5), no conspicuous similarity can be found between α and β subunits. Moreoever, good similarity to the σ region 2.2 can also be found overlapping with the σ region 3 similarity domain (residues 168–182). It is intriguing that a putative promoter recognition region and an RNA polymerase II binding domain are located together.

TFIIE-β is a highly basic protein of pI 9.5 with several basic amino-acid clusters through which it could easily complex with TFIIE-α, a very acidic protein with several acidic clusters[5]. One of the basic clusters in TFIIE-β lies in the serine-rich region (Fig. 3*a*) where several putative phosphorylation sites exist, suggesting that complex formation might be regulated by protein kinases. Another potential protein interaction site in TFIIE-β is a putative leucine repeat[20] from residues 154 to 176, of interest as TFIIE-α also has a potential leucine repeat[5] (Fig. 3*d*). Two subunits in the heterodimer may interact through this domain.

Previous studies suggested that partially purified TFIIE fractions have an ATPase activity that might be involved in an ATP hydrolysis event needed for transcription initiation[21,22]. But recent analyses failed to detect such an activity in a homogeneous preparation of natural TFIIE[3,4,23]. Consistent with this observation, neither TFIIE-α (ref. 5) nor TFIIE-β (this study) has any of the characteristic sequence motifs found in ATP-binding proteins (including ATPases and helicases)[24].

Surprisingly, we also found a region of TFIIE-β with sequence similarity to part of the basic region–helix–loop–helix (BR-HLH) domain of the c-Myc-related family of enhancer binding proteins (such as Myo-D1 and E12)[7–9]. In addition to the similarity in primary structure (Fig. 3*e*), the putative secondary structure of TFIIE-β seems to be consistent with such a motif, whereas the composition of residues 258–273 of TFIIE-β resembles basic regions of the type found in enhancer-binding proteins. Further, the Chou–Fasman algorithm predicts that residues 274–284 (the horizontal bar in Fig. 3*d*) have a high probability of forming an amphipathic helix. Consistent with the possibility of a loop structure[7,25], residues 285–291 have the potential to form a β turn and contain loop-favouring residues such as serine and aspartate. The BR-HLH domain may also be functionally bipartite, with the HLH segment being involved in homo- and heterotypic protein–protein interactions and the basic region in DNA binding[7–9]. Hence the presence of related structures in TFIIE-β raises the possibility that it might be a DNA-binding protein with limited sequence specificity, possibly recognizing the DNA in conjunction with other factors, or that it might interact directly with enhancer bound BR-HLH proteins and thus have a role in transducing their effects to the basic transcription machinery.

Analysis of the protein sequence of TFIIB revealed structural features and potential homologies both to general and to gene-specific transcription factors from prokaryotes and eukaryotes. It should now be possible to analyse these features in relation to the role of TFIIE-β in preinitiation complex assembly, function and regulation. □

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Requirement of yeast fimbrin for actin organization and morphogenesis *in vivo*

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THE *SAC6* gene was found by suppression of a yeast actin mutation[1]. Its protein product, Sac6p (previously referred to as ABP67), was independently isolated by actin-filament affinity chromatography and colocalizes with actin *in vivo*[2]. Thus Sac6p binds to actin *in vitro*, and functionally associates with actin structures involved in the development and maintenance of cell polarity *in vivo*[2,3]. We report here that Sac6p is an actin-filament bundling protein 43% identical in amino-acid sequence to the vertebrate bundling protein fimbrin[4]. This yeast fimbrin homologue contains two putative actin-binding regions[5] homologous to domains of dystrophin, β-spectrin, filamin, actin-gelation protein and α-actinin. Mutants lacking Sac6p do not form normal actin structures and are defective in morphogenesis. These findings demonstrate an *in vivo* role for the well-documented biochemical interaction between fimbrin and actin.

To elucidate the molecular nature of the interaction between Sac6p and actin, we looked at the effect of Sac6p on assembly and organization of actin filaments *in vitro*. Thus, Sac6p was mixed with yeast actin under assembly-inducing conditions, and the resulting products were examined by electron microscopy. When pure yeast actin was assembled, a meshwork of actin filaments, with occasional aggregates, was observed (Fig. 1*a*). But when Sac6p was added at a molar ratio of one Sac6p per 20 or 10 actins, single filaments were not observed, all the actin appearing in filament bundles (Fig. 1*b*). This result was confirmed by anti-actin immunofluorescence microscopy (not shown), and indicates that Sac6p can bundle actin filaments.

SAC6 was sequenced to determine whether it is related to any of the known actin-filament bundling proteins (Fig. 2). A single long open reading frame was identified that encodes a protein of 642 amino acids, with a predicted relative molecular mass of 71,700. A search of the protein sequence database indicated that Sac6p is 43% identical to the chicken actin-filament bundling protein fimbrin[4], and 44 and 38% identical, respectively, to human T- and L-plastin[6,7], previously shown to be human homologues of chicken fimbrin[4]. Sequence alignments for chicken fimbrin, yeast Sac6p and human L-plastin (Fig. 3*a*) show that the homologies are extensive. Like fimbrin[4], Sac6p is composed of two repeated domains of 254 and 257 amino acids

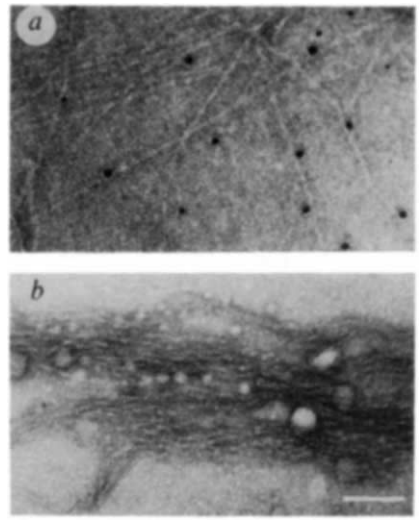
that are 26% identical to each other, preceded by a unique region (131 amino acids for Sac6p) at the amino terminus. In each repeated domain lies a region of 27 amino acids that is very similar to putative actin-binding regions[5] identified in ABP-120 (actin-gelation protein)[8], fimbrin[4], β-spectrin[9], ABP-280 (filamin)[10], dystrophin[11] and α-actinin[12] (Fig. 3*b*). In the amino terminus, as in fimbrin, there are two potential calcium-coordinating EF hand sequences[13] (Fig. 3*c*). But in contrast to fimbrin, the positions of calcium-coordinating residues do not fit the consensus well enough to make it likely that these regions of Sac6p bind to calcium ions[13].

To test the function of Sac6p *in vivo*, a complete *SAC6* gene deletion was constructed (see legend to Fig. 4). The null mutant is viable, but grows at a reduced rate at 23 °C (150–215 min doubling time for the mutant compared with 115 min for wild type; the ranges in growth rate are due to variations seen in three different mutant strains with related, but nonidentical genetic backgrounds, described in the legend to Fig. 4). The null mutant fails to grow at 37 °C, undergoing less than a 1.4-fold increase in cell number after the shift. At 37 °C, the mutant cells show a slight increase in the proportion of unbudded cells, but arrest at all stages of the cell cycle. In addition, these cells are somewhat more heterogeneous in size than at 23 °C, and many of them appear lysed. These results indicate that Sac6p is advantageous to cells at lower temperatures and has an essential function at high temperature, and suggest there may be some other protein(s) that can functionally substitute for *SAC6* at the lower temperatures. Similar suggestions of partial functional redundancy in the actin cytoskeleton have been made for *Dictyostelium*[14].

By immunofluorescence microscopy, the *sac6* null mutant has an altered actin cytoskeleton, even at 23 °C. As a temperature shift affects even the wild-type actin distribution (our unpublished observations), we have characterized the phenotype at 23 °C, using strains AAY1040, AAY1041, and AAY1042 (*sac6::LEU2/sac6::LEU2*) and AAY1043 (*SAC6*$^+$/*SAC6*$^+$) (see legend to Fig. 4). First, we have found that whereas extensive actin cables are seen in 90% of wild-type cells (Fig. 4*b*), cables are visible in only 22% of the mutant cells (strain AAY1041), and those cables that are seen are drastically reduced in intensity, frequency and length (Fig. 4*d*, *e*; note that the wild-type actin cables visible in the microscope are very under-represented in Fig. 4*b*, as most actin cables are in a different focal plane from the more cortically located actin patches). This observation supports a role for Sac6p in cross-linking actin filaments *in vivo*. Second, the cortical actin patches normally concentrated at the growing surfaces of the bud (Fig. 4*b*)[15,16] show this asymmetry in only 3% of the mutant budded cells (strain AAY1041). Instead, the actin patches are fairly uniformly distributed at the cortex (Fig. 4*d*) of 79% of the mutant cells and are aberrantly clustered (Fig. 4*e*) in the remaining 18% of the cells. In this latter class, the clusters are aberrant in that they cover an abnormally large surface area and, in contrast to wild type, do not occur exclusively in the bud (compare Fig. 4*b*, cells a–d with the corresponding cells a′–d′ in *e*). The actin distribution described for AAY1041 is similar to that seen in AAY1040 and AAY1042, although there is some variability in relative frequencies; however, even in AAY1042, the strain most different from AAY1041, 56% of the cells have no detectable actin cables, and 79% show the abnormal distributions of actin patches shown in Fig. 4*d*, *e*.

Interestingly, the mutant cells are much rounder than wildtype (Fig. 4*a*, *c*). As this phenotype is reversed by *SAC6* on a low-copy-number plasmid (not shown), the defect is caused by the *sac6* null mutation. This observation supports the notion that the polarized distribution of actin seen in normal cells is important in the polarization of growth[15].

Fimbrin had prevously been found in vertebrate cells and shown to bundle actin filaments *in vitro*[17–20]. Our findings suggest that fimbrin homologues are likely to be found in association



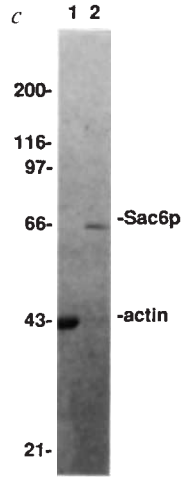


FIG. 1 Electron micrograph of negatively stained yeast actin filaments (*a*) and yeast actin filament bundles formed by the addition of Sac6p at a molar ratio of one Sac6p per 10 actins (*b*). All fields (8/8) containing Sac6p lacked individual (non-bundled) filaments, whereas all fields (5/5) lacking Sac6p contained individual filaments. Scale bar, 0.1 μm. *c*, Coomassie blue stained SDS-polyacrylamide gel showing purified yeast actin (lane 1) and Sac6p (lane 2). In some Sac6p preparations, a small amount of yeast actin from the yeast actin column (see below) copurifies

METHODS. Yeast actin was isolated by DNase1 affinity chromatography[16] and, after desalting on a Sephadex G25 column (Pharmacia), was further purified using a Mono Q (Pharmacia) FPLC column. The actin eluted at roughly 0.2 M KCl using a 0 to 0.4 M KCl gradient (complete procedure to be described in detail by S. Kron, D.G.D., D.B. and J. Spudich, manuscript in preparation). Sac6p was isolated from a wild-type or *abp1* null mutant[22] by actin-filament affinity chromatography as described by Drubin *et al.*[2]. Sac6p was sometimes concentrated in a dialysis bag placed in Ficoll (M_r 400K) and was dialysed into 5 mM HEPES, pH 7.4, 1 mM EDTA, 20 mM KCl, 10% glycerol, 0.5 mM DTT, 1 mM ATP and a 1X protease inhibitor cocktail[2]. To determine effects of Sac6p on actin filament assembly and organization, Sac6p (or dialysis buffer alone for control experiments) was brought to 5 mM $MgCl_2$ and 10 X concentrated monomeric yeast actin (30 μM, stored in 5 mM Tris, pH 7.5, 0.2 mM ATP, 0.2 mM DTT and 50 μM $MgCl_2$) was added to a final concentration of 3 μM. After 90 min at 23 °C, samples were applied to ionized carbon stabilized formvar grids and negatively stained with 1% aqueous uranyl acetate for viewing by electron microscopy.

with actin in all eukaryotes; thus this association arose early in the evolution of eukaryotes. Our results also suggest that there is a functional association of these fimbrin homologues with actin in all cells, not just the highly specialized microvillar actin bundles where fimbrin was first discovered and characterized.

Our experiments also give us insight into the function in the living cell of the fimbrin–actin interaction. The failure to properly organize actin results in a defect in morphogenesis, particularly as manifested by cell shape, even at the nominally permissive temperature of 23 °C. But the fact that the cells are able to grow at this temperature suggests that proper actin organization might be most important under stressful conditions, such as extreme temperature. We speculate that individual actin filaments can direct transport of materials to regions of growth

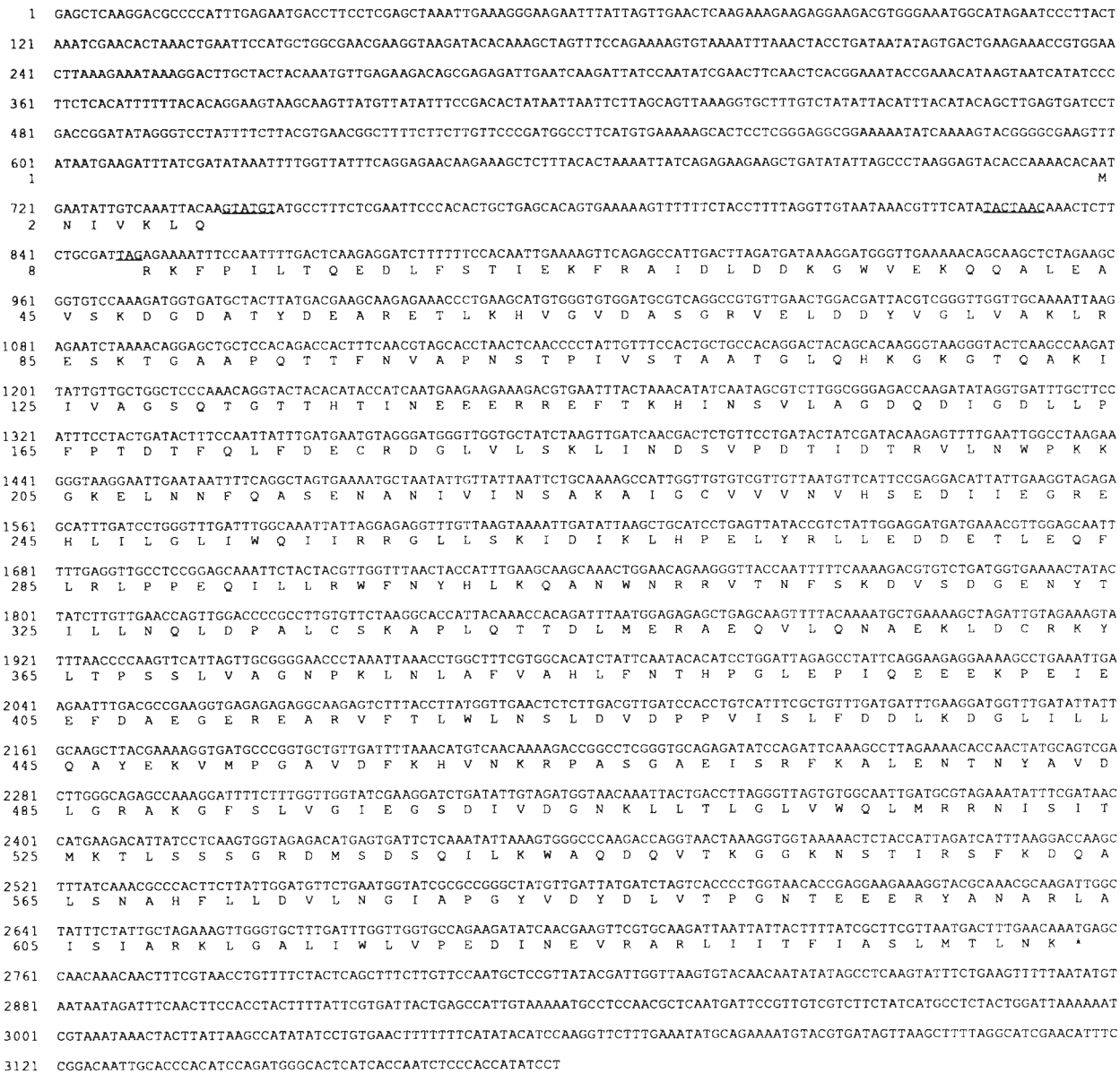


FIG. 2 Nucleotide and predicted amino-acid sequence of *SAC6*. Underlined nucleotides are splicing consensus sequences in the intron[23].

METHODS. To sequence *SAC6*, it was first necessary to isolate a complete, wild-type copy of the gene; previously, complete mutant and incomplete wild-type copies had been obtained[1,3]. To this end, plasmid pRB1276 (ref. 1), containing an incomplete copy of wild-type *SAC6* in YIp5, was integrated into the *SAC6* locus of DBY2057 *(MATa ura3-52 SAC6$^+$)*. Genomic DNA was isolated, cut with *Aat*II, and circularized by ligation. Amp^r transformants were selected in *Escherichia coli*, and plasmid DNA containing the whole of *SAC6* and most of YIp5 was isolated. The *Sac*I–*Nco*I fragment, containing the complete *SAC6* gene[3], was subcloned into pUC118 and pUC119 (ref. 24). Two sets of overlapping unidirectional deletions were generated as described previously[25] except that exonuclease VII was used instead of S1 nuclease. Overlapping sequence of both strands was obtained between nucleotides 325 and 2,987 (Fig. 2). The remaining sequence was obtained on one strand only. Preliminary analysis of the *SAC6* sequence indicated there was an intron near the 5′ end of the gene. To locate the splice junctions precisely, poly(A)$^+$ RNA (kindly provided by S. Mayer) from strain D273-10B (ref. 26) was used to generate cDNA as described[27]. Primers designed to flank the putative intron were used to amplify this region of the cDNA by the polymerase chain reaction (PCR). The primer at the 5′ end corresponded to nucleotides 694 to 710 of the sequence shown, and the primer at the 3′ end corresponded to nucleotides 1,005 to 1,038. The PCR product was then cloned into plasmid pRS416 (ref. 28) and sequenced, confirming the existence of the intron, and the indicated splice junctions.

at low temperature and cortical actin acts to strengthen actively growing regions of the cell surface that otherwise might be weakened during cell growth, as has been suggested for tip extension in pollen tubes[21]. Consistent with this possibility, we have noted in processing these cells for immunofluorescence microscopy that they appear more fragile than wild type, even when grown at 23 °C.

Our evidence is most consistent with the view that the fimbrin homologue Sac6p is an actin-filament bundling protein that acts in yeast to 'fine-tune' the structure of the actin cytoskeleton, making it possible for the actin cytoskeleton to function optimally even when the cell is stressed. □

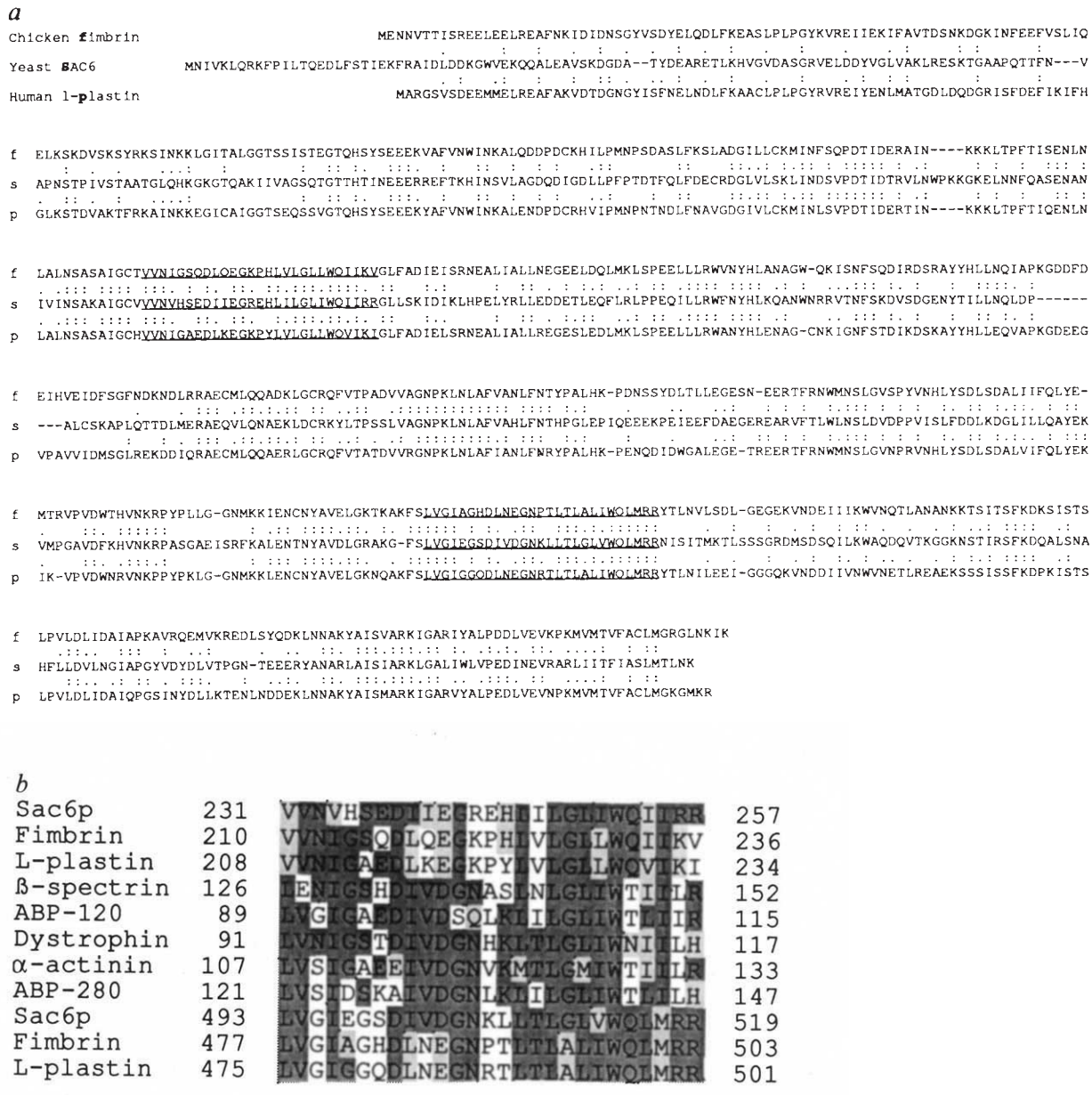


FIG. 3 Sac6p homologies. *a*, Alignment of chicken fimbrin[4], yeast Sac6p and human L-plastin[6,7] sequences. Double dots, identities; single dots, similarities according to the following groupings: D, E; S, T; I, L, V, M; A, G; F, W, Y; N, Q; H, R, K; C; P. Underlined amino acids are the two putative actin-binding domains shown in *b*. *b*, Putative 27 amino-acid actin-binding domains of indicated actin-crosslinking proteins. Dark shade, identities; light shade, similarities (groupings as in *a*). Dark shading for groups of four or more identities; light shading for residues similar to the group of identical residues. Numbers indicate residue positions. References to amino-acid sequences: chicken fimbrin, ref. 4; human L-plastin, refs 6, 7; *Drosophila* β-spectrin, ref. 9; *Dictyostelium* ABP120, ref. 8; human dystrophin, ref. 11; human α-actinin, ref. 12; human ABP280, ref. 10. *c*, Potential EF hands of Sac6p and chicken fimbrin[4] compared with EF hands (helix–loop–helix I, in each case) of bovine brain calmodulin and chicken skeletal troponin C (see ref. 13). Following the convention for calcium-binding sites, the X, Y, Z, −X, −Y and −Z positions correspond to residues that coordinate with calcium in calmodulin and troponin C (ref. 13). Numbers indicate residue positions.

c

	Helix	Loop (X Y Z-Y-X)	Helix (-Z)	
15	QEDLFSTIEKFRAI	DLDDKGWVE	KQQALEAVSKD	Sac6p
50	DATYDEARETLKHV	GVDASGRVE	LDDYVGLVAKL	Sac6p
10	REELEELREAFNKI	DIDNSGYVS	DYELQDLFKEA	Fimbrin
50	YKVREIIEKIFAVT	DSNKDGKIN	FEEFVSLIQEL	Fimbrin
6	EEQIAEFKEAFSLF	DKDGDGTIT	TKELGTVMRSL	CaM
16	EEMIAEFKAAFDMF	DADGGGDIS	TKELGTVMRML	TnC

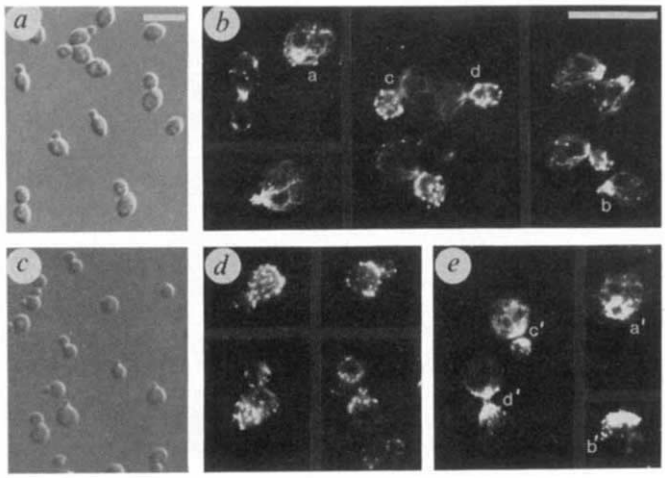


FIG. 4 Phenotypes of strains lacking Sac6p. Differential interference contrast (DIC) and fluorescence micrographs of wild-type strain AAY1043 (*a*, *b*) and *sac6*-null mutant strain AAY1042 (*c*–*e*) cells grown at 23 °C. For fluorescence microscopy, cells were strained with anti-actin antibodies (*b*, *d*, *e*). Scale bar, 10 μm.

METHODS. Generation of the *sac6* null mutation. A *LEU2* -containing fragment was placed between *SAC6*-flanking regions obtained by PCR amplification of nucleotides 143–710 and 2,759–3,120 (fig. 2) and inserted into pUC218 (Genentech). The *LEU2* gene bordered by the *SAC6*-flanking sequences was cut out of the plasmid and used to transform diploid yeast strain TPS507 (*Mata/MATα ade2/+ his3/his3 leu2/leu2 lys2/lys2 ura3/ura3*). Leu^+ tranformants were selected, and the replacement of one of the copies of *SAC6* by *LEU2* in one isolate (AAY1069) was confirmed by Southern blotting of genomic DNA. Further confirmation was obtained by tetrad analysis. Thus, 14/14 tetrads derived from AAY1069 showed 2:2 cosegregation of temperature sensitivity and the *LEU2* marker. Southern-blot analysis of genomic DNA from all four segregants from each of three tetrads showed the expected pattern of restriction fragments: the Leu^- Ts^+ segregants had the wild-type pattern, and the Leu^+ Ts^- segregants the replacement pattern (not shown). Further, immunoblot analysis of each of the 12 segregants showed that Sac6p was present in each of the six Ts^+ Leu^- segregants, but absent from the six Ts^- Leu^+ segregants (not shown). $SAC6^+$ segregants derived from AAY1069 were crossed to each other to generate strain AAY1043 *(MATa/MATα SAC6⁺/SAC6⁺ lys2/lys2 ade2/+ leu2/leu2 ura3/ura3 his3/his3). sac6::LEU2* segregants derived from AAY1069 were crossed to AAY220 (*MATα leu2)* or AAY221 (*MATa leu2),* and segregants from these crosses were mated in various combinations to generate strains AAY1040 (*(MATa/MATα ura3/ura3 his3/+ leu2/leu2 sac6::LEU2/sac6::LEU2 ade2/+),* AAY1041 *(MATa/MATα leu2/leu2 sac6::LEU2/sac6::LEU2 lys2/+ ura3/+*), and AAY1042 (*MATa/MATα sac6::LEU2/sac6::LEU2 lys2/+ ade2/ade2 leu2/leu2 ura3/+).* These four strains were used in the phenotypic analyses described in the text. Immunofluorescence microscopy was done as described prevously[29], using affinity-purified polyclonal anti-yeast actin antibodies[2]. Fluorescence micrographs were obtained using hypersensitized Kodak Technical Pan 2415 film (Lumicon; see ref. 29).

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A 'molten-globule' membrane-insertion intermediate of the pore-forming domain of colicin A

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THE 'molten' globular conformation of a protein is compact with a native secondary structure but a poorly defined tertiary structure[1,2]. Molten globular states are intermediates in protein folding and unfolding[3–5] and they may be involved in the translocation or insertion of proteins into membranes[6]. Here we investigate the membrane insertion of the pore-forming domain of colicin A, a bacteriocin that depolarizes the cytoplasmic membrane of sensitive cells[7–9]. We find that this pore-forming domain, the insertion of which depends on pH (refs 10, 11), undergoes a native to molten globule transition at acidic pH. The variation of the kinetic constant of membrane insertion of the protein into negatively charged lipid vesicles as a function of the interfacial pH correlates with the appearance of the acidic molten globular state, indicating that this state could be an intermediate formed during the insertion of colicin A into membranes.

Models based on the X-ray structure of the colicin A pore-forming domain have been proposed for the first step of its electrostatic membrane insertion mechanism and for the resulting structure of the inserted monomer in the absence of membrane potential[12,13]. These are largely supported by experiment[13–17], but the intervening stage is not well understood. By analogy with enzyme reactions, the kinetics of membrane insertion of colicin A into lipid vesicles should strictly depend on the proportion and the energy state of this insertion intermediate.

We have previously shown that the three tryptophan residues of the pore-forming fragment become accessible to the lipid phase on membrane insertion[11]. Addition of brominated dioleoylphosphatidylglycerol (Br-DOPG) liposomes to the protein in solution results in an exponential decrease of the tryptophan fluorescence due to quenching by the bromine in the middle of the phospholipid fatty acyl chain. We have determined the kinetic constant of insertion of the pore-forming domain into vesicles by recording the quenching of the intrinsic fluorescence by Br-DOPG as a function of time (Fig. 1). Transformation of the initial fluorescence decay according to the equation given in Fig. 1 legend, as shown in the inset of Fig. 1, gives a straight line, so it is reasonable to assume first-order kinetics, at least for the initial period of the insertion. The slope of the straight