Actin and Myosin Function in Directed Vacuole Movement during Cell Division in *Saccharomyces cerevisiae*

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Abstract. During cell division, cytoplasmic organelles are not synthesized de novo, rather they are replicated and partitioned between daughter cells. Partitioning of the vacuole in the budding yeast *Saccharomyces cerevisiae* is coordinated with the cell cycle and involves a dramatic translocation of a portion of the parental organelle from the mother cell into the bud. While the molecular mechanisms that mediate this event are unknown, the vacuole's rapid and directed movements suggest cytoskeleton involvement. To identify cytoskeletal components that function in this process, vacuole inheritance was examined in a collection of actin mutants. Six strains were identified as being defective in vacuole inheritance. Tetrad analysis verified that the defect cosegregates with the mutant actin gene. One strain with a deletion in a myosin-binding region was analyzed further. The vacuole inheritance defect in this strain appears to result from the loss of a specific actin function; the actin cytoskeleton is intact and protein targeting to the vacuole is normal. Consistent with these findings, a mutation in the actin-binding domain of Myo2p, a class V unconventional myosin, abolishes vacuole inheritance. This suggests that Myo2p serves as a molecular motor for vacuole transport along actin filaments. The location of actin and Myo2p relative to the vacuole membrane is consistent with this model. Additional studies suggest that the actin filaments used for vacuole transport are dynamic, and that profilin plays a critical role in regulating their assembly. These results present the first demonstration that specific cytoskeletal proteins function in vacuole inheritance.

The cytoplasm of eukaryotic cells is distinguished by the presence of numerous membrane-bound organelles that carry out specific and essential cellular functions. These organelles are complex structures that are not readily synthesized de novo. Hence, during cell division each new cell does not synthesize its own set of organelles, rather the organelles present in the parental cell are replicated and then partitioned between daughter cells before cytokinesis (54). The coordination of organelle partitioning with the cell cycle, together with the accuracy and efficiency of this process, strongly suggests the involvement of active partitioning mechanisms. A few proteins have recently been implicated in organelle inheritance through biochemical (19, 20, 60) and genetic approaches (34, 44, 53, 58, 61). However, the underlying molecular mechanisms remain to be established.

Organelle inheritance has been studied in a variety of systems and it appears that the mechanisms involved vary among different organisms, as well as for different organelles within the same cell. During mitosis in mammalian cells, the ER and Golgi apparatus become fragmented, forming many small vesicles that are specifically divided between daughter cells and then reassembled, leaving each new cell with its own complete set (54). Fragmentation is thought to aid in the equal partitioning of these organelles, although it is not clear whether this partitioning is stochastic or whether it requires the function of specific proteins. In the budding yeast *Saccharomyces cerevisiae*, organelles such as the nucleus, vacuole, and mitochondria do not undergo fragmentation. Therefore, the faithful inheritance of these low copy structures absolutely requires that they be actively partitioned between the mother and daughter cell before cytokinesis (54). Interestingly, although partitioning of these organelles might be expected to use common components, many of the proteins involved appear to be unique, since mutants that are defective in the inheritance of one organelle often show normal partitioning of others (34, 44, 53, 56, 58).

Inheritance of the vacuole in *S. cerevisiae* serves as an excellent model for studying organelle inheritance. This event begins early in the cell cycle and is marked by the formation of a tubular-vesicular "segregation structure" that extends from the parental organelle into the bud (55, 57). Translocation of the segregation structure from the mother cell into the bud proceeds along a specific path at a rate of 0.1–0.2 μm/s (Weisman, L.S., unpublished). Once established, the segregation structure allows for the transfer of vacuolar material between mother and daughter...
were labeled as described above, and then washed and incubated in fresh chase period. Cells were collected by low-speed (800 g) centrifugation and at least one cell doubling as verified by OD600 measurements. After the chase period, cells were rinsed twice, resuspended in 5 ml of fresh medium, and incubated for 1 h. The cells were then harvested, plated onto YEPD plates, and analyzed using an infrared confocal microscope.

Materials and Methods

In Vivo Labeling of Vacuoles

For measuring vacuole inheritance, vacuoles were labeled in vivo with N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) (FM4-64) (Molecular Probes, Eugene, OR) according to the method of Vida et al. (50). Briefly, 0.1-0.2 OD600 U of cells was harvested from log phase cultures, resuspended in 0.25 ml of YEPD medium containing 80 μM FM4-64, and incubated for 1 h at 24°C with shaking. After labeling, cells were washed twice, resuspended in 5 ml of fresh medium, and incubated for an additional 2-5 h. This chase period was sufficient for allowing for at least one cell doubling as verified by OD600 measurements. After the chase period, cells were collected by low-speed centrifugation and examined by fluorescence microscopy using a Zeiss Axioskop fluorescence microscope, equipped with an MC 100 35-mm camera.

For zygote experiments, 2 OD600 U of the indicated parental strains were labeled as described above, and then washed and incubated in fresh medium for 1 h at 24°C with shaking. After 1 h, labeled cells (~1 OD600 U) were mixed with an equal number of unlabelled cells of the opposite mating type, incubated with shaking at 24°C for 1 h, and then plated onto YEPD plates and placed in the dark. After 2.5-3.5 h in the dark, zygotes were scraped from the plate, resuspended in 4 μl of YEPD medium on a glass slide and examined by fluorescence microscopy as above.

For the cell sorting experiment shown in Fig. 2, 1-ml samples were removed at the indicated time points during the chase and analyzed using an EPICS 753 Fluorescence-Activated Cell Sorter (Coulter Electronics, Inc., Hialeah, FL). Cells were excited at 488 nm and emitted light was detected with a 670/14 band pass filter. For each sample 10⁴ cells were analyzed.

Phalloidin Staining of F-Actin

Phalloidin labeling of F-actin in fixed cells was done according to the method of Adams and Pringle (2). Briefly, log phase cells were fixed with 3.7% formaldehyde in growth medium for 60 min at room temperature, then washed three times with PBS (2) and resuspended in 0.1 ml of PBS. FITC-phalloidin (Molecular Probes) was added from a methanolic stock solution to a final concentration of 1.1 μM and cells were incubated in the dark at room temperature for 2.5-3 h. Labeled cells were washed five times with PBS before examination by fluorescence microscopy.

Indirect Immunofluorescence Localization

For all experiments, cells were fixed by the addition of 37% formaldehyde directly to the growth medium to a final concentration of 3.7%. Fixation was carried out with minimal shaking for 3 h. Spheroplasts were made by incubating fixed cells in 1.2 M sorbitol, 0.1 M potassium phosphate, pH 6.5, 1% β-mercaptoethanol with 10 μg/ml oxytetracycline (Easogenetics, Eugene, OR) for 10-15 min (80-90% of cells formed spheroplasts as assessed by phase contrast microscopy). Washed spheroplasts were attached to 1% polylysine-coated multiwell slides (ICN Biomedicals, Aurora, OH). Standard blocking buffer and wash conditions were used (25). In all cases, localization of the vacuole membrane was achieved using an anti-60-kD ATPase mouse monoclonal antibody (Molecular Probes) at a dilution of 1:50 followed by Lissamine rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Labs, Inc., West Grove, PA) at a dilution of 1:200.

We found that the actin cables could not be adequately detected with fluorescein phalloidin conjugates in cells which had been fixed and spheroplasted. Consequently, we tested the use of rabbit anti-yeast actin antibody for simultaneous localization of actin and the vacuole membrane. Clear visualization of actin cables by immunofluorescence requires that the cells be pretreated with cold (-20°C) methanol for 6 min, followed by -20°C acetone for 30 s (10). Although the methanol/acetone treatment is not compatible with the immunofluorescence localization of several antigens, the ATPase staining was not affected by this treatment. Actin was visualized by a 2-h incubation with rabbit anti-yeast actin antibody (32) (1:10 dilution) followed by a 1-h incubation with Oregon Green 488 conjugated goat anti-rabbit IgG (Molecular Probes) (1:200 dilution). Affinity purification of anti-actin antibody was performed with nitrocellulose blots as described (39).

Simultaneous visualization of Myo2p and the vacuole membrane was performed in yeast zygotes. 2 OD600 U each of mid-log phase MATa and MATα strains were mixed together in YEPD medium and shaken at 24°C for 1 h. The cells were then harvested, plated onto YEPD plates, and allowed to incubate at 24°C for an additional 3-4 h. Cells were gently washed from the plate with YEPD and fixed as described above. Myo2p labeling was visualized using affinity-purified rabbit anti-Myo2p antibody followed by antibody amplification as described (32) except that Oregon Green 488 conjugated goat anti-rabbit IgG was used as the final antibody. Affinity-purified anti-Myo2p antibody was generously provided by Drs. S. Lillie and S. Brown (University of Michigan, Ann Arbor, MI). The amplification antibodies were preabsorbed two times to fixed yeast cells and were used at a final dilution of 1:250. In all experiments, omission of the primary antibody resulted in no detectable fluorescence. No cross-reactivity was observed between the anti-mouse and anti-rabbit antibodies.

Stained cells were visualized using an MRC 1024 Scanning Confocal head mounted on a Nikon Optiphot equipped with either a 60x or 100x oil immersion objective, 1.4/NA BioRad Labs (Hercules, CA). The excitation light source used was a mixed gas krypton/argon laser, passed through a dual dichroic filter, allowing excitation at both 488 nm and 568 nm. Dual detection was performed with separate photomultiplier tubes and the resultant images merged using Laser Sharp software. For each field, a z-series of 3-7-μm steps was scanned, and projected to a single view. Single steps were also analyzed.

1. Abbreviations used in this paper: SC, synthetic complete medium; vacuole inheritance mutant; YEPD, yeast extract peptone, dextrose medium.


**Table I. Yeast Strains Used**

| Strain   | Genotype                                                                 | Reference |
|----------|---------------------------------------------------------------------------|-----------|
| TDyDD    | MATa/MATa, leu2-3, 112//leu2-3, 112, ura3-52/ura3-52, lys2+/-, ade2+/-, act1Δ::LEU2+/- | (8)       |
| LWY1412* | MATa, leu2-3, 112, ura3-52, ACT1                                             | This study|
| LWY1425* | MATa, leu2-3, 112, ura3-52, ACT1                                             | This study|
| LWY1408* | MATa, leu2-3, 112, ura3-52, lys2, act1Δ::LEU2, pADSE(URA3)                  | This study|
| LWY1419* | MATa, leu2-3, 112, ura3-52, act1Δ::LEU2, pADSE(URA3)                        | This study|
| DDY338   | MATa, ura3-52, leu2-3, 112, hisΔ3200, canl-1, tub2-201, cty1, act1-101::HIS3 | (59)      |
| DDY339   | MATa, ura3-52, leu2-3, 112, hisΔ3200, canl-1, tub2-201, act1-102::HIS3      | (59)      |
| DDY356   | MATa, ura3-52, leu2-3, 112, hisΔ3200, canl-1, tub2-201, act1-105::HIS3      | (59)      |
| DDY341   | MATa, ura3-52, leu2-3, 112, hisΔ3200, canl-1, tub2-201, ade2-101, cty1, act1-11::HIS3 | (59) |
| DDY365   | MATa, ura3-52, leu2-3, 112, hisΔ3200, tub2-201, canl-1, cty1, act1-121::HIS3 | (59)      |
| DDY655   | MATa, ura3-52, leu2-3, 112, hisΔ3200, tub2-201, canl-1, cty1, act1-122::HIS3 | (59)      |
| DDY336   | MATa, ura3-52, leu2-3, 112, hisΔ3200, canl-1, tub2-201, cty1, act1-133::HIS3 | (59)      |
| DDY355   | MATa, ura3-52, leu2-3, 112, hisΔ3200, tub2-201, ade2-101, act1-135::HIS3    | (59)      |
| RH2069   | MATa, his4, leu2-3, 112, ura3-52, bar1, end7-1                              | (36)      |
| JPTA     | MATa, ura3-51, met6, ade1, his6, leu2-3, 112, myo2-66                       | (23)      |
| 21R      | MATa, ura3-52, leu2-3, 112, ade1, MYO2                                      | (23)      |
| myo4ΔU5-2A | MATa, ura3-51, his3, leu2, trpl, myo4Δ::URA3                              | (17)      |
| DC5      | MATa, ura3-52, lys2, his3, leu2, ade2-201, ade3, PYFI                       | (18)      |
| BHY31    | MATa, ura3-52, lys2, his3, ade2-201, ade3, pyf1-112::LEU2                   | (18)      |

*These strains were generated by sporulation of TDyDD after transformation with plasmid pADSE as described by Cook et al. (8).

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**35S-Labeling and Immunoprecipitation**

35S in vivo labeling and immunoprecipitation of carboxypeptidase Y and proteinase A were performed using a modification of the procedure described by Horazdovsky and Emr (21). Cultures were grown to log phase in synthetic complete (SC) medium supplemented with 0.2% yeast extract. Spheroplasts were prepared from 20 OD600 U of cells as described by Vida et al. (51), except that SC medium was used instead of WIMPYE, and zymolyase (10 mg/ml) (ICN Pharmaceuticals, Costa Mesa, CA) was used instead of oxalase. Spheroplasts were harvested (3,000 g, 3 min), resuspended at 10 OD60U in SC-methionine, cysteine medium containing 1 M sorbitol, 1 mg/ml BSA, and 0.1 mg/ml a2-macroglobulin (21). Labeling with 150 μCi/ml Trans35S-label (ICN Pharmaceuticals) was carried out for 10 min at 24°C with gentle shaking. The chase was initiated by adding unlabeled methionine (5 mM), cysteine (1 mM) and yeast extract (0.2%); and then incubated for an additional 30 min at 24°C. Aliquots taken before and after the chase period were separated into intracellular and extracellular fractions by centrifugation (15,000 g, 30 s), and then TCA was added to a final concentration of 10%. Dried TCA pellets were resuspended by sonication in resuspension buffer (28). CPY and PrA immunoprecipitations were carried out as described (28), using one wash each with "urea buffer" (100 mM Tris [pH 7.5], 200 mM NaCl, 2 M urea, 0.5% Tween-20) and 0.1% SDS instead of the 1% β-mercaptoethanol wash. Samples were analyzed by SDS 11% polyacrylamide gel electrophoresis and fluorography.

**Results**

**Actin Is Involved in Vacuole Inheritance**

Vacuole inheritance was examined by labeling cells with the styryl dye FM4-64. This vital fluorophore specifically stains the vacuolar membrane, and is stable for several generations (50). Labeled cells were viewed by fluorescence microscopy after incubation for a minimum of one cell doubling time in fresh medium without the fluorophore. In this type of "pulse-chase" experiment, buds that did not receive the fluorophore exclusively through inheritance. Previous studies have shown that daughter cells that do not inherit a vacuole are nonetheless able to generate a normal vacuole (53, 58), perhaps through Golgi to vacuole vesicle traffic and/or endocytosis. This ability to generate a normal-sized vacuole may account for the virtually wild-type growth rate of act1-ΔDSE.

Actin filaments in S. cerevisiae cells are found in two characteristic forms, cortical patches at the cell surface and cytoplasmic cables that extend between the mother cell and the bud (1, 27). The cortical patches are most often found in areas experiencing active growth and are thought to function in endocytosis and secretion (35). The function of the cables is unknown. The distribution of filamentous actin in act1-ΔDSE cells is similar to wild-type (Fig. 1, c and d). Therefore, the act1-ΔDSE mutation does not block vacuole transport by simply disrupting the yeast cytoskeleton. Rather, the inheritance defect in this mutant is probably a direct consequence of impaired actin function.

To probe the extent of the vacuole inheritance defect in act1-ΔDSE, we sought to analyze a large population of cells. Logarithmically growing cultures were labeled with FM4-64, and then washed and chased in fresh medium. Samples taken during the chase period were analyzed by Fluorescence Activated Cell Sorting (Fig. 2). As expected, wild-type cells behave primarily as a homogeneous population in which the average fluorescence per cell gradually decreases (53). Vacuole inheritance mutants (vac) examined by this method exhibit a characteristic bimodal fluorescence distribution, in which two peaks are observed that correspond to a population of weakly fluorescent
Figure 1. Deletion of the three NH$_2$-terminal amino acids of actin blocks vacuole inheritance but does not disrupt the overall organization of the actin cytoskeleton. (a and b) Wild-type (a) and act1-ADSE (b) cells were labeled with a vacuole-specific fluorophore as described in Materials and Methods. After labeling, cells were washed two times with fresh medium, resuspended in 5 ml of medium without the fluorophore, incubated for an additional 3 h, and then visualized by fluorescence microscopy. Wild-type buds inherit a labeled vacuole from the parental cell, but buds in the actin mutant do not. Arrowheads mark cells with a segregation structure. (c and d) To visualize filamentous actin, wild-type (c) and act1-ADSE (d) cells were fixed and stained with FITC-phalloidin according to the protocol of Adams and Pringle (1991). The pattern of F-actin staining is similar in wild-type cells and the actin mutant. Bar, 5 μm.

daughter cells that have not inherited a fluorescent vacuole, and a population of highly fluorescent mother cells that have retained their fluorescent vacuoles (53). As seen in Fig. 2, act1-ADSE cells display a fluorescence profile that is characteristic of vac mutants. Furthermore, the fluorescence intensity of the mother cells in this mutant remains almost unchanged over the course of the experiment (approximately two doubling times), indicating that
the mother cells retain almost all of their original vacuolar material.

To verify that the vac phenotype of act1-ΔDSE is due to the mutant actin gene, a hemizygous (act1Δ::LEU2/ACT1) diploid, carrying the act1-ΔDSE actin gene on a URA3-containing plasmid, was sporulated and vacuole inheritance was quantitated in the meiotic progeny (Fig. 3a). The results demonstrate that the inheritance defect cosegregates with the act1-ΔDSE gene. Furthermore this phenotype is recessive, since cells carrying both a mutant and a wild-type actin gene exhibit normal inheritance (see for example, tetrads 2C and 2D in Fig. 3a). The fact that the vac phenotype is recessive is consistent with in vitro studies which demonstrated that wild-type actin can attenuate defects associated with ΔDSE actin when assembled as a copolymer (8).

**Vacuole Inheritance Defects in Additional act1 Alleles**

We examined vacuole inheritance in several additional actin mutants. Table II shows the results of this analysis and summarizes other phenotypes that have been reported for

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**Figure 2.** Fluorescence-activated cell sorting profiles of wild-type (WT) and act1-ΔDSE (ΔDSE) cells. Cells were labeled with a vacuole-specific fluorophore and chased in fresh medium as described in the legend to Fig. 1. At the indicated time points 1-ml samples were removed and analyzed by fluorescence-activated cell sorting. 10⁶ cells were counted for each sample. These results are representative of two independent experiments.

**Figure 3.** Tetrad analysis demonstrates that a vacuole inheritance defect cosegregates with mutant actin alleles act1-ΔDSE, act1-101, and act1-105. (a) A diploid strain (TDyDD) in which one chromosomal copy of the ACT1 gene has been replaced with the LEU2 gene, was transformed with a URA3-containing centromeric plasmid (pΔDSE) carrying the act1-ΔDSE actin gene (8). A Ura+ transformant was selected and sporulated. The meiotic progeny were scored for vacuole inheritance as well as for the presence of the pΔDSE plasmid (Ura+) and the disrupted (act1::LEU2) chromosomal actin gene (Leu+). (b and c) Mutant strains carrying the act1-101 (b) or act1-105 (c) alleles were back-crossed to congenic wild-type strains. The resulting diploid strains were sporulated and scored for vacuole inheritance as above. The presence of the mutant (His+) or wild type (His-) actin allele was also determined. To quantitate vacuole inheritance, random fields of cells with a labeled vacuole in the mother cell were analyzed. A minimum of 50 cells were scored for each strain. (Open bars) Buds that inherited normal amounts of the parental vacuole. (Stippled bars) Buds that inherited very little (<10% of normal) parental vacuole. (Solid bars) Buds that received no detectable vacuole from the mother cell. Similar results were obtained with an additional six (act1-ΔDSE) and four (act1-105) tetrads (not shown).
Table II. Relationship of Vacuole Inheritance Phenotypes to Previously Reported Defects in Actin Mutants

| Allele     | Amino acid substitution | Vacuole inheritance* | Previously reported phenotypes* |
|------------|-------------------------|-----------------------|---------------------------------|
| act1-101   | D363A, E364A            | –                     | ts (59)                        |
|            |                         |                       | bud scar delocalization (24)    |
|            |                         |                       | delocalized actin patches (24)  |
|            |                         |                       | short/faint actin cables (24)   |
|            |                         |                       | minor nuclear inheritance defect (24) |
|            |                         |                       | clumped mitochondria (24)       |
| act1-102   | K359A, E361A            | +                     | wt growth (59)                  |
| act1-105   | E311A, R312A            | –                     | ts, cs (59)                     |
| act1-111   | D222A, E224A, E226A     | –                     | ts (59)                        |
|            |                         |                       | bud scar delocalization (24)    |
|            |                         |                       | delocalized actin patches (24)  |
|            |                         |                       | thin actin cables (24)          |
|            |                         |                       | nuclear inheritance defect (24) |
|            |                         |                       | morphological defects (24)      |
| act1-121   | E83A, K84A              | +                     | ts, cs (59)                     |
|            |                         |                       | morphological defects (24)      |
|            |                         |                       | delocalized actin patches (24)  |
|            |                         |                       | actin bars (24)                 |
|            |                         |                       | minor nuclear inheritance defect (24) |
| act1-122   | D80A, D81A              | +                     | cs, ts (59)                     |
| act1-133   | D24A, D25A              | –                     | cs, ts (59)                     |
|            |                         |                       | morphological defects (24)      |
|            |                         |                       | delocalized actin patches (24)  |
|            |                         |                       | faint actin cables (24)         |
|            |                         |                       | clumped mitochondria (24)       |
|            |                         |                       | mitochondrial motility defect (45) |
| act1-135   | E4A                     | –                     | wt growth (59)                  |
| act1 (end7-1) | G48A                 | +                     | ts (36)                        |
|            |                         |                       | endocytosis (36)                |

*Vacuole inheritance was examined as described in Fig. 3.
†References for previously reported phenotypes are indicated in parentheses.

these strains. We initially examined 20 strains from the charged-to-alanine mutant collection of Wertman and colleagues (59). Strains that grew poorly or exhibited significant morphological abnormalities were omitted from further study, since these phenotypes make interpretation of our inheritance assay difficult. The strains listed in Table II are those for which vacuole inheritance was reproducibly and unambiguously scored as either normal or defective in a quantitative assay (described in the legend to Fig. 3). Two of the mutants in Table II, act1-101 and act1-105, were back crossed to appropriate wild-type strains, and, as with act1-ΔDSE, the vacuole inheritance defect cosegregated with the mutant actin allele (Fig. 3, b and c).

The Location of Actin Cables Relative to the Vacuole Membrane Is Consistent with Actin Playing a Primary Role in Vacuole Movement

Both forms of filamentous actin, the cortical patches and the cables, undergo defined rearrangements at specific stages of the cell cycle (2, 27). The cables, which are aligned along the mother-bud axis, become visible just before bud emergence, and then disappear as the bud reaches full size and cytokinesis occurs. Vacuole inheritance also initiates early in the cell cycle (14, 55, 57). Hence, the time of appearance, position, and orientation of actin cables are consistent with a role for these cables in vacuole movement. To further explore the location of vacuoles relative to actin cables, double immunofluorescence labeling studies were performed. When actin and vacuole membranes are visualized simultaneously, it is evident that many of the actin cables are associated with vacuoles (Fig. 4). These results provide compelling evidence in support of a direct role for actin in vacuole inheritance.

Actin Filaments Used for Vacuole Inheritance Are Dynamic Structures

During zygote formation in S. cerevisiae, a portion of each parental vacuole is transferred to the bud. Although the parental vacuoles never fuse directly, their segregation structures join in the bud and their contents mix (55). We exploited this phenomenon to gain a better understanding of the nature of the actin filaments involved in vacuole inheritance. Zygotes formed from the homozygous mating of two act1-ΔDSE strains fail to transfer the vacuole to the bud (Table III). If vacuole transfer requires stable actin filaments that are pre-existing in each parental strain, then in
a heterozygous mating (act1-ΔDSE × ACT1) vacuole transfer should remain defective in the act1-ΔDSE portion of the zygote. However, if the actin in ΔDSE filaments can exchange with wild-type actin, then wild-type actin should rescue the act1-ΔDSE inheritance defect. Zygotes from heterozygous matings show primarily wild-type vacuole transfer (Table III), indicating that actin from the wild-type parent can assemble into filaments in the act1-ΔDSE parent. This finding is correlated with in vitro studies which demonstrate that copolymerization of wild-type actin and ΔDSE actin increases the ability of the copolymer (relative to the ΔDSE polymer) to activate myosin S1-ATPase activity (8). Moreover, these findings suggest that actin filaments in yeast cells are in dynamic equilibrium with monomeric actin (see Discussion).

**The Organelle Inheritance Defect of act1-ΔDSE Is Specific for the Vacuole**

Cell proliferation requires proper segregation of several types of cytoplasmic organelles. It has recently been reported that actin is required for mitochondrial (45) as well as nuclear (10, 38) inheritance in *S. cerevisiae*. The distribution of these organelles between mother cells and buds was examined in act1-ΔDSE and found to be qualitatively similar to wild-type (not shown). Furthermore, quantitative analysis of nuclear inheritance as a function of bud size verified that the time of nuclear migration into the bud was normal in act1-ΔDSE (not shown). Thus, actin’s role in vacuole inheritance can be distinguished from its role in the inheritance of other organelles. These findings are consistent with earlier observations that many mitochondrial inheritance mutants exhibit normal vacuole inheritance, and likewise, that mitochondrial inheritance is normal in several vacuole partitioning mutants (34, 44, 58).

**The Vacuole Inheritance Defect in act1-ΔDSE Is Not Due to Mislocalization of Vacuolar Proteins**

We and others have reported the isolation of vacuole in-
inheritance mutants (44, 53, 58). A subset of these mutants exhibit defects in vacuole protein sorting (58). Likewise, several mutants isolated on the basis of defects in vacuole protein sorting were subsequently found to be defective in vacuole inheritance (40, 41). The vacuole protein sorting pathway overlaps with the secretory pathway and endocytosis. Moreover, actin functions in both secretion and pathway overlaps with the secretory pathway and endocytosis and the total number of zygotes scored is indicated in parentheses. The vacuole inheritance defect in heterozygous zygotes. Moreover, actin functions in both secretion and pathway overlaps with the secretory pathway and endocytosis. Furthermore, actin functions in both secretion and pathway overlaps with the secretory pathway and endocytosis.

**Table III. Wild-type Actin Rescues the act1-ΔDSE Vacuole Inheritance Defect in Heterozygous Zygotes**

| Cross                        | %   | %   | %   |
|------------------------------|-----|-----|-----|
| LWY1412 × LWY1425 (n = 115)  | 89  | 6   | 5   |
| LWY1419 × LWY1408 (n = 111)  | 1   | 0   | 99  |
| LWY1412 × LWY1419 (n = 142)  | 70  | 13  | 17  |
| LWY1419 × LWY1412 (n = 96)   | 73  | 18  | 9   |

For each cross the strain written first was labeled and then mated with an unlabeled strain of the opposite mating type. The table shows the percentage of zygotes with the indicated distribution of labeled vacuoles. Each cross was performed two to three times and the total number of zygotes scored is indicated in parentheses.

The vacuole inheritance defect in act1-ΔDSE cells is not due to mislocalization of vacuolar proteins. Spheroplasts from log-phase cultures of wild-type and act1-ΔDSE cells were pulse-labeled with Trans35S-label for 10 min and then chased for 30 min as described in Materials and Methods. Aliquots taken before (P) and after (C) the chase period were separated into intracellular (I) and extracellular (E) fractions by centrifugation, and then precipitated with trichloroacetic acid. Carboxypeptidase Y was immunoprecipitated from each fraction and analyzed by SDS-PAGE 11% and fluorography. p1, p2, and mCPY refer to the ER-modified, Golgi-modified, and mature forms of carboxypeptidase Y, respectively. A similar analysis of Proteinase A synthesis revealed that it too is processed normally in act1-ΔDSE cells.

**A Class V Myosin Functions in Vacuole Inheritance**

To further characterize the role played by actin in vacuole partitioning, we examined yeast strains carrying mutations in genes that encode known actin-binding proteins. We were particularly interested in the class V unconventional myosins, which are actin-based molecular motors implicated in organelle transport in a variety of organisms (47). Two class V myosins (Myo2p and Myo4p) have been identified in *S. cerevisiae* (17, 23). Both of these proteins function in the asymmetric localization of cellular components between the mother cell and the bud. The Myo4 gene product is required for the daughter cell-specific localization of Ash1p, a negative regulator of HO expression (22). Myo2p activity is required for polarized growth (7, 15, 23, 32). Time course studies with a temperature-sensitive Myo2 mutant (myo2-66) showed that a short shift to the restrictive temperature leads to the accumulation of small cytoplasmic vesicles, 80–100 nm in diameter, that are specifically localized to the mother cell and are not present in the bud (15, 23). Paradoxically, under these same conditions, many secreted and vacuolar proteins are properly targeted with wild-type kinetics, indicating that transport of many secretory pathway vesicles is unimpaired. It was concluded that Myo2p function is required for transporting a subset of cytoplasmic vesicles specifically from the mother cell into the bud, although the cargo in these vesicles remains unknown (15). As described below, our current studies indicate that Myo2p is also required for the transport of the vacuole into the bud.

The myo2-66 mutation encodes an amino acid substitution in the actin-binding domain of Myo2p (32). As shown in Table IV, this mutation is associated with a defect in vacuole inheritance (even at the permissive temperature). This defect is rescued by transformation with the wild-type MY02 gene. No vacuole inheritance defect was observed in a myo4Δ deletion mutant (not shown). Most myo2-66 cells exhibit a normal actin cytoskeleton at the permissive temperature.

**Table IV. A Class V Myosin Is Required for Vacuole Inheritance**

| Strain                      | %   | %   | %   |
|-----------------------------|-----|-----|-----|
| JP7A (myo2-66) (n = 50)     | 8   | 6   | 86  |
| LWY1656 (myo2-66 + pMYO2) (n = 68) | 74  | 13  | 13  |
| LWY1657 (myo2-66 + pMYO2) (n = 68) | 71  | 12  | 17  |

Vacuole inheritance was examined as described in Fig. 3. LWY1656 and LWY1657 are independent Ura+ transformants generated by transformation of JP7A with the wild-type MYO2 gene on a URA3, CEN plasmid (23), and were grown in SC-ura medium before labeling. Random fields of cells were examined and the percentage of cells with the indicated inheritance phenotype is shown. The total number of cells examined is given in parentheses.
temperature (23, 32) (Catlett, N.L., and L.S. Weisman, unpublished observations), yet nearly 100% display a vacuole inheritance defect. Therefore, the myo2-66 mutation does not simply perturb vacuole transport by grossly disrupting the actin cytoskeleton.

Consistent with a role for myosin in vacuole partitioning, is the observation that three actin mutations which impair vacuole inheritance (actl-ΔDSE, actl-101, and actl-135) specify changes in regions of actin that are important for myosin binding (46, 48). Moreover, we observed genetic interactions between actl-ΔDSE and myo2-66. When we sought to construct a myo2-66, actl-ΔDSE double mutant, only one complete tetrad germinated out of 35 potential tetrads. Two of the spores in this tetrad contained the myo2-66 mutation alone and two spores contained actl-ΔDSE alone. In most of the incomplete tetrads, we could unambiguously surmise the genotype of the missing strains, and these were predicted to have the genotype actl-ΔDSE, myo2-66. One strain, obtained from an incomplete tetrad, was actl-ΔDSE, myo2-66. However, this strain grew extremely poorly even at 24°C. We were able to successfully construct a heterozygous diploid from this strain that yielded meiotic progeny with single mutations, either actl-ΔDSE alone or myo2-66 alone. However, again none of the new progeny were actl-ADSE, myo2-66 double mutants. Hence, the myo2-66 and actl-ΔDSE mutations are at least synthetically sick, if not synthetically lethal. This synthetic interaction is consistent with the hypothesis that Myo2p interacts with the amino terminus of actin (46), and supports the idea that the vac defect observed in myo2-66 relates to Myo2p functioning in conjunction with actin.

In small budded cells, much of the Myo2p is localized to the tips of emerging buds (7, 32). To further explore the relationship between Myo2p and vacuole movement, we sought to determine where Myo2p resides in the cell, relative to vacuole membranes. Double label immunofluorescence studies were performed on yeast zygotes because of the large volume occupied by vacuoles, which extend all the way to the tip of the small budded cell, double label immunofluorescence studies were performed with DBY1398, the wild-type strain used in our earlier studies (Fig. 7). We observe that, as found in our current wild-type strains, the Myo2p cap is located at the site of bud emergence and on tips of small buds. In cells with fully extended segregation structures, colocalization of the vacuole with Myo2p occurs in the bud tip as demonstrated in other strains (not shown, see Fig. 6). Interestingly, Myo2p also localizes to the tip of the extending segregation structure (arrowhead in Fig. 7). This localization of Myo2p on both the tip of the segregation structure and the bud tip is consistent with a role for Myo2p in both the directed transport of vacuoles and other vesicles into the bud. Note that there is some vacuolar membrane in the bud, but it is likely that this would not have been detected with the fluorescein derivatives (39, 57) used in previous studies.

**Actin-Profilin Interactions Are Important for Vacuole Inheritance**

Profilin is an actin-binding protein that is an important regulator of actin filament assembly. Two of the actin alleles associated with vacuole inheritance defects (actl-101 and actl-111) have been shown to inhibit the interaction of actin with profilin in vivo, via the “two-hybrid” assay (4). Furthermore, Glu364 (which is changed to Ala in actl-101) contacts profilin in the actin profilin cocrystal structure (43) and has been cross-linked to profilin in vitro (49). The overlap between amino acids in actin that are important for both vacuole partitioning and profilin binding, suggests that actin-profilin interactions may be important for the function of actin in vacuole inheritance. We examined a yeast strain (BHY31) carrying a mutation in the profilin gene. The pfy1-112 mutation specifies an amino acid substitution (R76G) (18) in a region of profilin that contributes to one of the primary contacts between profilin and actin (43). This mutation gives rise to a vac phenotype (Fig. 8) without disrupting actin localization or producing any other discernible phenotypes (18). It is not immediately clear what role profilin might play in vacuole partitioning. However, given the dynamic nature of actin filaments used for vacuole transfer (see above), the role of profilin in regulating filament assembly may be critical to the function of these filaments in vacuole inheritance.

The crystal structure of the bovine profilin-actin complex demonstrates two major contact sites (43). One contact includes a salt bridge between R74 of bovine profilin (analogous to R72 or R76 of yeast profilin based on sequence alignment) and the COOH-terminal carboxylate group of actin. The other contact includes a salt bridge between K112 of profilin and E364 of actin. Interestingly, the pfy1-112 mutation (R76A) alters a profilin amino acid that participates in the first salt bridge, while the actl-101 mu-
Figure 6. Specific regions of the vacuole colocalize with Myo2p. Wild-type zygotes (from mating of X2180-1A and X2180-1B, Yeast Genetic Stocks Center) were prepared for immunofluorescence and stained with anti-Myo2p (green) and anti-60-kD vacuolar ATPase (red) antibodies in order to simultaneously visualize Myo2p and the vacuolar membrane (see Materials and Methods). (A) 60 kD ATPase, (B) Myo2p, (C) combined image; regions where the vacuole membrane and Myo2p overlap appear yellow-green or yellow. The arrowheads in C and D indicate four examples of small buds with the Myo2p cap clearly colocalizing with a portion of the vacuole membrane. Arrows in C and D point to examples of small Myo2p spots along the vacuole membrane. (D) Enlarged view of zygote from (C). Bars: (C) 10 μm. (D) 2.5 μm.
Division Is Mediated by the Actin Cytoskeleton

rowhead indicates the segregation structure. Bar, 5 μM.

Discussion

Transfer of the Yeast Vacuole into the Bud during Cell Division Is Mediated by the Actin Cytoskeleton

In the present work we provide the first demonstration that a specific cytoskeletal component, actin, is required for vacuole transport in dividing yeast cells. Since a variety of functions have previously been ascribed to the actin cytoskeleton of S. cerevisiae (10, 29, 37), it is important to differentiate between a direct or indirect role for actin in vacuole inheritance. Several lines of evidence support a direct role. (a) Most of the actin mutants that have vacuole inheritance defects exhibit normal morphology and bud growth. (b) In the act1-ΔDSE mutant the vacuole inheritance defect occurs in the absence of any defects in vacuole protein sorting or F-actin localization. (c) Wild-type actin rapidly suppresses the act1-ΔDSE inheritance defect in heterozygous zygotes. (d) This suppression correlates with the ability of wild-type actin to attenuate defects of ΔDSE actin in vitro (8). (e) The time and position of vacuole transfer coincide with the formation of actin cables during the cell cycle (27). (f) Indeed, double label immunofluorescence studies show a close association of vacuole membranes and actin cables. (g) The rate of vacuole movement, 0.1–0.2 μm/s (Weisman, L.S., unpublished observation), is consistent with rates of other actin-based motility events (6, 30). We therefore suggest that actin functions directly in vacuole inheritance, presumably serving as a track that allows for the precise delivery of the vacuole first to the site of bud emergence, and then from the mother cell into the bud.

Vacuole Inheritance Is Defective in a Variety of Actin Mutants

Several act1 mutants exhibit a vacuole inheritance defect (Table II). When considered in the context of available structural data, these results offer insight into the mechanism by which actin facilitates vacuole inheritance. For example, act1-ΔDSE, act1-101, and act1-135 each alter residues that are important for actin-myosin interactions (46, 48). The E364A substitution in act1-101 is also predicted to destroy one of two primary contact sites between actin and profilin (43), and has recently been shown to impair actin-profilin interactions in a two-hybrid assay (4). The vac phenotypes of these mutants might therefore arise from the requirement for myosin and profilin function in vacuole inheritance. The act1-102 and act1-111 mutations also block the binding of profilin to actin in a two hybrid assay (4). While act1-111 displays a vac phenotype, act1-102 does not. This presumably reflects a difference in the
requirements for interaction in the two-hybrid assay vs function in vacuole inheritance. In this regard, it is worth noting that the act1-102 mutation also does not exhibit the temperature-sensitive growth phenotype that is associated with other mutations at profilin contact sites (59).

The act1-105 mutation has previously been shown to block interactions between actin and Srv2p, a bifunctional protein that functions in Ras-mediated signal transduction and actin cytoskeleton organization (4, 11, 12, 52). The vac phenotype of this mutant may represent further demonstration of the functional overlap between Srv2p and profilin (52). Finally, although the amino acids altered by the act1-133 mutation have not specifically been implicated in binding to any particular protein, this mutation disrupts actin cables and is associated with defects in mitochondrial localization and motility (10, 45). Hence, the vac phenotype associated with act1-133 may result from a more general defect in the actin cytoskeleton.

Actin functions in a wide variety of cellular processes. These activities generally require interactions with specific proteins that use overlapping and/or distinct binding sites on the actin molecule (4). Therefore, it is not surprising that vacuole inheritance is normal in some mutants with defects in other membrane trafficking pathways (e.g., end7-1) or that exhibit temperature sensitive growth (e.g., act1-121 and act1-122). Likewise, it is to be expected that some actin mutations that block vacuole inheritance do not lead to other discernible phenotypes (e.g., act1-135 in Table II).

**Actin Cables Colocalize with Vacuole Membranes**

Simultaneous indirect immunofluorescence localization of both filamentous actin and vacuole membranes reveals that many actin cables within the mother cell are associated with vacuole membranes. This association is most prominent immediately before and during vacuole partitioning. Notably, in unbudded cells with actin cortical patches organized at the site of bud emergence, a portion of the mother vacuole is also localized to that area. It appears that this region of the vacuole then transits into the bud as the bud emerges. These observations of the location of the vacuole just before bud emergence, are quite similar to what has been observed for other endomembranes (for review see reference 9). Colocalization of actin with the vacuole membrane corroborates the genetic evidence suggesting that actin is directly involved in vacuole inheritance.

**Myo2p Is Involved in Vacuole Inheritance**

Myo2p is a class V unconventional myosin that is required for polarized growth in S. cerevisiae (23). This requirement has been attributed to its proposed role in the transport of small cytoplasmic vesicles from the mother cell into the bud (15, 23). Several observations suggest that Myo2p also functions in vacuole inheritance. (a) A temperature-sensitive mutation in the actin-binding domain of Myo2p blocks vacuole inheritance, even at the permissive temperature. This defect is observed in essentially all cells and is not due to a disruption of the actin cytoskeleton. (b) The myo2-66 mutation does not affect other actin-dependent membrane trafficking pathways known to intersect with the vacuole

![Figure 9. Suggested model for actin-mediated vacuole inheritance in S. cerevisiae. In an unbudded cell (top) the vacuole (gray) is an oval, lobed structure and is associated with actin cables (black lines) (see Fig. 4). Myo2p (black, multi-lobed structure) may be acting as a motor, attached to both the vacuole and actin cables. (Some of the Myo2p is distributed in the mother cell and is often found colocalized with vacuole membrane [Figs. 6 and 7]). Most of the Myo2p is found in a cap at the site of bud emergence (7, 32) (Figs. 6 and 7). Cortical actin patches (small dark balls) are found at the plasma membrane and are present at the site of bud emergence (10). As the bud emerges, actin cables extend into the bud, as does the vacuole. Also at this time, the vacuolar segregation structure forms and may be transported by myosin along the actin filaments. As the bud enlarges, vacuole transfer continues until nuclear migration begins (not shown). For simplicity, other organelles are not depicted, also actin cortical patches in the small bud are not shown. For a discussion of other roles for Myo2p, see Results and Discussion.](image-url)
and also in small budded cells. This is based on the observation that vacuoles are juxtapositioned with both actin cortical patches (Fig. 4) and Myo2p caps (Fig. 6) at the site of bud emergence in as yet unbudded cells. One question that arises is why does Myo2p accumulate at caps in the bud? It may be that this location corresponds to other functions of Myo2p, which is also required for polarized growth (15, 23). Alternatively, perhaps these caps do not reflect the site of Myo2p function, but rather are the site that Myo2p resides after it has brought its cargo both to the site of bud emergence and also into the emerging bud. Unlike microtubule-dependent motility, all actin-dependent myosin-mediated vesicle movements characterized to date occur exclusively toward the barbed end of actin filaments (31). In this scenario, Myo2p would be moving along actin cables that have a single polarity and thus allow movement in a single direction. Once Myo2p arrives at the bud tip, it would not be able to go in the reverse direction. An alternative model to explain the location of Myo2p caps is that the protein is not working as a conventional motor, but rather is forming a site for the attachment and organization of actin cables and vacuoles. Our finding that Myo2p is found on the mother cell vacuole membrane is more consistent with the first model.

We also cannot rule out the possibility that Myo2p functions to target another protein to a specific subcellular location, and that proper targeting of this protein is required for partitioning of the vacuole and for polarized growth. Such a model would be consistent with the observation that myo2-66 is synthetically lethal with several late-acting sec mutations (15, 33). However, we favor the idea of a direct role for the reasons discussed above, and because the targeting of several proteins to a variety of subcellular locations have all been shown to be normal in myo2-66, even at the restrictive temperature (15, 23).

In a detailed analysis of the myo2-66 mutant by electron microscopy, Govindan and coworkers observed small (80–100 nm diameter) vesicles that accumulate in mother cells (but not in buds) after a short shift to the restrictive temperature (15). No secretory defect was observed and the identity of the vesicles could not be determined. Because early (but not late) SEC genes are required for their formation, the authors speculated that the 80–100-nm vesicles represent a subset of late secretory vesicles, or that they result from a defect in the inheritance of the Golgi complex. Based on those observations and our results presented here, we suggest that the latter possibility be explored further. Myo2p-66 vesicles correspond to a heritable unit of the organelles in the secretory pathway (e.g., the Golgi) and Myo2 function is required for targeting them specifically to the bud. The fact that transport of the 80–100-nm vesicles is blocked only at the restrictive temperature, while vacuole inheritance is defective at the permissive temperature may reflect their relative sizes, since transport of the vacuole is expected to be more physically demanding than transport of much smaller vesicles.

**Actin Filaments That Function in Vacuole Transport Are Dynamic Structures**

Examination of vacuole inheritance in newly formed zygotes allowed us to probe the capacity of the yeast actin cytoskeleton to undergo dynamic rearrangements during the cell cycle. In wild-type yeast zygotes, portions of each parental vacuole are transferred to the bud as well as to the other parent (55). This vacuole transfer event occurs normally in act1-ΔDSE/ACT1 zygotes, but not in act1-ΔDSE/act1-ΔDSE zygotes (Table III). Normal vacuole transfer in the heterozygote indicates that actin from the wild-type parent is able to assemble into filaments in the act1-ΔDSE parent. This actin is probably derived from a pre-existing source in the wild-type parent rather than from de novo expression of the wild-type gene, since the defect is rescued shortly after zygote formation and it is unlikely that gene expression in the newly formed diploid nucleus has contributed enough actin to rescue the defect. This argument is supported by the fact that many other recessive vac mutations are not rescued in this assay (53, 58).

Recently, Karpova and coworkers examined the relative abundance of filamentous and monomeric actin in yeast cells (26). They discovered that the majority of actin in wild-type cells exists as polymerized filaments, and that the steady-state level of free actin monomers is very low. Our zygote studies therefore imply that yeast actin filaments can be disassembled into soluble monomers that are able to diffuse into both parents of the zygote and be incorporated into filaments required for vacuole transfer. The coordination of vacuole transfer with the cell cycle implies that actin filament rearrangement is a regulated process and therefore protein-mediated. If this is indeed the case, it might explain the involvement of profilin in vacuole inheritance (Fig. 8), since profilin is thought to regulate actin filament assembly.

**Summary**

We have demonstrated the involvement of actin and myosin in vacuole inheritance. In addition to providing insights into the molecular mechanisms of this process, our results present a new in vivo assay for monitoring actin and myosin function. Unlike other functions ascribed to yeast actin (e.g., endocytosis and secretion), vacuole partitioning can be easily visualized in living cells. Moreover, the distance that the vacuole moves in yeast zygotes, over 5 μm, suggests that even subtle differences in rates of movement may be detected.

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