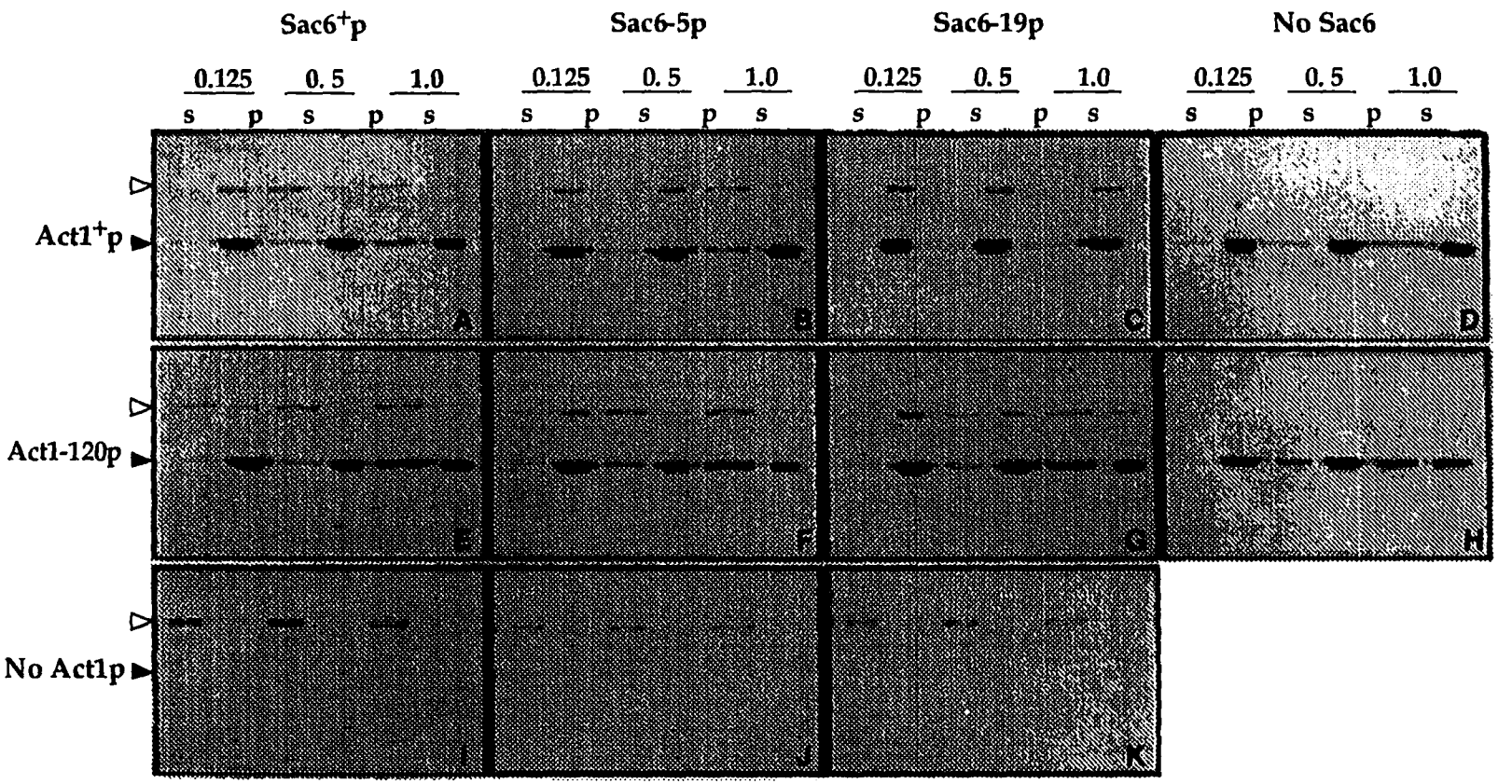

Sac6+p
Sac6-5p
Sac6-19p
No Sac6
0.125
0. 5
1.0
s
p
Act1+p
Act1-120p
No Act1p
A
B
C
D
E
F
G
H
I
J
K

FIGURE 4.3. Cross-linking of various combinations of wild-type and mutant actin and Sac6p, assayed by SDS-PAGE of supernatants and pellets after low-speed centrifugation (left of each panel), and by electron microscopy of the mixture before centrifugation (right of each panel). Portions of a single reaction mixture were used for both analyses. Cross-linking assays employing low-speed centrifugation and SDS-PAGE were performed as described previously (45), except that the concentration of NaCl in the actin-Sac6p mixture was 0.125 M, and centrifugation was in a TLA-100 rotor at 18,000 rpm for 15 min. at 22° C. The position of Sac6p (hollow arrowheads) and Act1p (solid arrowheads) in the supernatant (s) and pellet (p) are shown. Samples of the mixtures prior to centrifugation were prepared for electron microscopy as described previously (45) except that samples were stained in a solution containing 2% uranyl acetate, 0.125 M NaCl, 22.5 mM Hepes pH 7.5, 1 mM $MgCl_2$, and 2 mM EGTA. Stained, carbon-coated samples were collected on 300-mesh grids and viewed using a JEOL 100cx microscope at 80 kV. The identity of each sample was unknown during scoring.

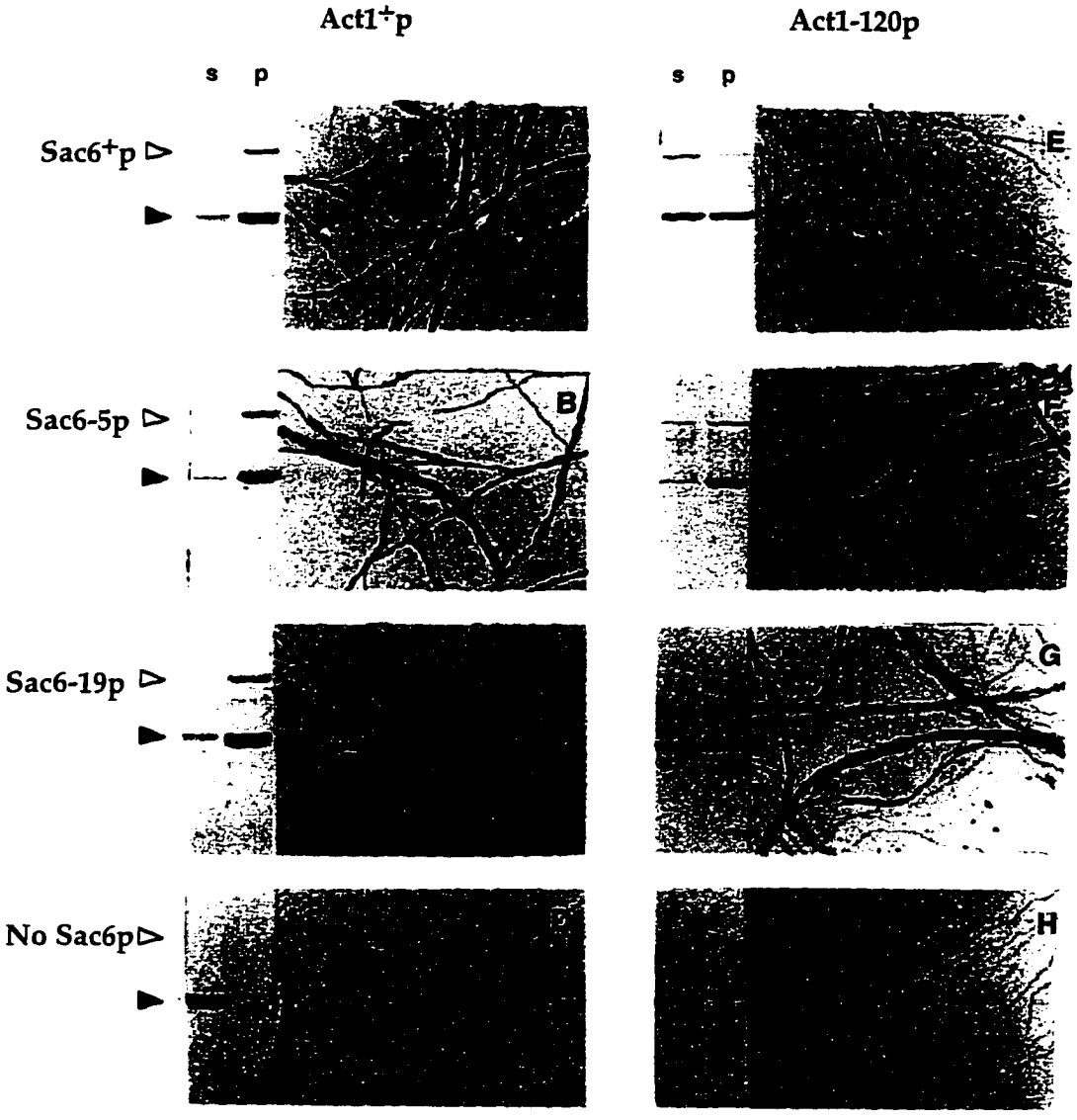
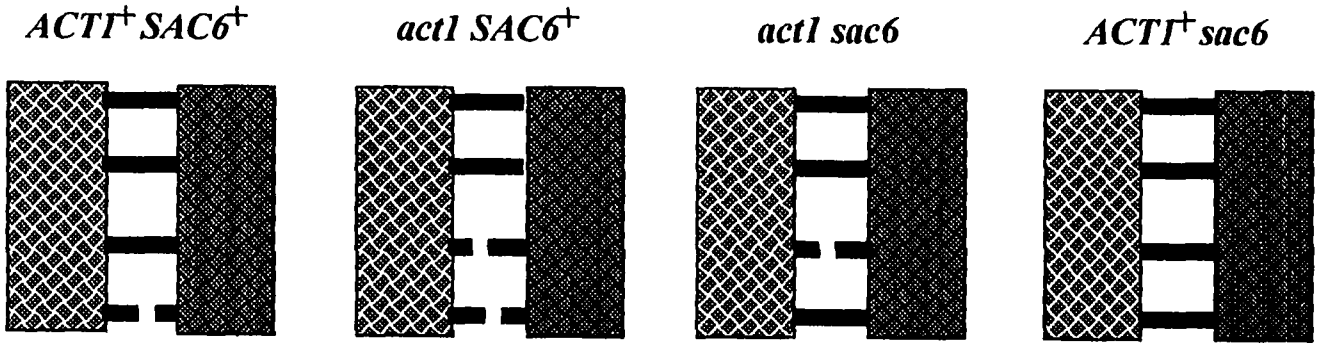

Act1+p
Act1-120p
s p
s p
Sac6+p
Sac6-5p
Sac6-19p
No Sac6p
B
E
G
H

FIGURE 4.4. Model of the mechanism of allele-specific suppression between actin and Sac6p. Actin is indicated with lighter patterned box, and Sac6p is indicated with darker patterned box. The loss or addition of black bars represent the reduction or increase in affinity between actin and Sac6p.


ACT1+ SAC6+
act1 SAC6+
act1 sac6
ACT1+ sac6

CHAPTER 5: Analysis and Suppression of Overexpression of Yeast Fimbrin.

INTRODUCTION

Actin has been implicated in many important cellular functions, such as secretion, endocytosis, mRNA localization and spindle pole orientation (40, 59, 88, 96, 116) A number of proteins modulate the structure and organization of actin [for review see (9, 107)]. Disruption, overexpression, or mutation of even one of many proteins controlling actin function can cause gross changes in actin organization, overall cellular morphology, and growth potential of cells. This suggests that a stoichiometric balance of the components is required.

Disruption of the actin cytoskeleton can occur by elevating the levels of actin or actin binding proteins beyond a threshold that a normal cell can tolerate. In yeast, actin (encoded by *ACT1*) and β-tubulin (encoded by *TUB2*), major proteins of the microfilament and microtubule cytoskeletons, are lethal when overexpressed (64). Overexpression of capping protein encoded by *CAP2*, which caps the barbed ends of actin filaments, results in an increase in the number of cells with an abnormal cytoskeleton and altered bud morphology, yet only minor defects in growth. Increasing actin binding protein I (encoded by *ABP1*) using a 2μ vector results in abnormal actin structures and causes cells to become temperature-sensitive (24). Further increases in actin binding protein I, via expression from a *GAL1* promoter results in lethality (64). In human cell lines, overexpression of the actin monomer binding protein profilin, results in an increase

in the concentration of filamentous actin (30). Futhermore, the distribution of actin differs in cells overexpressing profilin: actin is found close to the membrane, rather than in cables stretching across the cell (30). Thus, a proper balance of interactions in the actin cytoskeleton can be disrupted by increased concentration of one of its components.

Genetically, *SAC6* was identified as a dominant reciprocal suppressor of an *act1-3* mutation and biochemically the Sac6 protein was isolated by it's ability to bind to an F-actin affinity column (2, 24). Structurally, Sac6p contains two tandem actin binding domains similar to those in other actin binding proteins such as fimbrin, plastin, α-actinin, dystrophin and spectrin (4).

Here I describe the characterization of cells that overexpress Sac6p. Since the overexpression of Sac6p inhibited cell growth, I devised a genetic screen to isolate suppressors of the lethality. Since Sac6p was known to interact with actin, it was reassuring that we isolated an actin mutation from this screen, which suppressed Sac6p overexpression. Since over-expression of *SAC6* from the *GAL1* promoter inhibits cell growth, suppressors could be isolated on galactose plates with selection for the *GAL1-SAC6* plasmid. Those that had independent phenotypes in addition to only moderately reduced levels of expression from the *GAL* promoter were analyzed further. Notably, one of these suppressors, *sup56c*, shows a synthetic growth defect with a *sac6* null, possibly due to combination of two mutations that are unhealthy. The *sup56c* mutation is complemented by the previously identified gene, *SPT5*, which is thought to affect transcription *via* altering chromatin structure (118).

RESULTS

Phenotypic Characterization of Sac6p Overexpression.

Cells depleted of Sac6p have a more random distribution of actin and a spherical morphology compared to wild-type cells, indicating *SAC6* is required for proper organization of the actin cytoskeleton and the generation of normal cell shape (4). To determine the effects of altering the levels of Sac6p by overexpression, *SAC6* was placed under the control of the inducible *GAL1* promoter. Under inducing conditions, overexpression of *SAC6* inhibits cell growth (Figure 5.1). The same growth inhibition is observed in both haploid and diploid cells. In addition, the actin cytoskeleton is altered by overexpression of *SAC6*. Thus, some cells, (% was not determined) have randomly distributed cortical actin and 27% have abnormal actin bars, (Figure 5.3, Table 1). In addition, 39% of the overexpressing cells become multinucleated, and a similar number have multiple spindles (Figure 5.3). These results indicate that in cells overexpressing Sac6p, the nuclear cycle can continue in the absence of budding.

The *act1-3* Mutation Suppresses the Growth Defect due to *SAC6* Overexpression.

Various mutant actins have been shown to have decreased affinity for Sac6p (45). We reasoned that at least one of these, *act1-3*, might be able to suppress the growth defect of Sac6p over-production due to its reduced affinity for Sac6p. Therefore,*GAL1-SAC6 URA3* pAAB157 or a control plasmid pAAB121 was used to transform an *act1-3* strain

AAY131, and Ura$^+$ transformants were tested for growth on synthetic-galactose or glucose plates lacking uracil. As shown in Figure 5.2, the *act1-3* mutation can suppress the growth defect due to Sac6p overexpression. These results suggest that growth defects due to excess Sac6p are mediated through a stoichiometric imbalance with actin. Thus, reducing actin function or the ability to bind Sac6p negates the toxic effects of overproduction of Sac6p.

Genetic Screen to Identify Additional Suppressors of the Growth Defect due to *SAC6* Overexpression.

I envisioned possible ways in which Sac6p over-expression may inhibit growth. First, Sac6p could result in over-bundling of actin, which might disrupt such processes as secretion, endocytosis or asymmetric signaling. Consistent with this possibility is the observation that the cells contain abnormal actin bars (Figure 5.3), perhaps a consequence of excessive actin-bundling activity. Alternatively, an excess of Sac6p might fail to be modified appropriately, or could titrate out some factor necessary for cell growth. Yet another possiblity is that lethality may be the result of the budding cycle not being in synchrony with nuclear division. To better understand why *SAC6* over-expression is lethal, and to identify possible components that may regulate Sac6p post-translationally, or compensate for an excess of Sac6p, a genetic screen was devised to isolate genes that, when mutated, can suppress the growth defect due to *SAC6* over-expression. Since over-expression of *SAC6* from the *GAL1* promoter inhibits cell growth, suppressors could be isolated on galactose plates with selection for the *GAL1-SAC6* plasmid (see MATERIALS AND METHODS). Three hundred and sixy eight suppressor strains (some which come

from the same plate) were isolated and characterized further. Note that the number of the suppressor designates what plate the suppressor came from. The lower case letter is the isolate picked from that plate.

Plasmid-Linked Mutations.

I expected to obtain several classes of suppressors, including plasmid-linked mutations that no longer result in overproduction of wild-type Sac6p, through mutation of either the *GAL 1* promoter or the *SAC6* gene. To determine if suppression was due to plasmid-linked mutations, and to determine if the suppressors were dominant or recessive, the following approach was taken. Suppressor strains carrying a (*GAL1-SAC6 LYS2*) plasmid were crossed to a *SAC6* strain of the opposite mating type (AAY1947, AAY1948) containing a fresh plasmid (*GAL1-SAC6-LEU2*). Diploids were selected on synthetic-glucose plates lacking lysine and leucine. The resulting diploids, carrying two different *SAC6* overexpression plasmids, were purified and tested for their ability to grow on various galactose plates (see TABLE 5.2). If the suppressor is plasmid-linked and recessive, *i.e.*, due to a recessive mutation in the (*GAL1-SAC6 LYS2*) plasmid, then the resultant diploid should be able to grow on galactose plates lacking lysine (-lysine), but not leucine. As shown in Table 5.2, over 80% of the suppressors isolated fell into this catogory. If the suppressor is plasmid-linked and dominant *i.e.*, due to a plasmid mutation that can suppress even in the presence of a fresh plasmid, the resultant diploid should be able to grow on both -lysine gal and -leucine gal plates. However, growth on -leu galactose would be dependent on the presence of the (*GAL1-SAC6 LYS2*) plasmid. None of the revertants fell into this class (TABLE 5.2).

Genomic Suppressor Mutations.

The class of suppressor mutations that we wished to isolate in this screen were those that were genomic, either dominant or recessive. This class of suppressors could be distinguished from the plasmid-linked suppressors (above) in the same crosses described above. Thus, if the suppressor mutation is genomic and recessive, it will be masked by the wild-type copy in the resulting diploid strain. The diploid will then be unable to grow on synthetic-galactose plates lacking either lysine or leucine (Table 5.2). Fifty four suppressor mutations were of this class (Table 5.2).

If the suppressor mutation is genomic and dominant, however, the diploid should be able to suppress the lethality due to either the (*GAL1-SAC6 LYS2*) or the (*GAL1-SAC6 LEU2*) plasmid, and thus should grow on synthetic-galactose plates lacking either lysine or leucine. No suppressors of this type were obtained.

An additional genomic event that could lead to growth on galactose would be replacement of the *lys2:HIS3* locus in the genome with the *LYS2* marker from the plasmid. This event would allow cells that had lost the plasmid to grow on selective medium lacking lysine, even in the presence of galactose. Diploids resulting from a cross to wild-type carrying the (*GAL1-SAC6 LEU2*) plasmid would be able to grow on synthetic-galactose lacking lysine but not leucine, and thereby would appear similar to strains carrying recessive, plasmid-linked mutations (above). As neither category of mutation was of interest to us, however, the distinction was unimportant, but could be made by analysis of such strains on synthetic-galactose plates lacking histidine. *LYS2* replacements should be unable to grow under these conditions. Sixteen out of 368 suppressors were in this category (Table 5.2).

The analysis described above is critically dependent on the ability of the various suppressor strains to mate. Twenty-four of the suppressors, however, were unable to mate on plates and so were transformed with a fresh plasmid (*GAL1-SAC6 LEU2 CEN*) and tested for growth on synthetic-galactose plates lacking leucine. Growth indicated that suppression was not due to a plasmid-linked mutation. All 24 suppressors were confirmed genomic suppressors. This approach was also used to confirm that the genomic suppressors identified in the mating test were not plasmid-linked.

Genetic Analysis of the Genomic Suppressor Mutations.

Among the genomic suppressor mutations, we expected to obtain mutations in the galactose regulatory genes, which would result in decreased expression of *SAC6* to tolerable levels. Since a large number of genes affect galactose regulation (50), a reporter plasmid containing *GAL1-LacZ URA3* was included in the initial strain. This would allow assessment of the relative levels of induction from the *GAL1* promoter. Expression was measured using an X-Gal overlay assay, as described in MATERIALS AND METHODS. Briefly, suppressor strains were grown on synthetic-galactose plates lacking either lysine and uracil or just uracil, to maintain both the *GAL1-SAC6 LYS2* and *GAL1-LacZ URA3* plasmids, or just the *GAL1-LacZ URA3* plasmid, respectively. The plates were then covered with a molten agar mix containing X-GAL, and incubated for 1-2 h at 37° C. Suppressors were put into groups based on the relative level of blue-color development (due to levels of β-galactosidase activity) (TABLE 5.3). Only one mutant developed blue color as dark as, or darker than, the controls. The rest showed various levels of intensity from medium blue to white. Six mutants that were weak suppressors of

the growth defect due to *SAC6* over-expression were dark blue on synthetic-galactose plates lacking uracil, but were light blue on synthetic-galactose plates lacking lysine and uracil. This was presumably due to their ability to grow better in the absence than in the presence of the *GAL1-SAC6* plasmid.

Complementation Analysis of the Suppressor Mutations.

Genomic suppressors that were at least medium-blue on X-Gal plates were placed into complementation groups. *MATa* suppressors containing a *GAL1-SAC6 LYS2* plasmid were crossed to *MATα* suppressors containing a *GAL1-SAC6 LEU2* plasmid. Mating was carried out in liquid cultures, as some suppressors failed to mate on plates (see above). Diploids were selected on synthetic-glucose plates lacking lysine and leucine, and complementation groups were assigned on the basis of the ability of the diploids to grow on synthetic-galactose plates lacking lysine and leucine. The suppressor mutations fell into four complementation groups, A-D (TABLE 5.4).

Independent Phenotypes of Suppressors.

The existence of phenotypes in addition to the suppression phenotype can be helpful in determining which, of a collection of mutations, are most likely to be of interest. Suppressor mutations that decrease Sac6p function may have a phenotype, in a strain expressing normal levels of Sac6p similar to that of a *sac6* null strain. For example, these strains may exhibit temperature- or osmotic-sensitivity (4, 18, 88). Suppressors were therefore tested for their ability to grow on glucose (low levels of expression of *GAL1-*

SAC6) under conditions of high temperature or high salt (see TABLE 5.4). Interestingly, of the suppressors that were at least medium-blue in the X-GAL overlay test (complementation groups A to D), all were temperature-sensitive at 37° C on rich glucose plates (YEPD); however, no suppressors that were less than medium-blue (complementation groups not determined; see above) were temperature-sensitive. As shown in TABLE 5.4, the mutants in complementation groups A and B, but not C and D, were also osmotically-sensitive.

The Suppressor in Complementation Group A has a Mutation in *ACT1*.

Mutant *sup25b*, which was the darkest blue on X-Gal indicator plates, and had temperature-sensitive and osmotic-sensitive phenotypes (see Table 5.4), was analyzed further. First, I showed that all phenotypes were due to a single, nuclear mutation by crossing the mutant to a wild-type strain, and analyzing tetrads from the heterozygous diploid. Analysis of the diploid indicated that the mutation was recessive. *Sup25b* (AAY1944) was crossed to a wildtype strain (AAY1951). The temperature-sensitivity and osmotic-sensitivity cosegregated 2:2 in 8 tetrads consistent with the phenotypes resulting from a mutation at a single locus.

Since *act1-3* can suppress Sac6p overexpression, it was plausible that *act1* mutations may be isolated out of this screen. *act1* mutant strains are Ts and osmotically-sensitive (18, 88, 128). Since *sup25b* had phenotypes reminiscent of *act* phenotypes, *sup25b* was crossed to an *act1-3* strain. The resultant diploid showed temperature-sensitivity at 37° C and osmotic-sensitivity on 450mM NaCl indicating failure to complement. The diploid was sporulated and 16 tetrads were tested for osmotic-sensitivity

and temperature-sensitivity. Although the viability was only 75% all 42 live spores were osmotically-sensitive, and 41 spores were temperature-sensitive while only one of the spores was slightly Ts^+, consistent with *sup25* being tightly linked to the actin locus. 37/42 spores contained either a *LEU2 GAL1-SAC6* or *URA3 GAL1-SAC6* plasmid. All 37 segregants were able to support growth on galactose in the presence of the plasmid suggesting that both *sup25b* and *act1-3* can suppress Sac6p overexpression.

Sequencing of the sup25b mutation: To determine the location of *sup25b* in the actin gene, genomic DNA was isolated from a strain carrying the *sup25b* mutation; the actin gene was amplified using PCR, and the gene was sequenced. A single nucleotide change in the coding region of *act1* was found resulting in a Gly to Val change at amino acid 48. The *sup25b* mutation is located in the middle of the DNase binding loop (Figure 5.4).

A previouly characterized mutation, *act1-125*, alters residues 50 and 51, which lie two and three residues away from *sup25b* (128). Mutant actin purified from an *act1-125* strain, is defective in Sac6p-binding (45). In addition, *act1-125* is synthetically-lethal with a *sac6* null mutation, suggesting that *act1-125* has additional defects other than a reduction in affinity to Sac6p (44). Because of the proximity of *sup25b* to *act1-125*, I tested whether *sup25b* shows a similar synthetic-lethal phenotype with a *sac6* null mutation, and found that it does not. A strain carrying *sup25b*, AAY1785 was crossed to a *sac6::LEU2* null strain AAY1046 to create TSY410. The spores from the cross were 94.8% viable. From 22 tetrads tested there were five parental ditypes 0:4 osmotically-resistant (Osm^r) at 900 mM NaCl: osmotically-sensitive (Osm^s) at 900 mM NaCl, three nonparental ditypes 2:2

Osm^r to Osm^s and 14 tetratypes 1:3 Osm^r to Osm^s. Thus, *sup25b* is not synthetically lethal with a *sac6* null, unlike *act1-125* which is synthetically lethal with a *sac6* null (44), indicating there is a difference between how *sup25b* and *act1-125* affect the actin structure or interaction with other proteins.

The mechanism of suppression by *sup25b* may possibly be due to reducing the affinity of actin for Sac6p. Furthermore, this reduction in affinity to Sac6p may result in the independent phenotypes similar to the *sac6* null. Since the Sac6 suppressor proteins have been shown to have an increased affinity to actin (see Chapter 4, Figure 4.2) I hypothesized that these suppressors may also suppress the temperature-sensitivity and osmotic-sensitivity of *sup25b*. To test this hypothesis, *sup25b* was transformed with centromere plasmids containing the selectable marker *URA3* and either wild-type *SAC6* or *sac6* suppressor alleles (*sac6-4, sac6-5, sac6-14, sac6-17,sac6-2*). Six Ura+ transformants for each plasmid were selected and tested for their ability to suppress the temperature-sensitive phenotype of *sup25b*. The actin allele was suppressed by all five of the *sac6* alleles tested (data not shown). Interestingly, like *act1-125* (45), the phenotypes of *sup25b* were slightly suppressed by a single copy of wild-type *SAC6* consistent with the idea that Sup25b protein has a reduced affinity for Sac6p.

Characterization of *sup56c*.

Sup56c was crossed to an isogenic wild-type strain to determine if the phenotypes, (i) suppression of *GAL1-SAC6* overexpression, (ii) osmotically-sensitivity at 900 mM NaCl (Osm^S), (iii) resistant to 50 µg/ml benomyl at 30° C (Ben^r), and (iv) cold-sensitivity

on YEPD at 11° C (Cs), were linked. The viability of the spores was 75%. Twenty-three tetrads were analyzed and nine tetrads containing four spores segregated 2:2 Cs, Osm^S, Ben^r: Cs^+, Osm^r, Ben^S. 34/69 spores were Cs, Osm^S, Ben^r, of those that contained plasmid 25/26 suppressed the overexpression. 34/69 spores were Cs+, Osm^r and Ben^r, none of the 29 that contained a plasmid was able to suppress the overexpression and only 1/69 was Cs, Osm^r, Ben^S. The tetrad data strongly suggests that in *sup56c* that the Cs, Osm^S, and Ben^r phenotypes are conferred by a tightly linked or single locus.

Sup56c showed a reduction in expression from the *GAL1* promoter (TABLE 5.5), suggesting the mechanism of suppression may simply be through reduced levels of *SAC6* transcript. However, other phenotypes suggested the mutation in this strain may act through another mechanism. Particularly striking was the finding that reduction in *SAC6* expression enhances the phenotypes of sup56c. A *sac6:LEU2* null strain (AAY1046) was crossed to *sup56c* strain (AAY1945). The resultant diploid was dissected and the spores were tested for growth on YEPD plates at 11° C and on -leu plates. Twenty-five tetrads were analyzed. The viability was 61%. None of the 11 spores that were LEU+ were Cs indicating that the *sac6* null *sup56c* may be synthetically lethal. There were 12 tetratypes 1:1:1:1 leu- Cs+: leu- Cs-:leu+ Cs+: dead, 2 PD 2:2 leu+ Cs+: leu- Cs- and 5NPD 2:2 leu- Cs+: dead (see Figure 5.5). The genotype of dead spores were inferred assuming the Cs phenotype and *LEU2* each segregated 2:2. The *sac6:LEU2 sup56c* double mutants can germinate since the corresponding spores can undergo a few cell divisions as observed by analyzing the dissection plate. This genetic interaction suggests that sup56c requires the function of at least one copy of the *SAC6* gene.

Sup56c had phenotypes similar to *sla2*, (*s*ynthetically *l*ethal with *abp1*). *Sla2* mutants are also Cs, Ts and synthetically lethal with a *sac6* null. Hence a *SLA2 URA3* (AAB207), control *URA3* no insert (AAB35), 2μ *SAC6 URA3*(AAB144), *CEN SAC6 URA3* (AAB117) plasmids were transformed into *sup56c* (TSY256). Ura+ transformants were selected and tested for their ability to suppress the Cs and osmotic phenotype of *sup56c*. None of the plasmids were able to suppress the phenotypes suggesting that *sup56c* is not *SLA2*. Increasing the levels of Sac6p did not suppress the phenotypes of *sup56c*, as the Cs and Ts phenotypes were not suppressed by either a 2μ-*SAC6* or a *GAL1-SAC6* plasmid.

The wild-type gene was cloned by complementation of the Cs phenotype of *sup56c* (see Materials and Methods). Sequencing part of a complementing clone revealed an open reading frame corresponding to that of *SPT5*. The *spt5* mutant was originally isolated as a suppressor of Ty mediated gene expression (118). *SPT5* encodes a 115.6 kDa protein with two prominent features: a very acidic amino terminus and a carboxy terminus with the amino acid sequence (S-A/T-W-G-G-A/Q) repeated 15 times. A plasmid (pMS37) containing a deletion of the entire 15 repeats results in failure to complement *spt5::LEU2* and *spt5-194* allele (118). Interestingly, the Cs and osmotic phenotypes of sup56c are partially complemented by pMS37. To determine if *sup56c* carries a mutation in *SPT5*, AAY1946 (*sup56c*) was crossed to FY849 (*spt5::LEU2* deletion; as *SPT5* is essential, viability of FY849 was maintained with *SPT5* on a *URA3*, centromere-containing plasmid). The resultant diploid was sporulated and 24 tetrads were dissected. All viable spores that were Leu$^+$ were also Ura$^+$: these cells were unable to lose the *URA3 SPT5* plasmid as judged by lack of growth on 5-FOA. All viable spores that were not Leu$^+$ Ura$^+$ were osmotically-sensitive at 900mM NaCl, presumably due to the *sup56c* mutation . Of

24 tetrads tested, after removal of the plasmid by plating on 5-FOA, all segregated 2:2 osmotically-sensitive:lethal demonstrating that *sup56c* is tightly linked to *SPT5*. (The Ts and ben^r phenotypes were variable and could not be followed possibly due to strain differences.)

Suppressors in Complementation Group D are Complemented by *GAL11*.

Sup18b, which fell into complementation group D show approximately four fold reduction in production of Sac6p from the galactose promoter as judged by Western blots (not shown). This finding suggested that the mode of suppression is through reduction in the levels of transcription. However, members of complementation group C and group D had severe defects in budding at 37° C, observed by microscopic examination (using a dissection scope) of cells struck out onto a YEPD plate and grown at 37° C overnight. This suggested that group C and D mutations could also affect yeast morphogenesis as well as GAL induction. Since the mutants in complementation group C and D had phenotypes similiar to that of a *gal11* null mutant allele (28, 66, 76), I tested if members of group C or D could be complemented by a plasmid containing *GAL11*. Variation in the temperature-sensitive phenotype made analysis of complementation by *GAL11* difficult. However further analysis of AAY1952 and AAY1953 revealed that these mutants cannot grow on galactose plates at 11° C. Therefore, *sup33b* group C (AAY1952) and *sup35b* group D (AAY1953, an isolate not containing pRP127 was purified for transformation) were transformed with *GAL11 URA3* plasmids (pJF777 and pJF786) or control plasmids. Ura+ transformants were selected and tested for growth on YEP-GAL plates at 11° C.

The Cs Gal$^-$ phenotype of *sup35b*, but not *sup33b* was suppressed by *GAL11* on a

plasmid. These results are consistent with *sup35b* and *sup33b* being in two different complementation groups.

Actin Ring Structures in Strains That are Ts and Have a Reduced Mating Efficiency.

In budding yeast, actin is normally distributed in cortical patches, which are close to the surface of the membrane, and cables. During the cell cycle the actin cytoskeleton undergoes rearrangement. Early in the cell cycle actin is located in a ring prior to bud formation. As the bud emerges from the boundaries of the ring, actin concentrates at the tip of the growing bud. At the end of the cell cycle the actin again concentrates to the neck region between the mother and daughter cell.

The fact that group C and group D had budding defects at elevated temperature suggested that they might be defective in polarized secretion. Since actin has been postulated to be involved in budding and polarized secretion we hypothesized that the actin cytoskeleton may be disrupted in these mutants. Therefore, I examined the actin cytoskeleton of *sup33b* and *sup35b* at their restrictive temperature. After 12 hr at 37° C, ring structures were clearly apparent in mutant cells. These structures were brightly stained with anti-actin, anti-Sac6p (see Figure 5.6) or rhodamine phalloidin, but not with anti-tubulin (not shown). Staining with rhodamine phalloidin strongly suggests that these rings are composed of actin filaments. As shown in Figure 5.6 more than one ring can be found in one cell. In addition, the cortical patches appear absent in some of these cells; however, this could be due to incomplete digestion of the cell wall. To determine if these rings are remnants of bud scars, these cells were stained with Calcofluor. Calcofluor stains

chitin which normally is incorporated into bud sites. Preliminary results showed that Calcofluor staining of bud scars and the rhodamine phalloidin stained rings do not overlap. Hence, these rings may be a novel structure not seen before or; possibly the rings are potential bud sites, but at 37° C these cells are unable to deposit chitin properly. Although not as abundant, wild-type cells did give clumps of actin staining at 37° C but whether the concentrated actin areas are the same structures as in the mutant will require further analysis. Although these rings are intriguing, given that these mutations may be in transcription factors, the phenotypes are probably an indirect effect caused by a reduction in levels of one or many cytoskeletal components.

DISCUSSION

Cells overexpressing Sac6p behave similar to the defects observed in *cdc24,cdc42,cdc43*, *bem2*, and *act5* mutants (1, 98, 81) whereby they become multinucleated. Unlike damage to DNA (124), the cells may not have a mechanism to monitor disturbances in the actin cytoskeleton and subsequently pause nuclear division until the budding cycle is complete or the spindle is properly oriented with respect to the bud. However, key proteins that control the cell cycle can block the nuclear division as well as arrest the actin cytoskeleton at a certain organization predicted for that phase of the cell cycle (104).

The discovery that overexpression of Sac6p can be suppressed by certain actin mutations argues that the lethality is mediated through Sac6p interaction with actin, and not an accumulation of Sac6p aggregates poisoning the cell. If Sac6p is titrating out some factor other than actin, binding of Sac6p to actin is required for the second protein to bind to Sac6p. Alternatively, excess Sac6p may block the binding of another protein to actin. Residues in actin important for Sac6p binding are also required for binding of Fus1p to actin (8). We cannot rule out however that cells overexpressing Sac6p result in overbundling which could disrupt major processes such as assymmetric signalling, endocytosis, and/or secretion.

Interestingly, an actin mutation was isolated in a screen for endocytosis mutants (83). This allele, designated *end7-1*, alters the same residue as *sup25b* except *end7-1* results in a glycine to aspartic acid substitution (83). Since *SAC6* is required for

endocytosis (59) in yeast, the endocytosis defect of *end7-1* may result from a defect in binding of Sac6p to actin.

The observation that the actin mutation *sup25b* suppresses Sac6p overexpression, and the *sac6* suppressor mutations are able to suppress *sup25b* is consistent with the notion that Sup25bp has a decreased affinity for Sac6p. In addition, the results suggest that the actin mutation can affect the binding of the two actin binding domains and is thus also consistent with previous genetic data suggesting that the two domains bind to the same general region of actin (16). However, expression of either of the individual domains of plastin, the human homolog to Sac6p, is lethal in yeast (127). Furthermore, competition experiments suggest that the individual domains do not compete with each other for binding to actin, suggesting the separate domains have different binding sites on actin (127). Hence, the actin mutation could affect the binding of some positive Sac6p regulatory protein or cause more global disturbances in actin conformation. *Sup25b* is within the Dnase I-binding loop in subdomain 2 of actin (see Figure 5.4). Disruption of this region of actin by trypsin cleavage induces an increase in density between the 2 strands of the actin filament (94). Futhermore, positional changes in residues 33-70 (located in subdomain 2) of F-actin by 15° and 25° can occur with variation in buffer conditions (93). Thus, large allosteric effects caused by a mutation in this region cannot be ruled out. However, the *sup25b* mutation is located near other residues important for Sac6p binding and α-actinin binding (45, 77). Ultimately, it would interesting to study the properties of Sup25b filaments by electron microscopy to see if *sup25b* causes conformational abnormalities.

Synthetic lethal interactions have become increasingly common, and have been used to suggest functional redundancy and potential protein-protein interaction. For

instance, the loss of capping protein and fimbrin results in lethality (6) and indeed, actin filament assembly is disrupted in cells lacking either fimbrin or capping protein (53). Double mutations in *sla2* (*end 4*) and *sac6* also result in lethality. Both proteins are involved in a common process, cells containing mutations in either *sac6* or *sla2* are defective in endocytosis. The observation that *sac6* and *spt5* double mutants are inviable most likely does not indicate either functional redundancy or protein-protein interaction since Spt5p localizes to the nucleus and Sac6p localizes in the cytoplasm (24, 118). The phenotypic synergy seen in the double mutant may be due to Spt5p reducing the amount of a protein functionally redundant with Sac6p or may be an example of two different processes, which when reduced cause the cell to become sick. However, the importance of transcription factors being pivital control points for morphogenesis cannot be ruled out. Loss of HMG1-like chromatin associated proteins, *NHP6A*, and *NHP6B* exhibit budding and cytoskeletal defects. In addition, *NHP6A* and *NHP6B* have been suggested to function downstream of *Slt2p* MAPK pathway to help mediate growth and morphogenesis (20).

Suppressors in complementation group B through D reduce expression from a *GAL1* promoter, and given that group B and group D mutations are complemented by genes encoding proteins involved in transcription, Spt5 and Gal11, their mode of suppression may be due to reducing levels of Sac6p low enough to survive.

Limitations of the Screen and Possible Impovements.

The matings to determine whether the suppressor was plasmid-linked or genomic is the major time sink for this screen although it may be faster than transforming 300 cells with independent plasmids. To improve the screen, fusions of the plasmid to GFP (green

fluorescence protein) (102) to quickly visualize how much of the protein is produced and the localization of the protein *in vivo* would greatly enhance the fluidity of the screen. One could directly assay suppressors by picking cells from the plate and visualizing them under a microscope. For example, the *SPT5* and *sup18b* (complemented by *GAL11*) greatly reduced the amount of florescence seen *in vivo*, as expected they are involved in the general transcriptional apparatus. *GAL1-SAC6-GFP* expressed in *sup25b* resulted in the whole cell glowing brightly, although distinct patches and cables could not be seen in this suppressor possibly from the reduction in binding of Sac6p to actin. This screen was successful in isolating a *bona fide* suppressor of Sac6p overexpression, *sup25b*, that clearly did not reduce the levels of transcription. Other classes of suppressors may be obtained by reducing the amount of transcription from the *GAL1* promoter to the thresehold of lethality.

In vivo, a number of different proteins bind to actin structures forming a complex web of interactions. The proper stoichometry of some of these components are critical for proper function of actin *in vivo*. We have described a suppressor screen that should be useful in identifing suppressors of other genes that are lethal when overexpressed.

TABLE 5.1. Staining Patterns in a Sac6p Overexpression Strain.

Yeast Strain	%Multinucleated	%Multiple Spindles	%Actin Bars
Control	0	0	5
Sac6p	39.5	38	27

See MATERIALS AND METHODS for immunofluorescence procedure. Actin and tubulin structures were determined by immunofluoresence using anti-actin antibodies and anti-tubulin antibodies, respectively. Wild-type strain AAY1016 with a control plasmid (pAAB121) was compared to AAY1016 with a plasmid containing *GAL1-SAC6* (pAAB157). The cells were grown in 2% sucrose to midlog then washed once and shifted to 2% galactose for 12hrs. The number of nuclei was determined by DAPI staining of an asynchronous culture at 30°C.

TABLE 5.2. Summary of Suppressor Screen.

Analysis of Diploids	Overview of Screen		
	- lys gal	- leu gal	368total
Recessive plasmid-linked	+	-	~298
Dominant plasmid-linked	+	(+ or -)	0
Recessive genomic	-	-	54
Dominant genomic	+	+	0
Gene conversion of *Lys2* locus	+	-	16

Suppressors carrying pAAB329 (*GAL1-SAC6 LYS2 CEN*) were mated to a wild-type strain of the opposite mating-type carrying pAAB328 (*GAL-SAC6 LEU2 CEN*). Diploids were selected on -lys, -leu glucose plates. Individual colonies were tested for growth on -lys galactose plates and -leu galactose plates. Growth was scored as +, and no growth as -.

TABLE 5.3. Expression of *GAL1-LacZ* Fusion in Suppressor Strains.

X-GAL overlay assay	First	strain
Dark Blue	1	25b
Med-Dark Blue	24	33b,34c,24b,35b18b,48c, 19c,26c,40b,26b,41c,42a, 54c,63b,84b,21c,13b,51b, 49b,48a,33c,96c,80a,56c
Medium/Light Blue	3	39b,10b,93c
Light Blue	5	90c,27c,55c,87c,77c
White	15	7b,9c,13c,14b,20c,95c,78c,68b,65c,76c, 37c,38c,23c 12b(1st),14b(1st)
Dark blue only when not overexpressing SAC6*	6	4c,10c,52b,90b,71b,72c
Total	54	

* These were very weak suppressors of Sac6p over expression. Suppressors were grown on galactose plates selecting for plasmid maintanence. X-GAL overlay test was done as described in MATERIALS AND METHODS. Color development was determined visually after 2 hr at 37° C.

TABLE 5.4. Complementation Analysis.

Complementation Group	Linkage	No.	37° C YEPD	11° C YEPD	450mM NaCl	900mM NaCl
A. sup25b	*ACT1*	1	-	+	-	-
B. sup56c	*SPT5*	1	-	-	+	-
C. sup33b,34c,24b		3	-	+	+	+
D. sup35b*,18b,48c, 19c,26c,40b,26b,41c 42a,54c,63b,84b,21c 13b,51b,49b,48a,33c96c		19	-	+	+	+

*Only the representative member listed of complementation group D were crossed to a strain carrying the *gal11* null mutant allele.

+ Sup80a gave variable results and could not be assigned a group.

TABLE 5.5: Complementation of *sup56c* by *SPT5*.

plasmids	Not to scale	37°	11°	900 mM NaCl
pMS 18 FB1407 AAB272	NruI SalI SalI PvuII KpnI BamHI /_____/_____/_____/_____/_____/____/	-	-	-
pMS19 FB 826 AAB 273	NruI SalI SalI PvuII KpnI _____/_____/ Leu2 /_____/_____/	-	-	-
pMS 37 FB1408 AAB274	NruI SalI SalI PvuII _____/_____/____/_____/	+	+/-	+
pMS 4 FB 827	NruI SalI SalI PvuII KpnI BamHI _____/_____/____/_____/_____/____/	+	+	+
1D TSB84	NruI SalI SalI PvuII KpnI ..._____/_____/____/_____/_____/_____....	+	+	+
1d (*SacI* fill) frame shift.	NruI SalI SalI PvuII KpnI BamHI _____/_____/___/____x___/_____/____/ S a c I	+	+/-	+

pMS37 eliminates complementation of *spt5-194* and the null allele of *SPT5* but still partially complements *sup56c*.

FIGURE 5.1. Wild-type diploid AAY1016 and a *sac6* null strain AAY1046 transformed with AAB156 (cloning intermediate to AAB157 containing only the 5' 140 bp of *SAC6*), AAB157 (*GAL1-SAC6* full length), and AAB121 (plasmid no insert). Cells were grown at 30° C on -ura glucose (uninduced) or -ura galactose (induced) plates for 3 days.

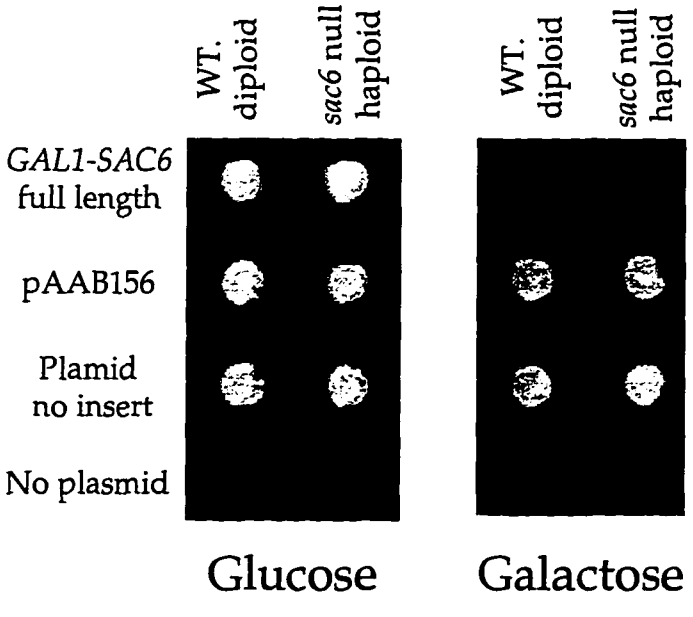
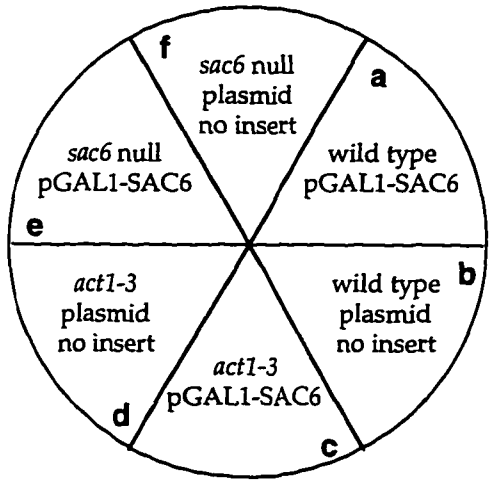

WT. diploid
sac6 null haploid
WT. diploid
sac6 null haploid
GAL1-SAC6 full length
pAAB156
Plamid no insert
No plasmid
Glucose
Galactose

FIGURE 5.2. Suppression of Sac6p overexpression lethality by *act1-3*. Growth of cells on -ura glucose (uninduced) or -ura galactose (induced) after 3 days at 30° C. (a), AAY1957 (*SAC6*$^+$ *ACT1*$^+$ strain transformed with AAB157, p*GAL1-SAC6*); (b), *AAY1959 (SAC6*$^+$ *ACT1*$^+$ strain transformed with AAB121, plasmid no insert); (c), AAY131 (*SAC6*$^+$ *act1-3* strain transformed with AAB157); (d), AAY131 (*SAC6*$^+$ *act1-3* strain transformed with AAB121); (e), AAY 1126 (*sac6* null *ACT1*$^+$ strain transformed with AAB157); (f), AAY1124 (*sac6* null *ACT1*$^+$ strain transformed with AAB121).



Glucose **Galactose**

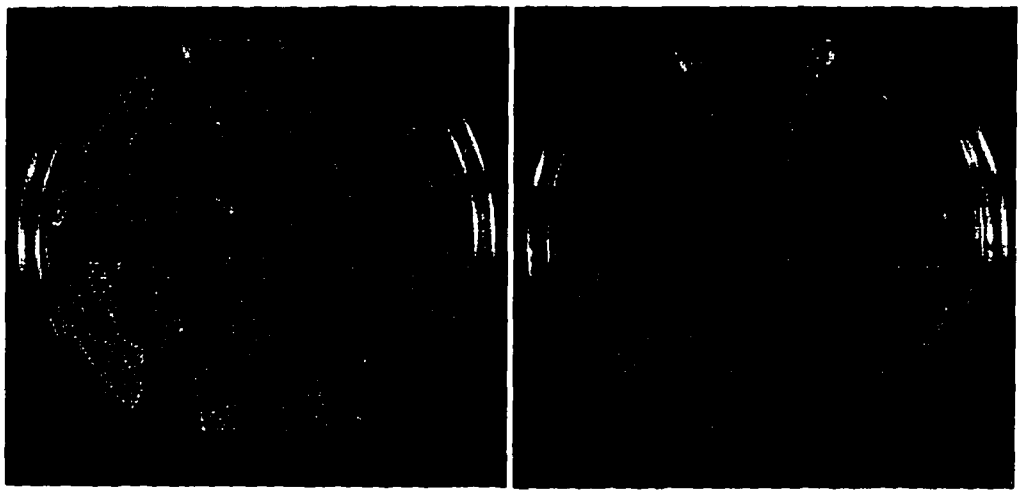


FIGURE 5.3. Fluorescence of wild-type diploid strain overexpressing Sac6p. (a), control, AAY1016 containing pAAB157, a *URA3 GAL1-SAC6* plasmid, grown in -ura glucose; (b), control, AAY1016 transformed with pPRP23, a *URA3* plasmid containing no insert, grown in -ura glucose; (e), control, AAY1016 containing pPRP23, grown in -ura galactose for 12 hrs; (c,d,f), AAY1016 containing pAAB157, after 12 hr. induction, grown in -ura glactose. Induction was performed as described in Table 5.1. Cells were stained with anti-actin, (a,b,c); anti-tubulin, (d); or DAPI, (e,f). Note that in cells overexpressing Sac6p (f), more than one DAPI stained body per cell is visible, also consistent with this result, these cells also have mutliple spindles (d). Also note the actin bars seen in (c) indicating disruption of the actin cytoskeleton.

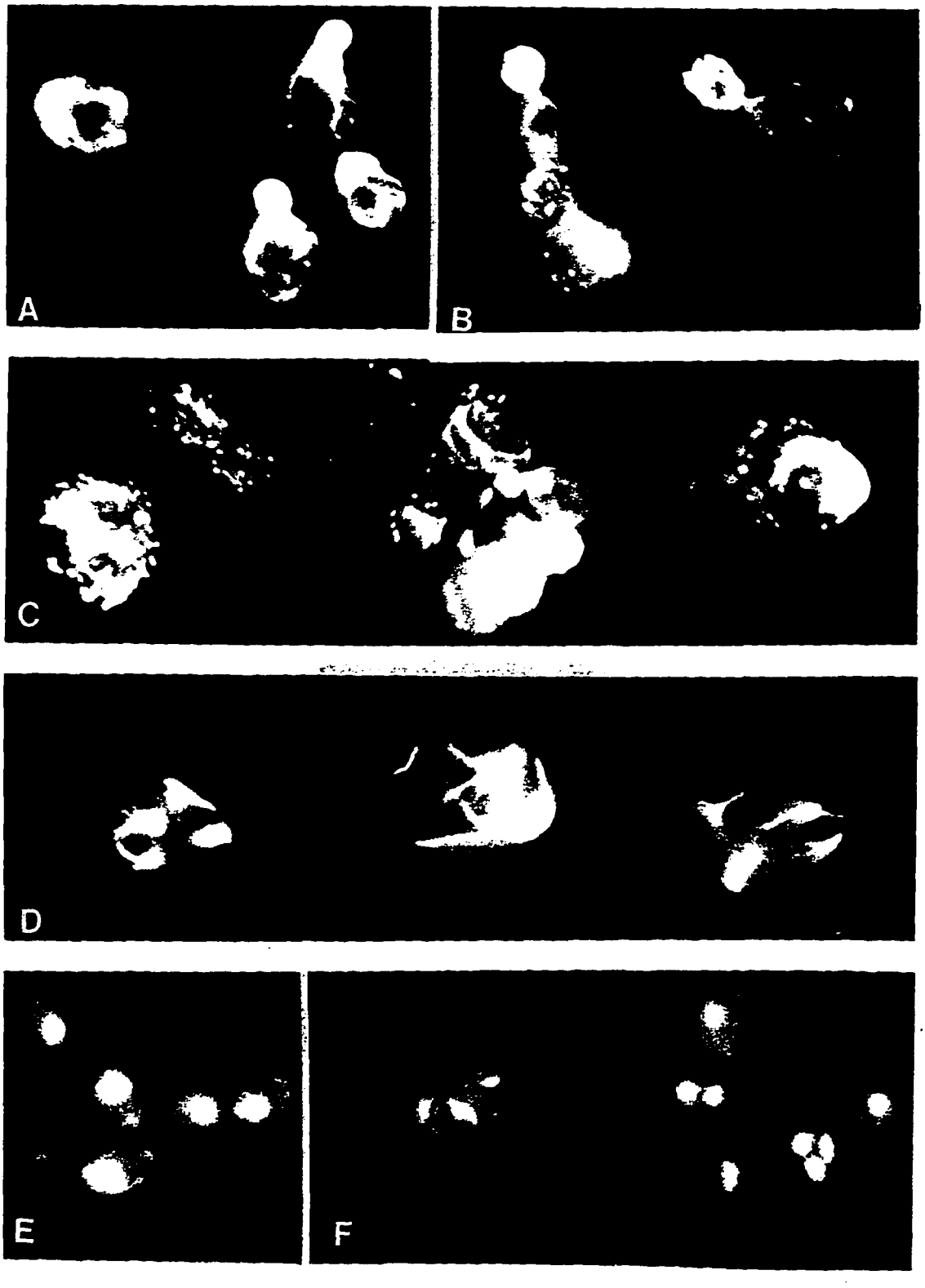
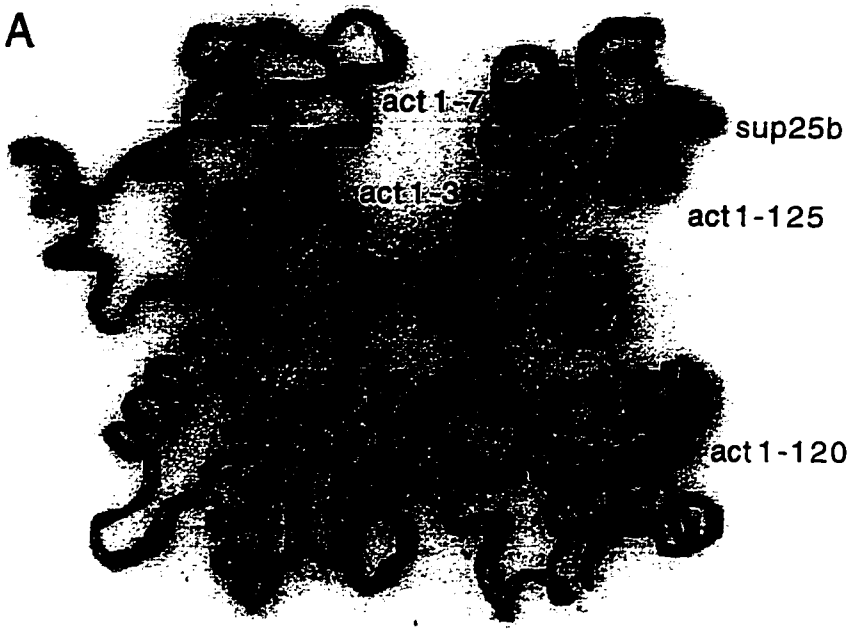

A
B
C
D
E
F

FIGURE 5.4. A. G-actin structure (52). B. F-actin structure(67) Location of residues that are important for Sac6p interaction with actin are indicated (45). Glycine, residue 48 is replaced with Valine and is shown in red using the program "Insight". *Sup25b* is within the Dnase I-binding loop in subdomain 2 of actin. Note the proximity of *sup25b* to the other highlighted residues and the relative change in position of *sup25b* in the two structures, F-actin and G-actin. 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 Insight II program (Biosym Technologies, San Diego, CA).


A
act1-7
act1-3
sup25b
act1-125
act1-120

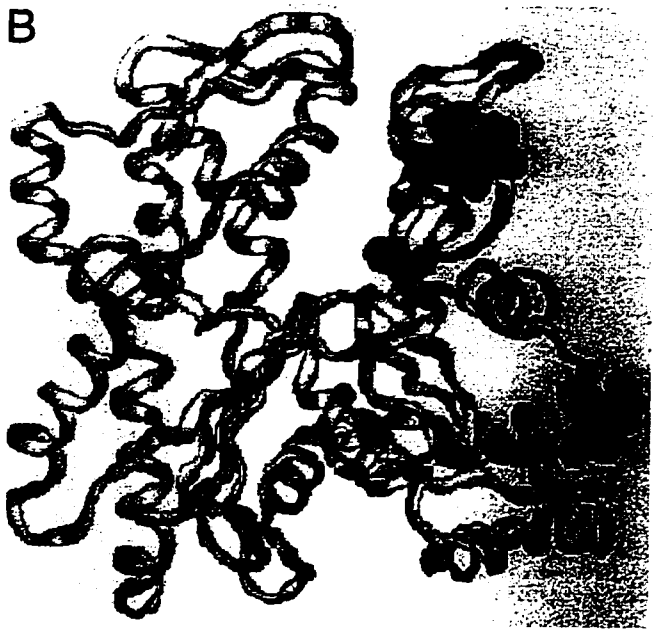

B

FIGURE 5.5. A dissection plate showing growth of *spt5 sac6* double mutants after 5 days at 25° C. A *sac6:LEU2* null strain (AAY 1046) was crossed to *sup56c* strain (AAY1945). A *sup56c* mutant is Cs. The resultant diploid was dissected and the spores where tested for growth on YEPD plates at 11° C and on -leu plates. Twenty-five tetrads were analyzed. The viabilility was 61%. None of the 11 spores that were Leu^+ were Cs indicating that the *sac6* null may be synthetically lethal with *sup56c*. Tetratypes segregated 1:1:1:1 Leu^- Cs^+: Leu^- Cs^-:Leu^+ Cs^+: dead, PDs segregated 2:2 Leu^+ Cs^+: Leu^- Cs^- and NPDs segregated 2:2 Leu^- Cs^+: dead. The genotype of dead spores were inferred assuming the Cs and Leu^+ phenotype each segregated 2:2. The *sac6:LEU2 sup56c* double mutants can germinate since the corresponding spores can undergo a few cell divisions as observed by analyzing the dissection plate. Although consistent, these results are tentative, further genetic analysis would be necessary to unequivocally determine that a *sac6 null* and *sup56c* have synthetic growth defects. It would be best to tag the *sup56c* mutation with an auxotrophic marker, such that both *sac6* and *sup56c* mutant allele can both be followed in analyzing spores from this cross based on growth on selective dropout plates rather than phenotypes.

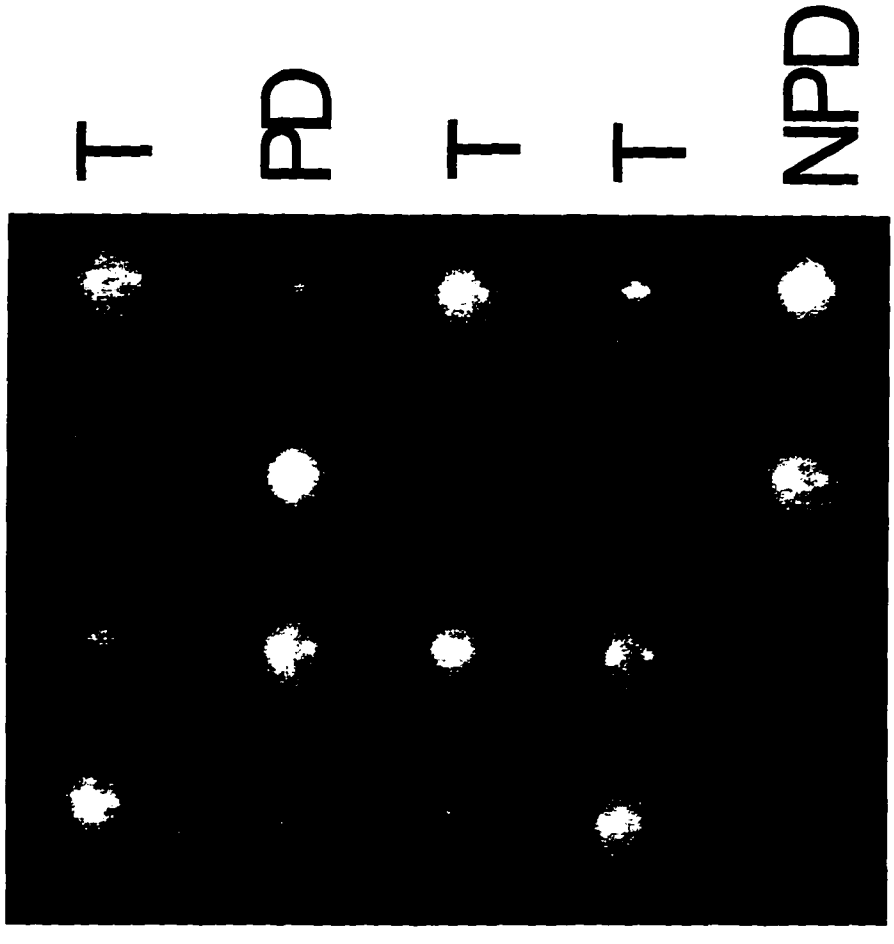
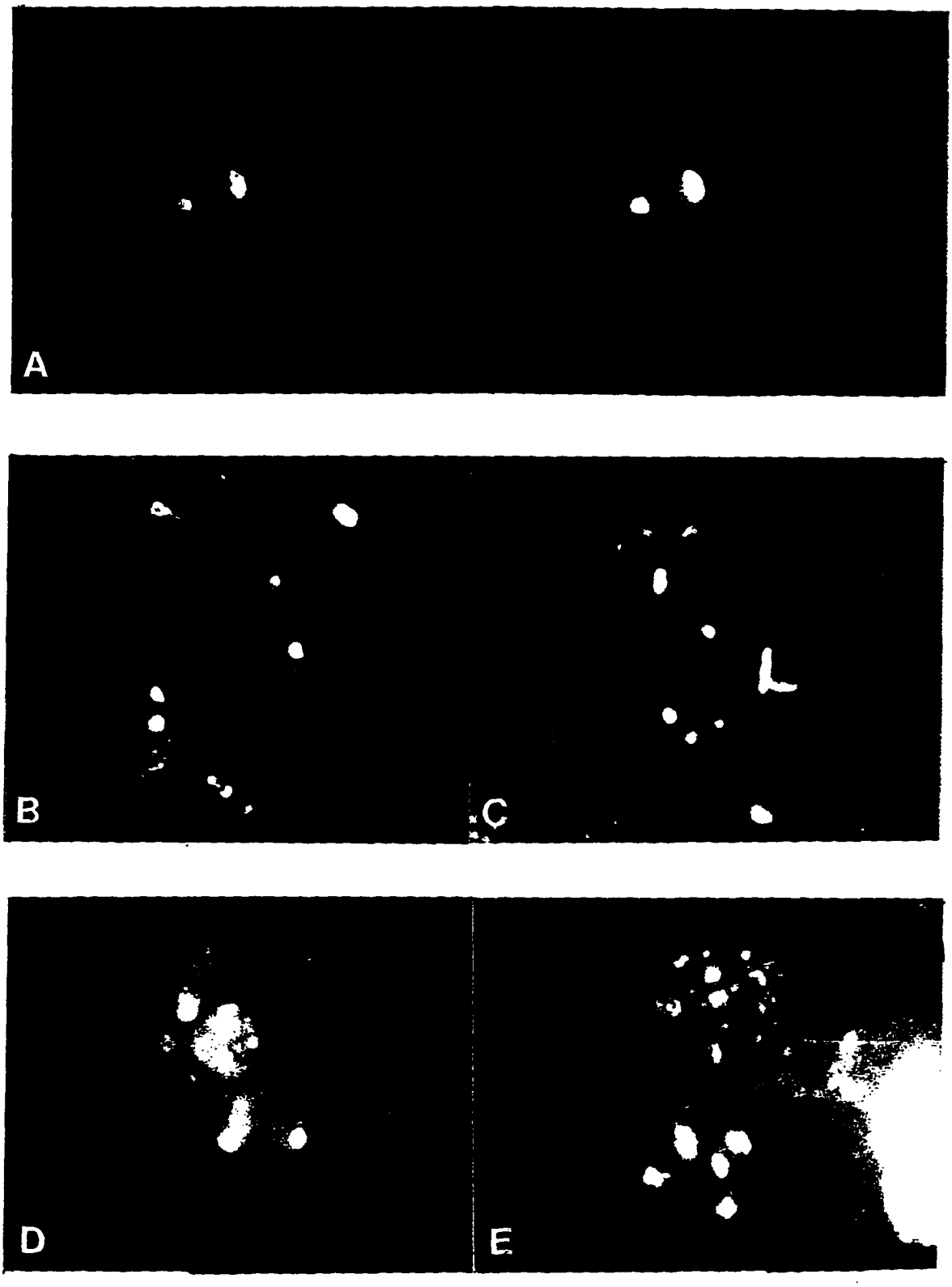

T
PD
T
T
NPD

FIGURE 5.6. Fluorescence of temperature-sensitive morphogenetic mutants after growth in YEPD 12 hours at restrictive temperature 37° C. (a), *sup33b*, (AAY1952), anti-Sac6p. Note: ring structures stained with anti-Sac6p antibodies. The left panel is a 3 minute exposure of the negative and the right panel is a 50 second exposure. A longer exposure clarifies the ring structure but it is harder to see the outline of the cell.. (b) and (c), *sup35b*, (AAY1953), anti-Sac6p; (d), *sup33b* (AAY1952), anti-actin; (e), *sup25b* (AAY1944), anti-actin.


A
B
C
L
D
E

CHAPTER 6: Summary.

In summary, I have identified a number of interesting characteristics of Sac6p including a trypsin-sensitive site that may be useful for future crystal studies. The N-terminus is sensitive to degradation, this may be one factor leading to the isolation of two Sac6p species eluting from a hydroxylapatite column. I have also isolated antibodies that are specific to the second peak. These antibodies may be useful for competition experiments if a protein is found that binds to the N-terminal region of Sac6p, or for future Western blot analysis if an *in vivo* role for Sac6p peak 1 is discovered. The observation that Sac6p yields multiple spots by 2-D gel analysis and that peak 1 can be phosphorylated by casein kinase I (personal communication, Namrita Dillian, Icos corporation) and casein kinase II but peak 2 is not phosphorylated as well (Ashok Bidwai, personal communication) forms precedence to determine if Sac6p is phosphorylated *in vivo* and may yield insight into how Sac6p is regulated.

Allele-specific suppression has been used in a wide variety of organisms to decipher interactions in many different biological processes. The interaction between Sac6p, an actin bundling protein, and Act1p has provided a useful model to examine the mechanism of allele-specific suppression. I have shown *in vitro* suppression of binding and crosslinking activity between Sac6 suppressor protein and mutant actin protein. In addition, Sac6 suppressor protein has an increased affinity to wild-type actin. This analysis, as well as previous genetic data, suggest that suppression may result from an overall increase in affinity to actin rather than a strict "Lock and Key" mechanism previously hypothesized. The *sac6-2* mutation causes cells to become sick, possibly due to

an increased affinity to actin. Since the residue altered in *sac6-2* is conserved between yeast fimbrin and L-plastin, it would be interesting to make the corresponding change in L-plastin to determine what phenotypic change would occur in a human cell line and also, if this change increased the affinity of L-plastin to actin.

Yeast fimbrin (Sac6p) is an actin bundling protein required for morphogenesis and maintenance of normal actin structures. To determine the effects of changing the stoichiometry of Sac6p, I placed the *SAC6* gene under the control of a galactose inducible promoter. Overproduction of Sac6p alters actin structures; in addition, cells fail to bud, yet nuclear division continues and the cells become multinucleated. I devised a screen to isolate genomic mutations that can suppress the *GAL1-SAC6* overexpression lethality. One suppressor, *sup25b*, defines a novel actin mutation, a Gly to Val change at amino acid 48. *In vivo*, a number of different proteins bind to actin structures forming a complex web of interactions. This analysis shows that the proper stoichometry of Sac6p is critical for proper function of actin *in vivo*. In addition, I have described a suppressor screen that should be useful in identifing suppressors of other genes that are lethal when overexpressed.

APPENDIX A: Yeast Strains Used in This Study.

Strain	Genotype	Plasmid	Source
AAY127	*MATa act1-3, ura3-52 (mod?)*		David Botstein
AAY131	*MATα act1-3 ura3-52*		David Botstein DBY1238
AAY215	*MATα his4-619*		David Botstein DBY877
AAY1015	*MATα ade2, his3Δ200, leu2-3.112, lys2-801, ura3-52, GAL*		David Botstein DBY4975
AAY1016	*MATa/α ade2/+, his2Δ200,/". leu2-3,112,/". lys2-801/". ura3-52/", GAL*		TPS507(?)
AAY1046	*MAT α sac6::LEU2 his3-Δ200 leu2-3,112 lys2-801 ura3-52* GAL		Adams *et al.* 1991
AAY1111	*MATα act1-3, sac6-5, his4-619*		Sharon Brower
AAY1116	*MATα act1-3, sac6-19, his4-619*		Sharon Brower
AAY1118	*MATα his3Δ200 leu2-3,112 lys2-801 ura3-52, ade2* GAL	pAAB35	this study
AAY1119	*MATα his3Δ200 leu2-3,112 lys2-801 ura3-52, ade2* GAL	pAAB156	this study
AAY1120	*MATα his3Δ200 leu2-3,112 lys2-801 ura3-52, ade2* GAL	pAAB157	this study
AAY1121	*MATa/α his3Δ200/" leu2-3,112/", lys2:-801/", ura3-52/", ade2/+* GAL	pAAB121	this study
AAY1122	*MATa/α his3Δ200/" leu2-3,112/", lys2:-801/", ura3-52/", ade2/+* GAL	pAAB156	this study

AAY1123	*MATa/α his3Δ200/" leu2-3,112/", lys2:-801/", ura3-52/", ade2/+* GAL	pAAB157	this study
AAY1124	*MATα sac6::LEU2 his3Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB121	this study
AAY1125	*MATα sac6::LEU2 his3Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB156	this study
AAY1126	*MATα sac6::LEU2 his3Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB157	this study
AAY1127	*MATa sac6::LEU2 his3Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB121	this study
AAY1128	*MATa sac6::LEU2 his3Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB156	this study
AAY1129	*MATa sac6::LEU2 his3Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB157	this study
AAY1561	*MATa ACT1::HIS3, can1-1, his3D200, leu2-3,112, ura3-52, tub2-201*		(128)
AAY1630	*MATa act1-120::HIS3, can1-1, ade4, his3D200, leu2-3,112, ura3-52, tub2-201*		(128)
AAY1632	*MATa act1-125::HIS3, can1-1, ade2, his3D200, leu2-3,112, ura3-52, tub2-201*		(128)
AAY1771	*sup 4c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1772	*sup 7b, MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1773	*sup 9c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1774	*sup 10b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1775	*sup 10c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1776	*sup 13b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study

AAY1777	*sup 13c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1778	*sup 14b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1779	*sup 18b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1780	*sup 19c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1781	*sup 20c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1782	*sup 21c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1783	*sup 23c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1784	*sup 24b MATα leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1785	*sup 25b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1786	*sup 26b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1787	*sup27c MATα leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1788	*sup33b MATα leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1789	*sup33c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1790	*sup35b MATα leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1791	*sup37c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1792	*sup38c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1793	*sup39b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1794	*sup40b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1795	*sup41c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1796	*sup42a MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study

AAY1797	*sup48a MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1798	*sup49b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1799	*sup51b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1800	*sup52b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1801	*sup54c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1802	*sup55c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1803	*sup56c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1804	*sup63b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1805	*sup65c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1806	*sup68b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1807	*sup71b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1808	*sup72c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1809	*sup76c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1810	*sup77c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1811	*sup78c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1812	*sup80a MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1813	*sup84b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1814	*sup87c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1815	*sup90b MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1816	*sup90c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study

AAY1817	*sup93c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1818	*sup95c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1819	*sup96c MATa leu2-3,112, ura3-52, ade2,lys2Δ::HIS3, his3Δ200*	pAAB329 pRP127	this study
AAY1860	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB159	this study
AAY1861	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB159	this study
AAY1862	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB158	this study
AAY1863	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB158	this study
AAY1864	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB121	this study
AAY1865	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB121	this study
AAY1866	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB162	this study
AAY1867	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB162	this study
AAY1868	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB117	this study
AAY1869	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB117	this study
AAY1870	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	p*GAL1-SAC6-GFP URA3*	this study
AAY1871	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	p*GAL1-SAC6-GFP URA3*	this study
AAY1872	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB160	this study
AAY1873	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB160	this study
AAY1874	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB161	this study
AAY1875	*sup25b MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB161	this study

AAY1876	*MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	p*GAL1-SAC6-GFP URA3*	this study
AAY1877	*MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	p*GAL1-SAC6-GFP URA3*	this study
AAY1878	*MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB121	this study
AAY1879	*MATa leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB121	this study
AAY1880	*MATa/α sac6::LEU2/", leu2-3,112/", ura3-52/", ade2/", lys2Δ::HIS3/", his3Δ200/"*	pAAB121	this study
AAY1881	*MATa/α sac6::LEU2/", leu2-3,112/", ura3-52/", ade2/", lys2Δ::HIS3/", his3Δ200/"*	p*GAL1-SAC6-GFP URA3*	this study
AAY1882	*MATa/α sac6::LEU2/", leu2-3,112/", ura3-52/", ade2/", lys2Δ::HIS3/", his3Δ200/"*	p*GAL1-SAC6-GFP URA3*	this study
AAY1883	*MATα sac6::LEU2, leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB121	this study
AAY1884	*MATα sac6::LEU2, leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	p*GAL1-SAC6-GFP URA3*	this study
AAY1885	*MATα sac6::LEU2, leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	p*GAL1-SAC6-GFP URA3*	this study
AAY1886	*MATα sac6::LEU2, leu2-3,112, ura3-52, ade2, lys2Δ::HIS3, his3Δ200*	pAAB121	this study
AAY1887	*MATa/α leu2-3,112/", ura3-52/", ade2/", lys2Δ::HIS3/", his3Δ200/"*	pAAB121	this study
AAY1888	*MATa/α leu2-3,112/", ura3-52/", ade2/", lys2Δ::HIS3/", his3Δ200/"*	p*GAL1-SAC6-GFP URA3*	this study
AAY1889	*MATa/α leu2-3,112/", ura3-52/", ade2/", lys2Δ::HIS3/", his3Δ200/"*	p*GAL1-SAC6-GFP URA3*	this study

AAY1911	*MATa/α act1-120::HIS3/act1-3, tub2-201/+, sac6-19/+, his4-619/+, can1-1/+, ade4/+, his3Δ200/+, leu2-3.112/+, ura3-52/+*		this study
AAY1912	*MATa/α act1-120::HIS3/act1-3, tub2-201/+, sac6-19/+, his4-619/+, can1-1/+, ade4/+, his3Δ200/+, leu2-3.112/+, ura3-52/+*		this study
AAY1915	*MATa sac6::LEU2, ura3-52, trp1, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1,6R,can1*		this study
AAY1916	*MATa sac6::LEU2, ura3-52, trp1, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1,6R,can1*	pAAB414	this study
AAY1917	*MATa sac6::LEU2, ura3-52, trp1, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1,6R,can1*	pAAB413	this study
AAY1918	*MATa sac6::LEU2, ura3-52, trp1, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1,6R,can1*	pAAB415	this study
AAY1937	*MATa/α act1-120::HIS3/act1-3, tub2-201/+, sac6-5/+, his4-619/+, can1-1/+, ade4/+, his3Δ200/+, leu2-3.112/+, ura3-52/+*		this study
AAY1938	*MATa/α act1-120::HIS3/act1-3, tub2-201/+, sac6-5/+, his4-619/+, can1-1/+, ade4/+, his3Δ200/+, leu2-3.112/+, ura3-52/+*		this study
AAY1942	*MATα ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB416 pAAB329	this study
AAY1943	*MATa ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB416 pAAB329	this study
AAY1944	*MATa sup25b ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB328	this study
AAY1945	*MATa sup56c ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52*		this study

AAY1946	*MATα sup56c ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52*		this study
AAY1947	*MA Ta his3 Δ200 leu2-3,112 lys2-801 ura3-52* GAL	pAAB328	this study
AAY1948	*MATα ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB328	this study
AAY1951	*MATα ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB329	this study
AAY1952	*MATα sup33b, ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB328	this study
AAY1953	*MATα sup35b, ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB328 pAAB329	this study
AAY1954	*MATα ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB149	this study
AAY1955	*MATα ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB416 pAAB329	this study
AAY1956	*MATα ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB415	this study
AAY1957	*MATα ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB157	this study
AAY1958	*MATα ade2 his3- Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB416	this study
AAY1959	*MATα ade2 his3-Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB121	this study
BJ2168	*MATa pep4::HIS3, prb1-1122,pep4-3,leu2 trp1 ura3*		(51)
BJ5465	*MATa ura3-52, trp1,leu2Δ1,his3Δ200, pep4::HIS3, prbΔ1.6R, can1, GAL*		(51)
BJ5465	*MATa/α ura3-52/", trp1/+, leu2Δ1/+, his3Δ200/", pep4::HIS3/", prbΔ1.6R/", can1/", GAL*		(51)

FY849	*MATa spt5::LEU2 lys2-128delta, trp1d-63, leu2d1,ura 3-52*	pMS4	(118)
TSY175	*MATα ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB329	this study
TSY193	*MATa ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB149 pAAB121	this study
TSY194	*MATa ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB107	this study
TSY195	*MATa ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB329	this study
TSY196	*MATa ade2 his3-Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB329 pRP127	this study
TSY197	*MATa ade2 his3 Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB329 pRP127	this study
TSY198	*MATa ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pRP127 pAAB149	this study
TSY199	*MATa ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB149	this study
TSY200	*MATa ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB328	this study
TSY256	*sup56c MATa ade2 his3Δ200 leu2-3,112 lys2::HIS3 ura3-52* GAL	pAAB328	this study
TSY410	*MATa/α+/sac6::LEU2, sup25b/+, ade2/+, his3Δ200/his3Δ200, leu2-3,112/leu2-3,112, lys2::HIS3/lys2-801, ura3-52/ura3-52*		this study

APPENDIX B: Plasmids Used in This Study.

Plasmid	Markers	Source
pAAB35	*GAL1-10 promoter CEN4 URA3*	Mark Johnson
pAAB107 pRS305	*CEN LEU2*	(115)
pAAB117	*SAC6 CEN URA3*	Alison Adams
pAAB121 pPRP23	*GAL1-10 promoter URA3 CEN*	Roy Parker, Dept. of MCB, Univ. of Arizona
pAAB144	*2μ SAC6 URA3*	Kurt Tonjes
pAAB149 pRS317	*CEN LYS2*	(115)
pAAB156	*GAL1-sac6(1-43)*	this study
pAAB157	*GAL1-SAC6 CEN URA3*	this study
pAAB158	*sac6-4 URA3 CEN*	Sharon Brower (16)
pAAB159	*sac6-5 URA3 CEN*	Sharon Brower (16)
pAAB160	*sac6-14 URA3 CEN*	Sharon Brower (16)
pAAB161	*sac6-17 URA3 CEN*	Sharon Brower (16)
pAAB162	*sac6-2 URA3 CEN*	Sharon Brower (16)
pAAB272 pMS18		(118)
pAAB273 PMS19		(118)
pAAB274 pMS37		(118)
pAAB328	*GAL1-SAC6 CEN LEU2*	this study
pAAB329	*GAL1-SAC6 CEN LYS2*	this study
pAAB413	*2μ GAL1-sac6-19 URA3*	Jerry Honts
pAAB414	*2μ GAL1-sac6-5 URA3*	Jerry Honts
pAAB415	*2μ GAL1-SAC6 URA3*	this study
pAAB416 pRP127	*GAL1-LacZ URA3 CEN*	Roy Parker, Dept. of MCB, Univ. of Arizona
pJF777	*2μ GAL11 URA3 AMP leu2d*	Greg Gingerich Jan Fassler, University of Iowa
pJF786	*CEN GAL11 URA3*	Robin Hockey Jan Fassler, University of Iowa
pMS4	*SPT5 CEN URA3*	(118)
pGAL1-SAC6-GFP	*pGAL1-SAC6-GFP*	this study

	URA3	
TSB84 clone 1d	*CEN SPT5 URA3*	this study

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