Phenotypic Analysis of Temperature-Sensitive Yeast Actin Mutants

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Summary

The consequences of two different mutations in the single essential actin structural gene of yeast (Saccharomyces cerevisiae) were studied. Both conditional-lethal actin mutants exhibit six phenotypes at the restrictive temperature: disruption of the asymmetric staining pattern of actin assembly; delocalized deposition of chitin on the cell surface; partial inhibition of secretion of the periplasmic protein, invertase; an intracellular accumulation of secretory vesicles; death of cells in the budded portion of the cell cycle upon prolonged incubation at the restrictive condition; and osmotic sensitivity. These results implicate actin in the organization and polarized growth of the yeast cell surface.

Introduction

Actin is a highly conserved protein present in all eukaryotes (Korn, 1978). The degree of conservation of the actin amino acid sequence is extreme; for example, 89% of the residues are identical when actin from yeast (Saccharomyces cerevisiae) is compared to actin from mammals (Gallwitz and Sures, 1980; Ng and Abelson, 1980). This degree of sequence conservation suggests a comparable conservation in vital functions common to all eukaryotic cells. The discovery that yeast actin is encoded by a single essential gene (Shortle et al., 1982) invited study of these common essential roles of actin by examination of the phenotypes of actin mutants. We report the effects on the cell of temperature-sensitive lethal actin mutations whose construction has recently been described (Shortle et al., 1984).

Actin in vivo is found in a wide array of structures in association with a variety of other proteins (Stossel, 1984). Purified actin can polymerize into 7 nm microfilaments similar to those found in vivo. Together, actin and its associated proteins are thought to play many roles in the eukaryotic cell. Actin's best established role is in muscle contraction, but it has been implicated in subcellular movements in all cells, including cytokinesis, chromosome segregation, and organelle transport (Shroeder, 1976; Mollenhauer and Morre, 1976; Brawley and Quatrano, 1979; Fowler and Pollard, 1982; Pollard et al., 1984). For some of these roles, evidence has necessarily been inferential. Using the yeast actin mutants, we have found more direct evidence for defects in some of these postulated functions.

The pattern of actin localization viewed by fluorescence microscopy (Kilmartin and Adams, 1984; Adams and Prin-

gle, 1984; see also Results) suggests several roles for yeast actin. A ring of chitin is formed in the cell wall in the region of the neck. Noting that this ring is apparently coincident with a ring of actin patches, Kilmartin and Adams (1984) proposed a role for the ring in localizing the synthesis of chitin to the neck. Adams and Pringle (1984) established a correlation between the growing regions of the yeast cell surface and the regions showing a concentration of actin patches. Based on this correlation they proposed a role for actin in the localization of surface growth. The apparent absence of actin from the yeast spindle suggests that actin is not involved in chromosome segregation.

The two temperature-sensitive lethal actin mutations used here were constructed by in vitro mutagenesis followed by gene replacement. The construction of these mutations was recently reported in detail (Shortle et al., 1984). One mutation (act1-1) changed proline at position 32 to leucine; the other mutation (act1-2) changed alanine at position 58 to threonine. Using these mutations, we found evidence supporting directly a role for actin in the organization, assembly, and function of the yeast cell surface.

Results and Discussion

Assembly of Actin in Wild-Type Cells

Assembly of actin into polymeric structures is expected to be central to actin function. Wild-type yeast forms actin-containing structures that can be visualized by use of a fluorescent derivative of the f-actin binding toxin phalloidin or by indirect immunofluorescence using anti-actin antibody (Kilmartin and Adams, 1984; Adams and Pringle, 1984). As a control in our studies on actin mutants we have repeated the localization of actin in the parental wild-type cells.

Wild-type cells growing exponentially at 23°C were fixed with formaldehyde and processed for indirect immunofluorescence as described by Kilmartin and Adams (1984), using affinity purified polyclonal anti-actin antibody kindly provided by Dr. Kilmartin. The pattern of assembly is cell cycle dependent. Growing, budded cells exhibit a strikingly asymmetric staining pattern. The mother cell shows cables of actin near the cell perimeter organized approximately along the long axis of the cells (Figure 1A). The bud, in contrast, does not exhibit cables; instead, brightly staining patches of actin-containing material lie near the cell surface. Throughout most of the budded portion of the cell cycle the level of asymmetry is high, with the mother cell showing few actin patches and the bud showing few actin cables. However, cells with small buds show scattered patches on the mother cell and some cells with very large buds show cables and patches equally distributed between mother and bud. Nuclear staining experiments (not shown) suggest that the loss of asymmetry characteristic of large budded cells is associated in time with nuclear division.
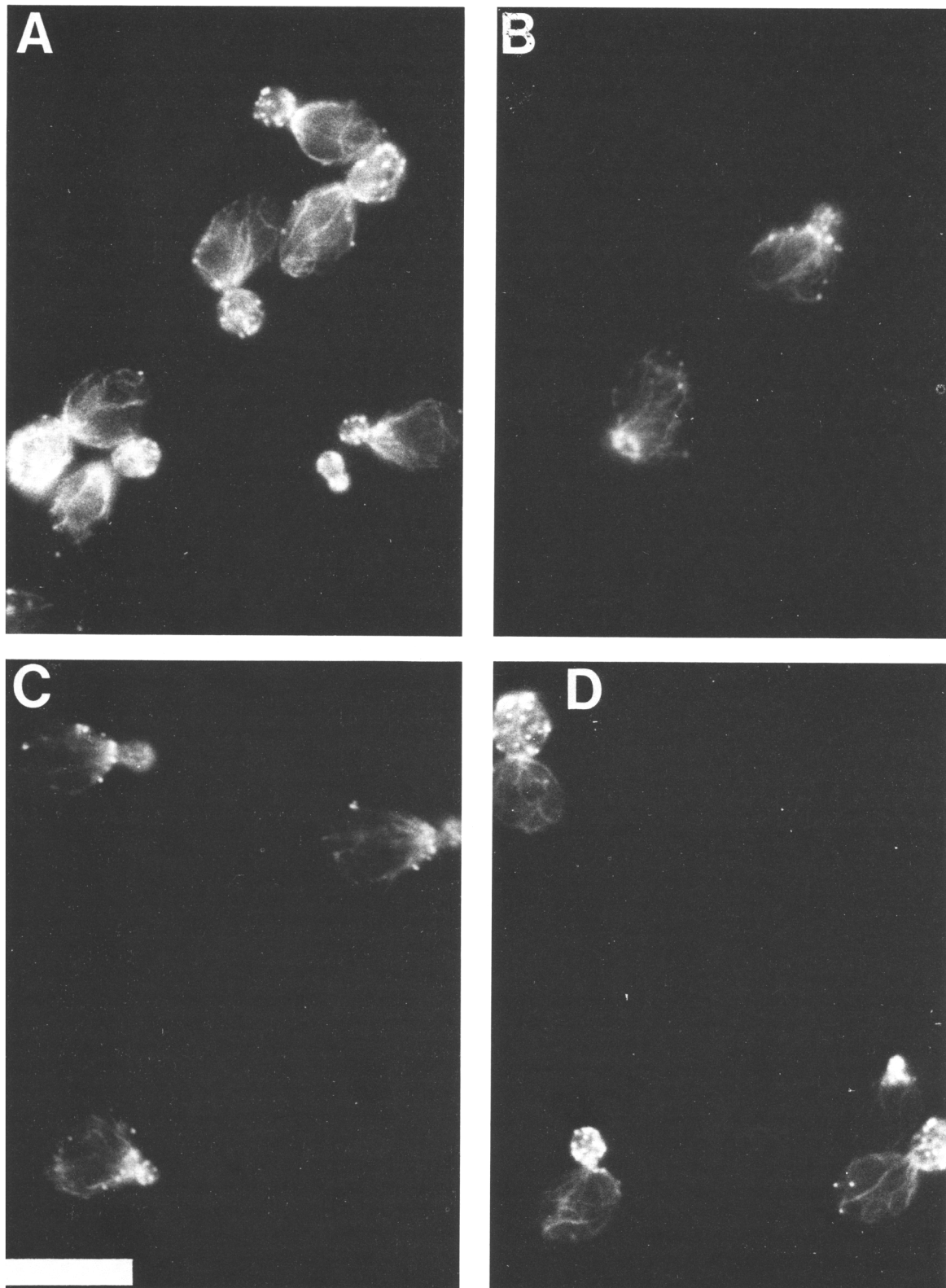
Figure 1. Localization of Actin in Wild-Type Cells

(A and B) Wild-type diploid cells DBY 1690 were grown at 23°C, fixed, and stained with anti-actin antibody. (C) Wild-type haploid cells DBY 640 were grown at 30°C; 4 × 10⁷ cells were harvested by filtration and resuspended in 20 ml of YEPD media that had been titrated to pH 4.0, and 200 units of α factor was added. After 130 min, cells were harvested by filtration, washed with fresh YEPD media, and resuspended in 16 ml of YEPD media. After 40 min of incubation without α factor, cells were fixed and stained with anti-actin antibody. (D) Wild-type diploid cells were grown at 23°C, fixed, and stained with rhodamine phalloidin. The bar corresponds to 5 μm.

In addition to the actin patches and cables, a ring of actin staining material is formed at or just before bud emergence (Figure 1B). The ring first appears as a series of spots arranged in a circle and persists as a continuous collar around the neck of the bud. This collar is most dramatically demonstrated by arresting the cells at the start of the cell cycle and then following the actin staining pattern as the cells proceed synchronously through one cell division. Exponentially growing wild-type cells of the a mating type were treated with the peptide hormone α factor. This hormone has the effect of arresting cells of the a mating type at the G1 stage of the cell cycle (Bucking–Throm et al., 1973). After 3 hr the cells were uniformly unbudded. The α factor was then removed, allowing the cells to proceed through the cell cycle in a synchronous fashion. After 40 min, 64% of the cells carried small buds. Essentially all of these cells showed a ring of actin dots after fixation and anti-actin staining (Figure 1C).

Rhodamine-tagged phalloidin has also been used to visualize actin distribution (Adams and Pringle, 1984). In our experiments, the staining pattern with this reagent is similar to that revealed by immunofluorescence (Figure 1D).

Actin Mutations Affect Actin Assembly

Because actin is an essential gene it was necessary to construct conditional-lethal actin mutants. Temperature-shift experiments using temperature-sensitive actin mutants revealed pleiotropic effects of the disruption of actin function. Since the mutants are temperature-sensitive, one can observe the effect of the mutation in two ways; by comparing mutant and wild-type cells at the restrictive temperature or by comparing mutant cells at the restrictive temperature to the same strain at the permissive temperature. As is often found with conditional-lethal mutations, such analysis shows that the mutants exhibit some phenotypic abnormalities even at the permissive temperature.

Two temperature-sensitive actin alleles, act1-1 and act1-2, have been used in temperature-shift experiments. Both mutants show optimal growth below 26°C. The optical density was followed during a period of exponential growth at 23°C and after a shift to 37°C. At 23°C act1-1 grows with a doubling time of 207 min, act1-2 doubles every 298 min, and an ACT+ culture doubles every 147 min. The slowed growth suggests that actin function is not normal in the mutants, even at the permissive temperature.

Following a shift in temperature to 37°C the absorbance of act1-1 and act1-2 cultures continues to increase for about 1 hr but then tapers off quickly (not shown). No increase is seen after 2 hr. These data indicate that with respect to growth, the alleles are completely restrictive at 37°C. Slow growth can be detected on solid media at temperatures as high as 35°C.

The most direct measure of in vivo actin function is the assembly pattern revealed by anti-actin antibody staining. In these experiments the actin mutants were grown at 23°C, then shifted to 37°C. At time points aliquots were removed, fixed in formaldehyde, and stained with anti-actin antibody. The mutants show altered patterns of actin assembly. At the permissive temperature act1-1 cells form only faint cables, difficult to record photographically, and bright patches appear on both mother and bud (Figure 2A). Often there is a concentration of patches at the neck of the bud. Permissively grown act1-2 cells exhibit a different phenotype. Bars or ribbons of actin appear to cross the mother cell diametrically; the bars do not pass through the nucleus, nor do they show an orientation toward the bud (Figure 2C). The aberrant staining pattern of the mutants at their permissive temperature is consistent with their slowed growth rate. The altered actin may not be fully functional at any temperature.

Following a shift from 23° to 37°C the actin staining pattern changes in the mutants. Cables are no longer visible in act1-1 after 30 min at 37°C, leaving only randomly distributed surface patches (Figure 2B). The actin bars seen in act1-2 at the permissive temperature are lost gradually over a period of 1 hr. These cells then exhibit disorganized fine cables, and patches are seen near the surface of both the mother cell and the bud (Figure 2D).

It is not clear how actin is organized at the molecular level in wild-type cells or how the mutant actins disrupt the normal interactions. Purified yeast actin can assemble into 7 nm filaments (Greer and Schekman, 1982). The cables in wild-type cells may represent bundles of such filaments. The failure of act1-1 to form visible cables suggests a defect in polymerization or bundling. The disorganization of cables in act1-2 suggests another defect, perhaps a failure to interact properly with the cellular components regulating assembly. In vitro studies on assembly of the mutant actins are necessary to resolve these possibilities. Neither mutant causes the disappearance of the actin patches seen in the growing regions of the cell. Their structure at the molecular level and the mechanism by which they are concentrated in the bud or at the neck remain unclear.

Chitin Deposition in the Actin Mutants

Wild-type strains deposit a ring of chitin in the cell wall at the base of the neck in budding cells (Hayashibe and Katohda, 1973; Cabib and Bowers, 1975). The chitin ring can be selectively stained with the fluorescent dye Calcafluor. The chitin ring remains on the mother cell as a bud scar after cell division. In haploid cells the site of bud emergence is usually adjacent to the previous site of bud emergence (Freifelder, 1960). The effect of these events is to leave a trail of chitin rings on the mother cell (Figure 3A). Kilmartin and Adams (1984) demonstrated a spatial correlation between the site of chitin deposition and the site of actin ring formation. On the basis of this correlation they proposed a role for the actin ring in the localization of chitin deposition.

We tested the effect of the actin mutations on the localization of chitin deposition. Mutant cells were grown at 23°C, then shifted to 37°C. Aliquots were removed at different times, fixed in formaldehyde, and stained with Calcafluor. At the permissive temperature the actin mutants show an altered staining pattern (Figures 3B and 3C). Rings are seen on the surface of the mutant cells, yet they are somewhat larger than the corresponding struc-
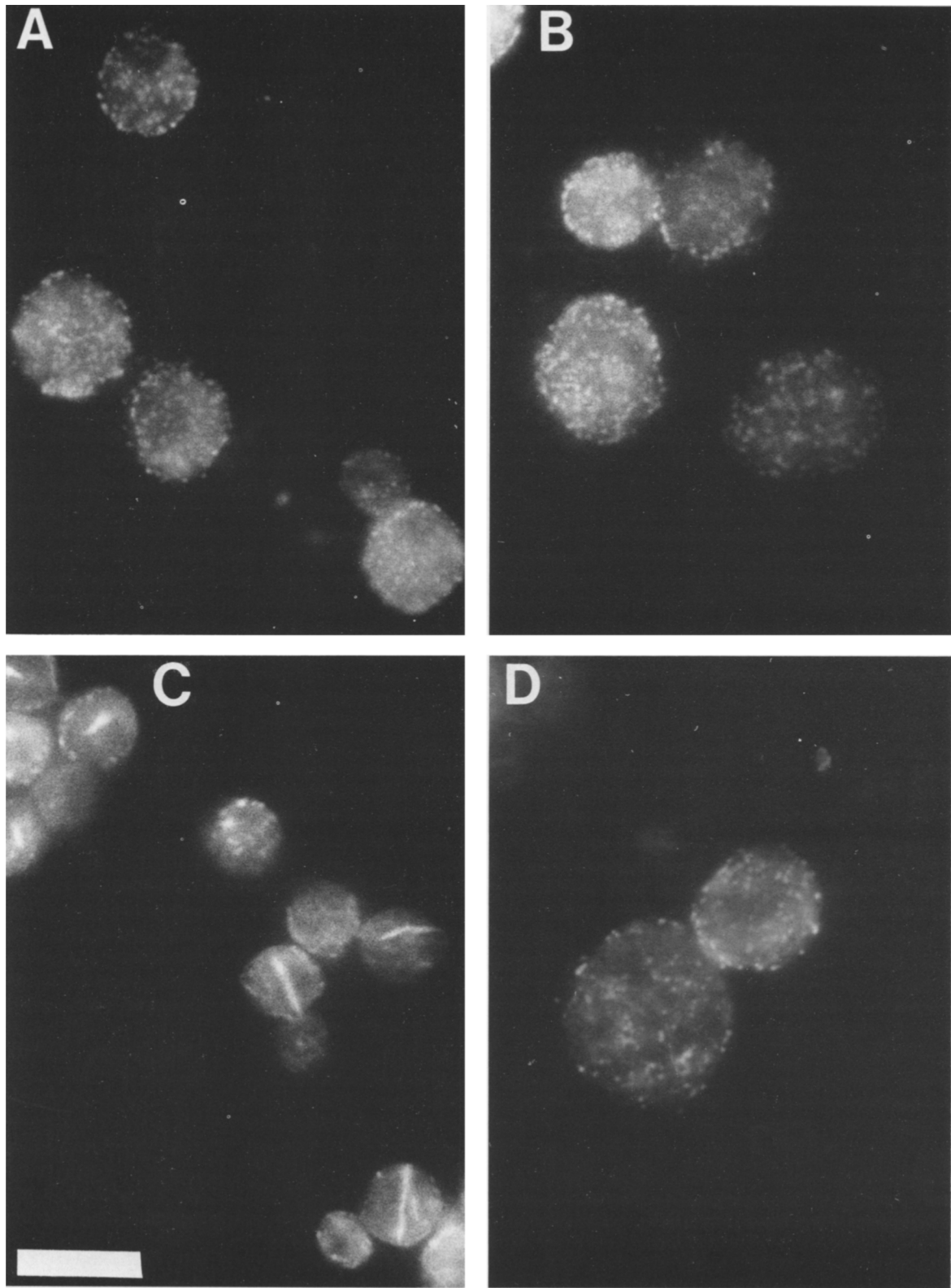
Figure 2. Actin Localization in Actin Mutant Cells

Actin mutant cultures were grown overnight at 23°C and then shifted to 37°C. At various times aliquots were fixed and stained with anti-actin antibody. (A) *act1-1* diploid DBY 1693 at 23°C. (B) *act1-1* diploid after 1 hr at 37°C. (C) *act1-2* diploid DBY 1696 at 23°C. (D) *act1-2* diploid after 2 hr at 37°C. The bar corresponds to 5 μm.
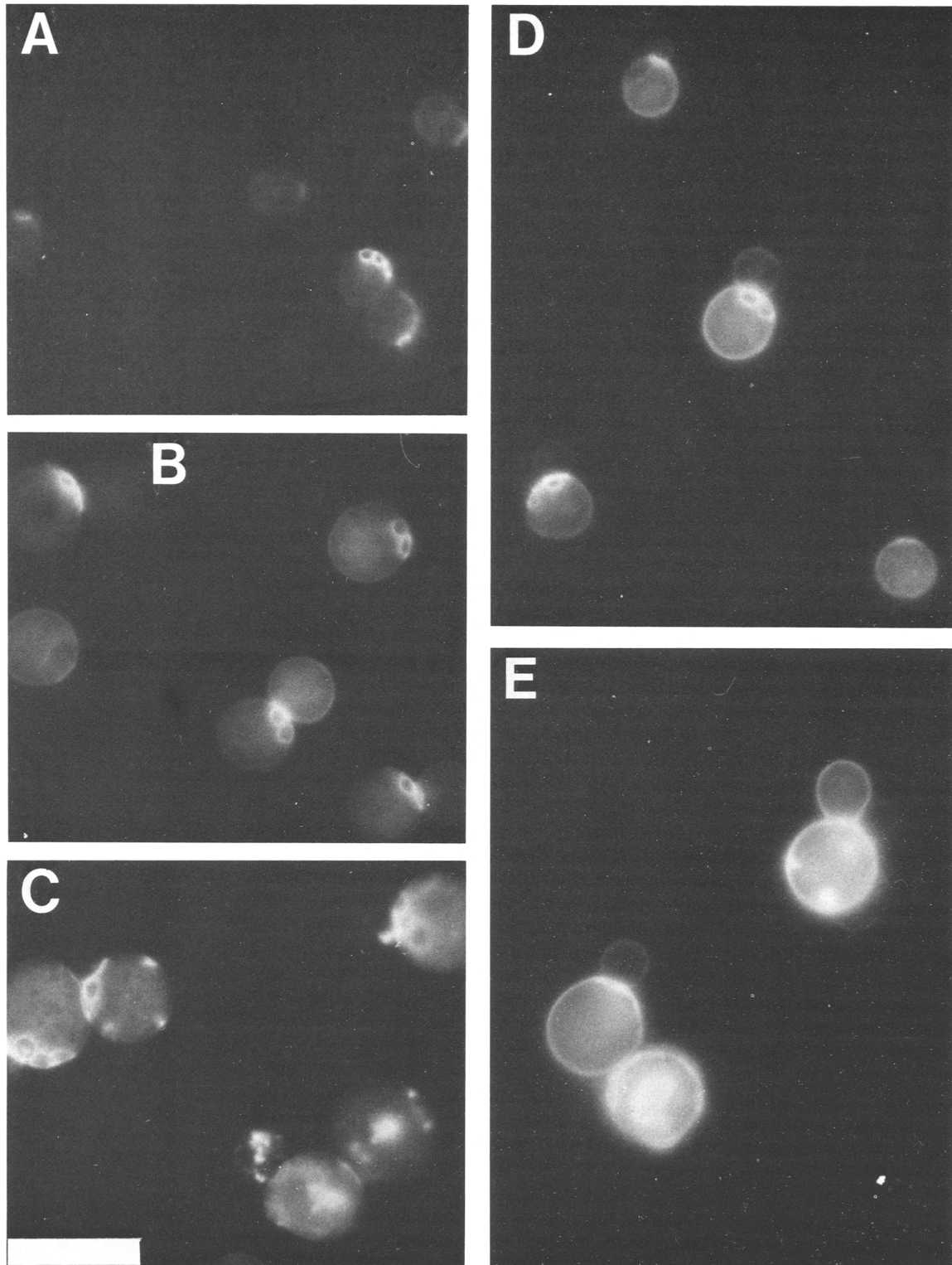
Figure 3. Chitin Localization in Wild-Type and Actin Mutant Cells

Cells were grown overnight at 23°C and then shifted to 37°C. At time points aliquots were removed, fixed, and stained with Calcafluor. (A) Wild-type DBY 877 at 23°C. (B) *act1-1* DBY 1692, at 23°C. (C) *act1-2* DBY 1694, at 23°C. (D) *act1-1* after 1 hr at 37°C. (E) *act1-2* after 1 hr at 37°C. The bar corresponds to 5 μm.

ture in wild-type cells. In addition, chitin is found in inappropriate locations; *act1-1* shows generalized staining of some mother cells, while *act1-2* shows patches of fluorescence (Figure 3C). Following a shift to the restrictive temperature the cells stain brightly over most of their surface. The partial effect at the permissive temperature is consistent with the partial disruption of actin function at this temperature.

We interpret these results as follows: the chitin is deposited outside the plasma membrane by the membrane-bound enzyme chitin synthetase, while the actin ring is assembled within the plasma membrane. Actin may be a member of a set of proteins that serve to define this specialized region of the plasma membrane. Chitin deposition may become delocalized in the actin mutants because the active enzyme is no longer constrained in the plane of the plasma membrane.

Another explanation is suggested by the work of Roberts et al. (1983). They demonstrated that in several cell division cycle (*cdc*) mutants, chitin deposition becomes delocalized. They propose that this is the result of the unbalanced conditions resulting from the cell cycle blocks. Similarly, delocalized deposition of chitin in the actin mutants may be an indirect effect of the mutation.

Invertase Secretion

Protein export and cell surface growth are both mediated by membrane bounded organelles (Novick et al., 1981). During the budded portion of the cell cycle, surface growth and protein secretion are primarily restricted to the bud (Tkacz and Lampen, 1972, 1973; Field and Schekman, 1980). Adams and Pringle (1984) noted a correlation between the site of surface expansion and the regions of concentrated actin patches. They suggested a possible role for the actin patches in polarized surface growth.

We tested the effect of the actin mutations on transport of a specific periplasmic protein. Invertase is the product of the *SUC2* gene. This gene encodes two transcripts; a constitutively synthesized transcript whose product remains freely soluble in the cytoplasm and a hexose repressed transcript (Carlson and Botstein, 1982). The protein product of the repressed transcript carries a hydrophobic signal sequence (Carlson et al., 1983). The polypeptide is inserted into the lumen of the endoplasmic reticulum. Here, nine carbohydrate core units are attached to asparagine residues. The partially glycosylated protein is then transported to a Golgi-like structure, where the carbohydrate cores are extended by addition of mannose residues (Esmon et al., 1981). The fully glycosylated protein is packaged into vesicles that are transported to the bud, where fusion of the vesicles with the plasma membrane releases the enclosed invertase. Secretion of invertase was followed in mutant and wild-type cells. Cultures were grown overnight, at the permissive temperature, in repressing medium, then the cells were shifted into derepressing medium at the restrictive temperature. The results of such an experiment are shown in Figure 4. The external pool can be detected in intact cells; the internal pool is detected after enzymatic digestion of the cell wall and lysis of the resulting spheroplasts. Following the
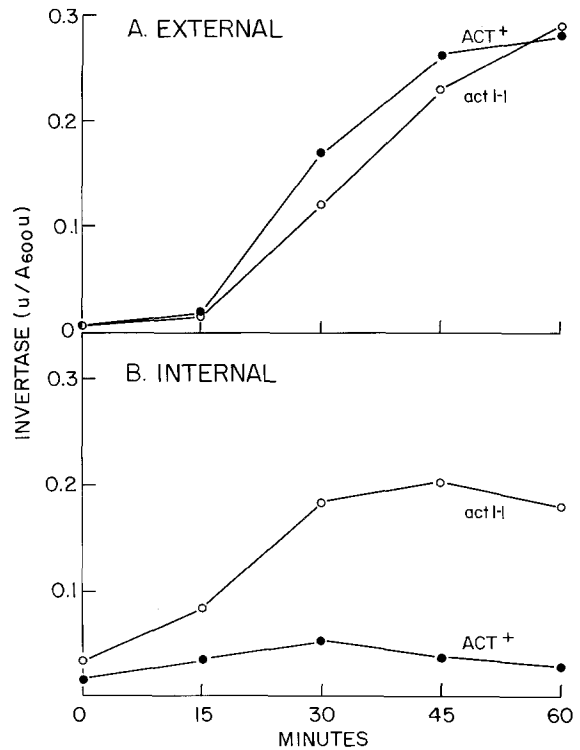

Figure 4. Invertase Secretion and Accumulation

Cells were grown overnight in YEPD media. At T = 0 cells were pelleted and then resuspended in YEP media containing 0.1% glucose and incubated at 37°C. At time points aliquots were removed and assayed for external and internal pools of invertase. The top shows external levels; the bottom shows internal levels. The *ACT⁺* strain was DBY 1033; the *act1-1* strain was DBY 1692.

shift from repressing to derepressing conditions synthesis of the secreted form of invertase could be detected. By 15 min the external pool of the enzyme began to rise. Only a transient rise in the internal pool was seen in wild-type cells, corresponding to the transit form of the secreted protein. Transit from the site of synthesis to the cell surface normally requires about 5 min (Novick et al., 1981). When *act1-1* or *act1-2* cells were shifted simultaneously to derepressing medium and to the restrictive temperature an abnormally large pool of invertase developed inside the mutant cells. Analysis of the accumulated invertase on an acrylamide gel demonstrated that it is of the fully glycosylated form (not shown). Derepression of the actin mutants at the permissive temperature resulted in internal levels of invertase that were intermediate between wild-type levels and the levels in the mutants at the restrictive temperature (not shown).

The buildup of fully glycosylated enzyme inside the mutant cells indicates a partial block late in the secretory pathway. A more dramatic accumulation of invertase can be demonstrated in *act1-1* cells grown at 14°C, a more completely permissive temperature for this allele. In this situation, approximately 80% of the total invertase is internal after 30 min of derepression at 37°C. This result suggests that the internal accumulation is the result of a slowing in the transport of this exported protein. The kinetic block of invertase transport may be incomplete because

both *act1-1* and *act1-2* happen to be incompletely defective in actin function or because the requirement for actin in secretion is indirect—i.e., the actin defect causes damage secondarily to the secretion apparatus in the cells as they die. Complementation analysis (not shown) indicates that *act1-1* is not allelic to any known secretory (*sec*) gene.

Accumulation of Vesicles

Previous work has demonstrated that a block in protein secretion is accompanied by a buildup of one of three membrane bounded organelles (Novick et al., 1980). Early blocks result in an exaggerated endoplasmic reticulum, intermediate blocks result in development of an enlarged Golgi, and late blocks result in an accumulation of vesicles. We examined the actin mutants for such effects. The mutant and wild-type cells were grown at 23°C, then shifted to 37°C. After 1 hr cells were fixed in glutaraldehyde and processed for electron microscopy. Thin section analysis showed that the actin mutants accumulated vesicles at the restrictive temperature (Figure 5). In addition to vesicles, *act1-1* accumulated aberrant doughnut-shaped structures similar to those seen in the Golgi-blocked secretory mutant *sec7* (Novick et al., 1981). A less dramatic accumulation of vesicles occurs in permissively grown mutant cells (not shown). These results are consistent with the observed partial block at a late stage of the secretory pathway and suggest a role for actin in the movement of secretory vesicles to the cell surface.

Viability and Cell Cycle Effects

Mutations that specifically block an event of the cell cycle exhibit uniform morphology upon prolonged incubation of an exponentially growing culture at the restrictive temperature (Hartwell, 1974). The actin mutants were examined for evidence of such a cell cycle arrest. Actin mutant cultures growing exponentially at 23°C were shifted to 37°C, and at time points aliquots were fixed in formaldehyde and briefly sonicated to disrupt cell clumps. Cells were examined by phase microscopy.

At the permissive temperature the mutants show a nearly normal distribution of budded and unbudded cells. Both *act1-1* and *act1-2* cells are somewhat larger and rounder than cells of the parental wild-type strain. In addition, the neck separating the mother and bud of mutant cells is often thicker than the wild-type neck. This is consistent with the observation of enlarged chitin rings on the actin mutants. A small fraction of the cells carry two or more buds.

Incubation of mutant cultures at the restrictive temperature results in an increase in the percentage of unbudded cells. By 2 hr at 37°C approximately 70% of the *act1-1* cells are unbudded. Further incubation at 37°C does not result in a higher percentage of unbudded cells. Cell lysis becomes prevalent after 4 hr; many cells appear very swollen, their vacuoles occupying nearly the entire cytoplasm, and cell debris is apparent in the medium.

These results are not consistent with the simple notion that the actin mutants are defective at a single point in the cell division pathway. There may be, nevertheless, a specific requirement for actin function early in the cell cycle
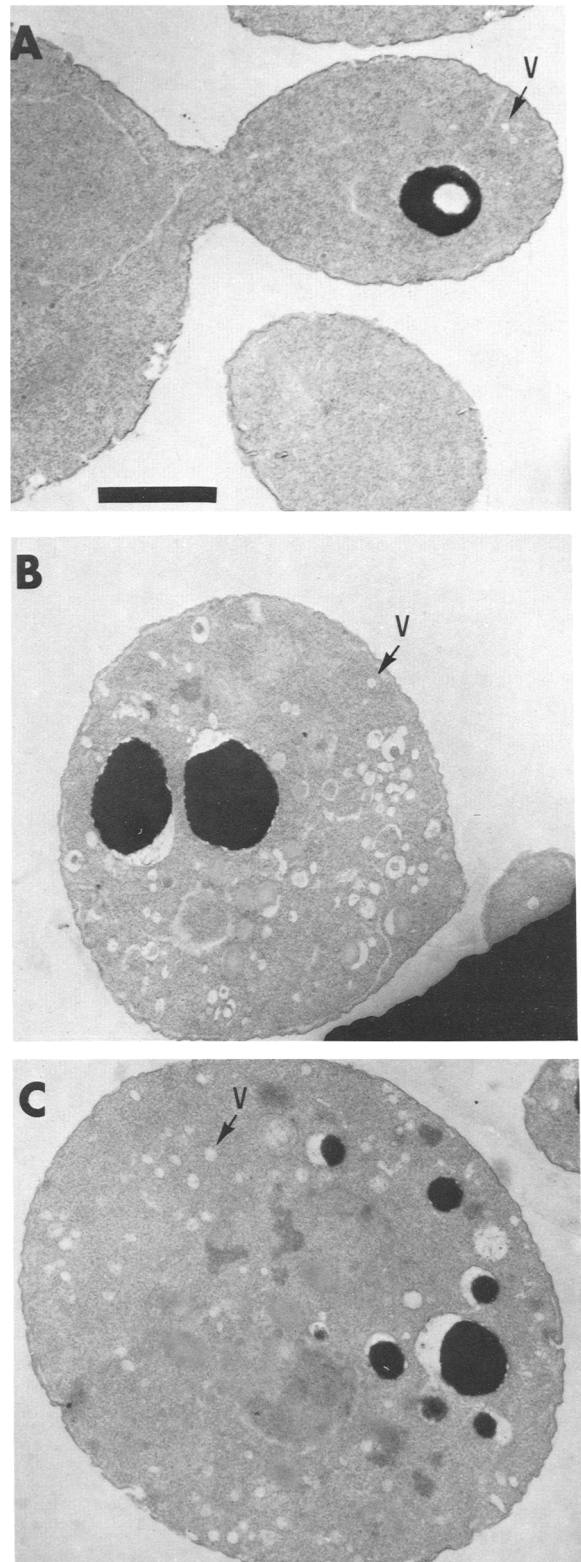

Figure 5. Thin Section Electron Microscopy of Wild-Type and Mutant Cells

Cells were grown overnight at 23°C and then shifted to 37°C for 1 hr. Cells were processed for electron microscopy and thin sections were photographed. (A) *ACT+*, DBY 877. (B) *act1-1*, DBY 1692. (C) *act1-2*, DBY 1695. The bar corresponds to 1 μm.
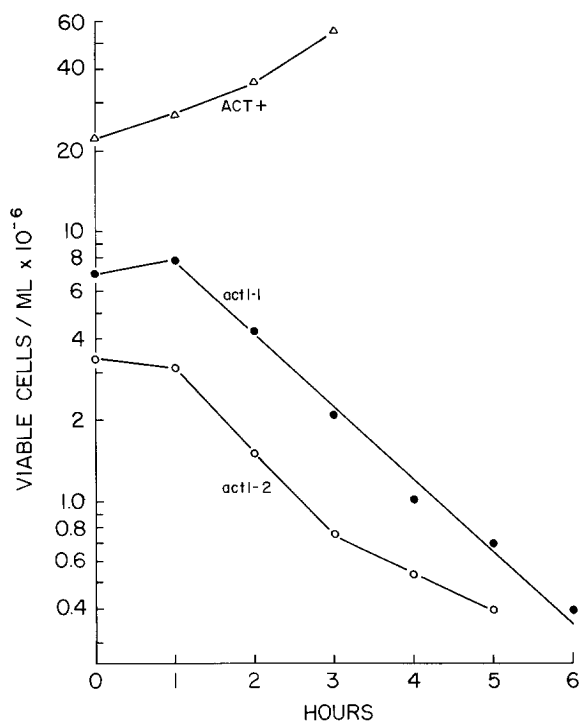
Figure 6. Kinetics of Death of Actin Mutants

Cells were grown overnight and shifted to 37°C at T = 0. Aliquots were removed at intervals, sonicated briefly, diluted with water, and plated for viability at 26°C on YEPD plates. The strains used were *act1-1*, DBY 1692; *act1-2*, DBY 1695.

as well as a more general requirement for actin that causes the mutant cells to die before a uniform unbudded morphology can be obtained.

The kinetics of cell death were followed after shifting a permissively grown culture to 37°C. Aliquots were removed, briefly sonicated, and plated on rich medium at 26°C. The number of viable cells per milliliter of culture remained essentially constant during the first hour and then dropped off by a factor of 10 during the next 3 hr (Figure 6). The drop in the number of viable cells per milliliter is consistent with the observation of lysed cells and cell debris following prolonged incubation. Delayed death by cell lysis is a characteristic phenotype of the actin mutants.

Death of the actin mutants could be independent of the growth state of the cells or it could be a function of the position in the cell cycle or the metabolic state of the cell. To address these questions, viability studies were performed with mutant cells in various growth states. A stationary culture of *act1-1* grown at 23°C was shifted to 37°C for 4 hr, and the cells were plated for viability on YEPD plates at 26°C. Only a 26% loss in viability was seen compared to the 88% loss observed with an exponential culture (Table 1). The increased viability could be the result of the slowed metabolic state of stationary cells or the uniform G0 arrest of the stationary culture. To distinguish between these possibilities an exponentially growing culture was treated with α factor at the time of the shift in temperature or for several hours prior to the shift in temperature. Addition of α factor at the time of the shift to 37°C results in a 65% drop in the viable cells per milliliter. Presynchronization of

Table 1. Viability and Cell Cycle Effects

Treatment	Ratio of Viable Cells per Milliliter at 4 hr to Viable Cells per Milliliter at 0 hr	
	act1-1	*ACT*[+]
No addition	0.12	7.5
Sorbitol[a]	0.12	5.4
Cycloheximide[b]	0.04	0.85
Stationary[c]	0.74	0.91
α Factor[d]	0.23	1.6
α Factor −2 hr[e]	0.43	1.5
α Factor −4 hr[f]	0.58	1.3
Hydroxyurea[g]	0.11	0.97
Hydroxyurea −2 hr[h]	0.12	1.4

Cells were grown overnight at 23°C to a density between 5 × 10^{5} and 5 × 10^{6}. At T = 0 cells were shifted to 37°C. At T = 0 and at T = 4 hr an aliquot was removed and briefly sonicated to disrupt cell clumps. The cells were diluted in water and plated on YEPD plates. After 3 days at 26°C colonies were counted to determine the viable cells per milliliter.
[a] Sorbitol was added at T = 0 to a final concentration of 0.5 M.
[b] Cycloheximide was added at T = 0 to a final concentration of 1 μg/ml.
[c] Cells in stationary phase were used.
[d] α Factor was added at T = 0 to a final concentration of 2.5 units/ml.
[e] α Factor was added at T = −2 hr.
[f] α Factor was added at T = −4 hr.
[g] Hydroxyurea was added at T = 0 to a final concentration of 0.1 M.
[h] Hydroxyurea was added at T = −2 hr.

the culture at the permissive temperature, by addition of the hormone 4 hr prior to the shift in temperature, results in only a 42% drop in viability. These results suggest that cells in the G1 stage of the cell cycle are less susceptible to "actinless death" and that it is necessary for the cells to be in G1 at the time of the shift for the full effect to be seen.

This finding can be interpreted in two ways. Actin mutants may die at one specific point in the cell cycle. By trapping the cells in G1 we prevent them from passing their execution point. Alternatively, they may be sensitive to "actinless death" at all stages of the cycle except G1.

To test these possibilities, the actin mutant cells were presynchronized at an alternative stage. Hydroxyurea inhibits DNA synthesis and therefore traps cells in the S phase of the cell cycle (Slater, 1973). If actin mutants had one execution point, at a stage other than S, presynchronization of cells at S would prevent them from passing their execution point and would prevent mutant death. If the mutants were sensitive at all stages except G1, presynchronization at S would not prevent death. Presynchronization with hydroxyurea yielded no increase in mutant viability. This result is consistent with a requirement for actin at all stages of the cell cycle, though with a lowered requirement in G1. The result does not rule out the possibility that the actin mutants have a single execution point at S, or that there are several execution points, including one at S.

Starvation of an inositol auxotroph also results in a dramatic loss of viability (Henry et al., 1977). Inositolless death can be prevented by inhibition of protein synthesis. On the basis of this observation Henry et al. (1977) have argued that inositolless death is the result of imbalanced cell growth—continued cellular metabolism without production of phosphatidyl inositol.

Table 2. Loss of Chromosome V

		can^R, HOM$^+$	can^R, hom$^-$
ACT$^+$	23°C	1.4 × 10^{-4}	2.1 × 10^{-5}
ACT$^+$	37°C	1.7 × 10^{-4}	9.6 × 10^{-6}
act1-1	23°C	1.2 × 10^{-4}	7.6 × 10^{-6}
act1-1	37°C	1.5 × 10^{-4}	2.0 × 10^{-5}

ACT$^+$/ACT$^+$ (DBY 1573) or act1-1/act1-1 (DBY 1576) diploids were grown overnight at 23°C. The cultures were split, one aliquot was maintained at 23°C, and the other aliquot was incubated at 37°C. After 4 hr, cells were pelleted, washed once with water, and resuspended in water. Appropriate volumes were plated on YEPD plates to measure the viable cells per milliliter and on synthetic media containing canavanine as well as leucine, lysine, histidine, methionine, and threonine. After 3 days at 26°C the canavanine plates were replica stamped onto synthetic media with and without methionine and threonine to determine the fraction of colonies that were hom$^-$. The values represent the fractions of viable cells that had undergone recombination or chromosome loss.

We tested the effect of inhibition of protein synthesis on the death of the actin mutants. Exponentially growing mutant cultures were shifted to 37°C in the presence or absence of cycloheximide. After 4 hr at 37°C the cells were briefly sonicated and plated on rich medium at 26°C to assess viability. Inhibition of protein synthesis did not rescue the actin mutants (Table 1). Actin mutant death appears to be a direct effect of the altered gene product and not the result of imbalanced growth. This result also implies that act1-1 is defective for actin function and not for actin synthesis; actin synthesized at 23°C is defective at 37°C in the absence of further protein synthesis.

Sensitivity to Osmotic Pressure

The yeast cell wall is essential for maintenance of the structural integrity of the cell in conditions of low osmotic support. Enzymatic removal of the cell wall of wild-type cells results in cell lysis, which can be prevented by addition of osmotic support. The lysis phenotype of the actin mutants suggested that their defect may be remedied by increased osmotic support.

The effect of osmotic support on actin mutant growth and viability was tested in two ways. In the first experiment various forms of osmotic support, KCl, sorbitol, or ethylene glycol, were added to solid media and growth of the mutants was assessed at a range of temperatures. Osmotic support did not raise the restrictive temperature of either mutant. Surprisingly, increased osmotic strength lowered the temperature at which growth failed. Both mutants grow slowly at 33°C on YEPD plates, yet fail to grow on YEPD plates containing 0.5 M ethylene glycol (data not shown). In the second experiment, mutant cells growing exponentially in YEPD media at 23°C were shifted to 37°C in the persence or absence of 0.5 M sorbitol. After 4 hr at

37°C an aliquot was removed and, briefly sonicated, and cells were plated for viability at 26°C on YEPD plates. The presence of 0.5 M sorbitol had no effect on cell viability (Table 1).

These results indirectly suggest that the lysis of the actin mutants is not due to a simple inability to withstand the normal turgor of the cell; such a defect should be at least partially remedied by addition of osmotic support. The unexpected osmotic sensitivity demonstrated by the actin mutants appears to be a property of the lowered concentration of functional actin rather than a property of the particular alleles isolated; a diploid carrying one wild-type copy of actin and one null allele also shows osmotic sensitivity (D. Shortle, personal communication). Actin expression or actin function may be unusually sensitive to the altered internal environment resulting from growth at high osmotic strength, or actin may play a role in the osmotic regulation of the cell.

Chromosome Stability

Little or no actin is seen inside the yeast nucleus by immunofluorescence (Kilmartin and Adams, 1984). This suggests that actin does not play a role in chromosome segregation. To assess independently the role of actin in chromosome segregation, we measured directly the effect of an actin mutation on the frequency of chromosome loss. This is a very sensitive test because of the high fidelity of chromosome segregation under normal conditions.

The frequency of loss of chromosome V was tested in act1-1/act1-1 and ACT$^+$/ACT$^+$ diploids using the test described by Wood (1982). Strains heterozygous for the recessive chromosome V markers, hom3 and can^R, were incubated at the restrictive or permissive temperatures and plated on canavanine-containing media. Canavanine

resistance can result from mitotic recombination or from the loss of the chromosome carrying the CAN^S allele. Mitotic recombinants were distinguished from presumptive chromosome loss events by screening the can^R colonies for the simultaneous appearance of another recessive phenotype, hom3, on the opposite arm of chromosome V. The frequency of chromosome V loss in permissively grown act1-1/act1-1 was close to the frequency in wild-type diploids and to the published value (see Table 2). Upon incubation at 37°C for 4 hr, neither the wild-type strain nor the mutant diploid showed a significant increase in loss, compared to the 34-fold increase resulting from disruption of microtubules (Wood, 1982).

Regulation of Assembly

The construction of conditional-lethal actin mutants facilitated the definition of several roles for actin in cell growth and division. The mutants should also be of use in addressing a second question: how is the complex cell cycle dependent pattern of actin assembly regulated? By analogy to higher eukaryotes one would predict the existence of a set of yeast proteins that control the site and extent of actin polymerization by means of physical interactions with the actin molecule. Such proteins could be identified genetically as suppressors of an actin mutant (Botstein and Maurer, 1982). The deleterious effects of an alteration in one member of a protein complex can be avoided by a compensating change in another protein of the complex. We have analyzed such extragenic suppressors and have identified three complementation groups that can yield suppression of act1-1. Identification of the suppressor gene products and analysis of conditionally lethal alleles of these genes may help in understanding the in vivo regulation of actin assembly at a molecular level.

Conclusion

Using conditional-lethal mutations in the single actin gene of yeast, we have been able to implicate actin in a variety of cell functions, mostly affecting the synthesis, assembly, and integrity of the cell surface. We suggest that cytoplasmic actin may have similar or identical roles in all eukaryotic cells. Finally, we believe our results support the notion that yeast, because of its powerful genetics, can play a central role in the elucidation of the cellular functions of highly conserved cytoskeleton proteins.
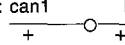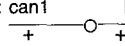
Experimental Procedures

Yeast Strains

The yeast strains used are listed in Table 3. The construction of the conditional-lethal alleles of ACT1, act1-1 and act1-2, has been described (Shortle et al. 1984). Standard genetic techniques of crossing, sporulation, and tetrad analysis were followed as described by Sherman, Fink, and Lawrence (1974).

Media

Unless otherwise indicated, YEPD medium, containing 1% yeast extract, 2% bactopeptone, and 2% glucose was used. YEP medium contains no glucose. Synthetic medium containing indicated supplements was prepared as described by Sherman, Fink, and Lawrence (1974). Solid media contained 2% agar.

Table 3. Yeast Strains Used

Strain	Genotype
DBY 640	MATa, ade2-1, ACT⁺
DBY 877	MATα, his4-619, ACT⁺
DBY 1033	MATα, ura3-52, ACT⁺
DBY 1573	Chr. V: can1 ———————— hom3; MATa/MATα, leu2/+, $\frac{+ \quad\quad -O- \quad\quad +}{lys2/+, \; his4/+, \; ACT^+/ACT^+}$
DBY 1576	Chr. V: can1 ———————— hom3; MATa/MATα, leu2/+, $\frac{+ \quad\quad -O- \quad\quad +}{lys2/+, \; his4/+, \; act1-1/act1-1}$
DBY 1689	MATa, his4-619, ACT⁺
DBY 1690	MATa/MATα, ade2-1/+, his4-619/+, ACT⁺/ACT⁺
DBY 1691	MATa, his4-619, act1-1
DBY 1692	MATα, his4-619, act1-1
DBY 1693	MATa/MATα, ura3-52/+, his4-619/+, act1-1/act1-1
DBY 1694	MATα, his4-619, act1-2
DBY 1695	MATa, his4-619, act1-2
DBY 1696	MATa/MATα, his4-619/+, ade2-1/+, act1-2/act1-2

Growth of Strains

Early exponential cultures were prepared by inoculation of liquid medium with stationary cells and overnight incubation with agitation at the indicated temperature. The absorbance was measured in a 1 cm quartz cuvette at 600 nm in a Beckman Model 25 spectrophotometer. One A_{600} unit corresponds to approximately 10^7 haploid yeast. Experiments were initiated with exponentially growing cells at a density of 1×10^6–3×10^7 cells/ml.

Immunofluorescent Staining of Yeast Cells

Fixation and staining of yeast cells were performed by a modification of the method described by Kilmartin and Adams (1984). Approximately 10^8 cells from an exponentially growing culture were harvested by filtration, washed once with 0.1 M potassium phosphate (pH 6.5), and resuspended in 5 ml of phosphate buffer containing 5% formaldehyde. After 1.5 hr at room temperature the cells were pelleted and washed twice with phosphate buffer. The cells were resuspended in 1 ml of a solution containing 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5), 24 mM 2 mercaptoethanol, and 0.05 mg Zymolyase 60,000. After 90 min at 30°C the cells were pelleted and resuspended in 1 ml of 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5). An aliquot of the cell suspension was added to a well of a polylysine-coated multiwell slide. After 30 sec the excess was removed and the slide was immersed in methanol at –20°C for 6 min, then in acetone at –20°C for 30 sec. Affinity purified anti-yeast actin antibody prepared in rat (a generous gift from Dr. Kilmartin) was diluted 1:30 with phosphate-buffered saline containing 1 mg/ml bovine serum albumine (PBS-BSA) and applied to the fixed and permeabilized yeast cells. After 90 min the antibody was removed and the slides were washed twice by immersion in PBS-BSA. The excess solution was removed, and affinity purified anti-rat IgG was applied. The anti-rat IgG (Miles Lab) was affinity purified using rat IgG (Miles Lab) coupled to Sepharose 4B as absorbant. After 60 min the slides were washed as before. Slides were mounted in p-phenylenediamine in 90% glycerol as described by Kilmartin and Adams (1984).

Rhodamine Phalloidin Staining

For labeling of actin with rhodamine phalloidin, cells were fixed as described above; however, cell walls were not removed. Staining was by the protocol of Adams and Pringle (1984). Rhodamine phalloidin was the generous gift of J. Pringle.

Calcafluor Staining of Chitin

For labeling of chitin, fixed and washed cells were incubated with Calcafluor White M2R at 1 μg/ml in 50 mM potassium phosphate for 5 min, then washed and resuspended in phosphate buffer. Calcafluor was the generous gift of J. Pringle.

Photomicroscopy

Cells were photographed using Kodak Tri X film on a Zeiss Standard microscope equipped for epifluorescence. A Zeiss Neofluor N.A. 1.3 objective was used with rhodamine selective filter 487715-9902 or dapi filter set 487702-7960. Exposure times were approximately 3 sec, and the film was processed in Diafine developer.

Viability Studies

To determine the number of viable cells per milliliter an aliquot was removed from cultures and sonicated for 5 sec to disrupt cell aggregates. The cells were diluted in sterile water and plated on YEPD media. Colonies were scored after 3 days of growth at 26°C.

Measurement of Invertase Activity

Approximately 2 × 10⁷ cells were harvested by centrifugation and resuspended in 1 ml of 10 mM sodium azide. External enzyme levels were measured using intact cells as described by Goldstein and Lampen (1975). Internal levels were measured in spheroplast lysates; 0.5 ml of the cell suspension was mixed with 0.5 ml of a solution containing 2.8 M sorbitol, 50 mM potassium phosphate (pH 7.5), 50 mM 2-mercaptoethanol, and 50 μg/ml Zymolyase 60,000. After 1 hr at 37°C, the spheroplasts were pelleted and then lysed by addition of 0.5 ml of 0.5% Triton X-100. N-ethylmaleimide, 0.2 mM, was added to the second stage of the enzyme assay to prevent interference by residual 2-mercaptoethanol.

Thin Section Electron Microscopy

Approximately 5 × 10⁸ cells were harvested by filtration, washed with 10 ml of 0.1 M cacodylate (pH 6.8), 1 mM EDTA, then resuspended in 9 ml of the same buffer containing 3% glutaraldehyde. After 2 hr of incubation at room temperature, the cells were washed twice with 50 mM potassium phosphate (pH 7.5) and resuspended in 2 ml of phosphate buffer containing 0.5 mg Zymolyase 60,000. After 60 min at 30°C the cells were washed in the cacodylate buffer and resuspended in cacodylate buffer containing 2% osmium tetroxide. After 1 hr of incubation at 0°C, the cells were washed in water and resuspended in 2% aqueous uranyl acetate. After 1 hr at room temperature, cells were washed in water, formed into agar blocks, dehydrated in graded ethanol, and embedded in SPURR epoxy medium (Polysciences). Sections were cut on a diamond knife, stained with lead citrate, and poststained with uranyl acetate.

Acknowledgments

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