Synthetic Lethality Screen Identifies a Novel Yeast Myosin I Gene (MY05): Myosin I Proteins Are Required for Polarization of the Actin Cytoskeleton

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Abstract. The organization of the actin cytoskeleton plays a critical role in cell physiology in motile and non-motile organisms. Nonetheless, the function of the actin based motor molecules, members of the myosin superfamily, is not well understood. Deletion of MY03, a yeast gene encoding a “classic” myosin I, has no detectable phenotype. We used a synthetic lethality screen to uncover genes whose functions might overlap with those of MY03 and identified a second yeast myosin I gene, MY05. MY05 shows 86 and 62% identity to MY03 across the motor and non-motor regions. Both genes contain an amino terminal motor domain, a neck region containing two IQ motifs, and a tail domain consisting of a positively charged region, a proline-rich region containing sequences implicated in ATP-insensitive actin binding, and an SH3 domain. Although myo5 deletion mutants have no detectable phenotype, yeast strains deleted for both MY03 and MY05 have severe defects in growth and actin cytoskeletal organization. Double deletion mutants also display phenotypes associated with actin disorganization including accumulation of intracellular membranes and vesicles, cell rounding, random bud site selection, sensitivity to high osmotic strength, and low pH as well as defects in chitin and cell wall deposition, invertase secretion, and fluid phase endocytosis. Indirect immunofluorescence studies using epitope-tagged Myo5p indicate that Myo5p is localized at actin patches. These results indicate that MY03 and MY05 encode classical myosin I proteins with overlapping functions and suggest a role for Myo3p and Myo5p in organization of the actin cytoskeleton of Saccharomyces cerevisiae.

The myosin superfamily of molecular motors encompasses at least eleven different classes of proteins (Cheney et al., 1993; Sellers and Goodson, 1995). While the conventional myosin (myosin II) has been studied in great detail in both muscle and nonmuscle cells, comparatively little is known about most unconventional myosin proteins. Myosin I proteins were the first unconventional mysins discovered (Pollard and Korn, 1973; Hammer et al., 1986). Members of the myosin I class have now been identified in phylogenetically diverse organisms (for review see Sellers and Goodson, 1995), suggesting that they are ubiquitous, ancient proteins with a central role in eukaryotic cell biology.

Myosin I proteins can be divided into at least two subclasses based on homologies in motor and tail domains (Hasson and Mooseker, 1995). “Classic” myosin I proteins have been found in fungi, ameboid cells, and metazoa (for review, see Sellers and Goodson, 1995). These proteins possess tail domains containing a positively charged region implicated in membrane binding, a proline-rich region, and an SH3 domain (for review, see Hammer, 1991). The proline-rich region contains sequences implicated in ATP-insensitive actin binding (Lynch et al., 1986; Doberstein and Pollard, 1992; Jung and Hammer, 1994; Rosenfeld and Rener, 1994). Between the motor and tail domains is a “neck” region containing one or two “IQ” motifs, sequences expected to bind to calmodulin or calmodulin-related myosin light chains (Cheney and Mooseker, 1992). In contrast, the brush border myosin I’s and related proteins have tails with only the putative membrane binding region and necks with 3–6 IQ motifs. Proteins clearly related to the brush border myosins have been found only in metazoa, although similar proteins exist in ameboid cells (reviewed by Sellers and Goodson, 1995).

The function of myosin I proteins is not well understood. One model proposes that myosin I proteins bind to both microfilaments and organelles and use the energy of ATP hydrolysis to drive organelle movement along actin tracks (Adams and Pollard, 1989). Alternatively, myosin I proteins could act to connect the plasma membrane to the...
actin cytoskeleton and drive movements of the cytoskeleton against the membrane. A classic myosin-I–specific variation of this model proposes that the second, ATP-insensitive, actin-binding site in the tail could be used to crosslink actin filaments and allow myosin-I to drive F-actin sliding on actin filaments, thus altering actin organization at the membrane interface.

Experimental observations obtained thus far are consistent with all of these proposed functions. In Dictyostelium, myosin I proteins have been implicated in cell motility, phagocytosis, and pseudopod formation (Jung and Hammer, 1990; Wessels et al., 1991; Jung et al., 1993; Titus et al., 1993). Syringle loading of inhibitory antibodies indicates that one myosin I protein, Acanthamoeba 1C, is essential for the function of the Acanthamoeba contractile vacuole (Doberstein et al., 1993). Finally, recent studies in Aspergillus nidulans indicate that the MyoA gene encodes an essential myosin I required for secretion and polarized growth (McGoldrick et al., 1995).

Previous studies identified in Saccharomyces cerevisiae a gene that encodes a classic myosin I protein, MYO3. Deletion of this gene had no discernible phenotypic effects under laboratory conditions, presumably for reasons of functional redundancy (Goodson and Spudich, 1995). However, deletion of MYO3 in combination with mutations of other known yeast myosin genes (MYO1, a myosin II, MYO2, a myosin V, or MYO4, a myosin V) has no detectable effect (Goodson and Spudich, 1995; Haarer et al., 1994; Lilie, S.L., and S.S. Brown, unpublished results). These findings indicate that the redundant protein is not one of the known yeast myosins. We used a genetic screen to identify mutations that create a requirement for MYO3. This strategy, termed a synthetic lethality screen, has been useful for identification of genes that are involved in a common process (for example, see Bender and Pringle, 1991). We present results of such a screen and demonstrate that it identifies a new yeast myosin I gene, MYO5. We demonstrate that yeast strains deleted for both MYO3 and MYO5 have a severe defect in growth, actin polarization, and actin-dependent processes including secretion, endocytosis, and polarity establishment. We suggest that the primary defect in the mutants bearing classic myosin I deletions is abnormal polarization of the actin cytoskeleton.

Materials and Methods

Yeast And Bacterial Manipulations

Yeast manipulations including cell culture, transformation, and tetrad analysis were carried out according to Guthrie and Fink (1991). Bacterial manipulations were carried out according to Sambrook et al. (1989).

Synthetic Lethality Screen

myo5 mutants were isolated using a synthetic lethal strategy based on selection against the URA3 gene. A yeast strain (HA10-1c) containing a deletion of MYO3 (Table I) was transformed with a centromere-based, pRS316-derived plasmid (P316SRMYO3) which contains the URA3 marker and the full coding region of MYO3 (Sikorski and Hieter, 1989). Transformants were mutagenized with ethylmethane sulfonate (EMS) until only ∼25% of the cells were viable (Lawrence, 1991). Transformants were replica plated to uracil-free plates and to plates containing 5-fluoro-

Cloning and Sequence Analysis

The MYO5 gene was cloned by complementation of mutant 37 with a plasmid library. The library consisted of genomic yeast DNA partially digested with Sau3A and sub-cloned into a centromere-based, LEU2-containing shuttle vector derived from YCP50 (Christianson et al., 1992; American Type Culture Collection, Rockville, MD; ATCC No. 77162). Of ∼12,000 Leu+ transformants, 46 colonies were no longer dependent upon retention of p316SRMYO3 (the MYO3-containing plasmid marked by URA3) as assayed by survival of these colonies at 30°C on solid, unbuffered minimal medium (SD) containing 5-FOA. Library plasmids were isolated from 40 of these colonies (Ward, 1990). 29 contained the MYO3 gene and 11 contained a different set of overlapping inserts which conferred 5-FOA resistance to synthetic lethal mutants. The DNA sequence of this insert was determined as described previously (Goodson and Spudich, 1995). Sequence analysis of the region which conferred FOA resistance revealed a new myosin I gene, MYO5. Similar results were obtained by complementation of mutant C11 with the same library. Sequences were analyzed with the GCG Package (Genetics Computer Group, Inc., Madison, WI).

Disruption of MYO5

A strain of yeast missing all of the MYO5 coding sequence (as well as 95 bases of 5' noncoding sequence) was created by a "delta deletion" (Sikorski and Hieter, 1989). 5' and 5' noncoding sequence of the MYO5 locus were inserted into the integrating plasmid pRS304 in a direct orientation to create the plasmid p304KO3 (see Fig. 1). p304KO3 was linearized with Sphi to cut between the 3' and 5' inserts and transformed into the diploid yeast strains CRY3 and HA20. Southern blot analysis revealed that sequences between the 3' and 5' inserts on one chromosome of the transformant were replaced by the plasmid DNA. Haploid strains bearing the MYO5 deletion were isolated by tetrad dissection. The Trp+ phenotype marking the deletion segregated 2:2 in both the CRY3 and HA20 transformants and was tightly linked to growth defects in the HA20 transformants (data not shown).

Construction of Epitope-tagged Myo5p

Myo5p was epitope tagged by insertion of three copies of the 11 amino acid epitope from human c-myc (Evan et al., 1985) at the extreme COOH terminus of the protein. To do so, the stop codon of MYO5 was replaced with a BamHI site using PCR. The 3' end of the modified MYO5 gene was then excised using BamHI and BstEII fragment which consists of the remainder of the MYO5 gene and 1.8 kb of its 5' noncoding region containing the MYO5 promoter region. The ligated products were digested with SpeI and BamHI, and subcloned into the KS Bluescript vector (Stratagene, La Jolla, CA). Three copies of the myc tag coding sequence were excised from the pKK-1 plasmid with BamHI and inserted in frame at the BamHI site at the 3' end of the MYO5 gene. An Nhel linker (New England Biolabs Inc., Beverly, MA) containing a stop codon in all three reading frames was inserted immediately downstream from the myc tag. The entire construct was excised with Sacl and XhoI and sub-cloned into the centromere-based yeast shuttle vector, pRS-Y2, a derivative of pRS316 which contains the multiple cloning site of pYES2 (Invitrogen, San Diego, CA). This construct is referred to as pRS5-Y2-myc-MYO5. A construct containing untagged MYO5 and 1.8 kb of its 5' noncoding region (pRS5-Y2-MYO5) was produced using a similar approach.

Light Microscopy

Actin cytoskeletal structure and chitin deposition were visualized using rhodamine-phalloidin, a rabbit polyclonal antibody raised against yeast actin (Duhub et al., 1988), and Calcofluor (Sigma Chemical Co., St. Louis, MO) according to published procedures (Adams et al., 1991; Pringle et al., 1991). Cell viability was determined using the FUN-1 cell stain (Molecular
Probes, Eugene, OR) according to manufacturer’s instructions. Other methods (fixation, mounting, DAPI staining) were as described by Pringle et al. (1991). Photomicroscopy was performed on a Leitz Dialux microscope using a 100× (NA 1.4) objective (Rockleigh, NJ). Images were collected using a cooled CCD camera (model # Star-1; Photometrics, Tucson, AZ). Light output from the 100W Mercury Arc lamp was controlled using a shutter driver (model # Uniblitz D122; Vincent Associates, Rochester, NY) and attenuated using neutral density filters (Omega Optical Corp., Brattleboro, VT). Image enhancement and analysis were performed on a Macintosh Quadra 800 computer (Cupertino, CA) using the public domain program NIH Image 1.55. Images were stored on a magnetic optical disk drive (Peripheral Land Inc., Fremont, CA).

Confocal images of cells were obtained with a laser scanning confocal microscope (model # MRC 600; Bio-Rad Microscience, Cambridge, MA) and Adobe Photoshop 2.5 (Mountain View, CA).

**Electron Microscopy**

Preparation of samples for transmission electron microscopy was carried out according to Stevens (1977). Yeast were fixed by addition of glutaraldehyde (Sigma Chemical Co.) to growth medium to a final concentration of 5%. After incubation for 3 h at room temperature, cells were concentrated by centrifugation (10,000 g, 10 min, room temperature), and washed two times with 0.9% NaCl. Samples were resuspended in 4% KMnO4 in 0.1 M Na-cacodylate, pH 7.4 (Electron Microscopy Sciences, Fort Washington, PA), and incubated at 4°C for 1 h with gentle rotation. After two washes with 0.9% NaCl, samples were resuspended in 2% uranyl acetate (Electron Microscopy Sciences) and incubated for 1 h at room temperature. Samples were washed three times, dehydrated in a graded series of ethanol solutions, infiltrated with propylene oxide for 10 min, and embedded in Epon-812 (Tousimis Research Co., Rockville, MD). Ultrathin sections were stained for 5 min with 1% lead citrate before viewing with a JEOL 1200 transmission electron microscope.

**Invertase Secretion Assay**

Secreted invertase was assayed as described (Goldstein and Lampen, 1975) in intact cells. Cells were grown to log phase in YEP media containing 2% glucose. Cultures were washed with YEP supplemented with 0.05% glucose, resuspended in this media, and incubated at 30°C with constant shaking. At the times indicated, cell densities were determined by OD600 measurements, and aliquots were removed from the culture. Cells were washed with ice cold 10 mM NaNO3, resuspended in 10 mM NaN3, and stored on ice until all aliquots had been collected. A portion of each aliquot containing equal numbers of cells was added to 100 mM sodium acetate, pH 5.1, and 125 mM sucrose and incubated for 20 min at 37°C. Reactions were terminated by addition of 2.5 volumes 200 mM K2HPO4 and stored on ice. An aliquot from each terminated reaction was diluted with 4 volumes 200 mM K2HPO4 and boiled for 5 min to destroy invertase activity. The quantity of glucose in each sample was determined by addition of Glucostat reagent (100 mM KPi, pH 7.0, 20 μg/ml glucose oxidase, 2.5 μg/ml peroxidase, and 15 μg/ml O-diaisidine) and incubated at 37°C for 30 min. Color development was initiated by addition of one volume 6 M HCl and measured using a spectrophotometer (model DU-50; Beckman Instruments, Fullerton, CA) at 540 nm. Levels of glucose released were calculated by comparing absorption readings to a standard curve. Units of invertase activity are defined as micromoles of glucose released/min/10 mg cell (dry weight), assuming that 1 OD600 equals 0.19 mg cells/ml.

**Lucifer Yellow Uptake Assay**

The uptake of Lucifer yellow CH was performed according to Riezman (1985) with slight modifications. Cells were grown to log phase in YEP + 2% glucose and Lucifer yellow CH (Sigma Chemical Co.) was added to 4 mg/ml. After 60 min incubation at 30°C with constant agitation, cells were washed three times with ice cold 50 mM succinate, pH 5.0, 100 mM NaCl, 10 mM MgCl2, 20 mM NaN3. An aliquot of each sample was embedded in 0.8% low melt agarose on a glass slide and placed under a cover slip. Optical images were collected using the Leitz Dialux microscope as described above. Cells were scored as positive if the vacuole was clearly stained with Lucifer yellow.
Results

Synthetic Lethality Screen Identifies a New Yeast Myosin Gene, MY05

Yeast deleted for the classic myosin I gene MYO3 have no discernible defects, suggesting that another protein(s) has overlapping function with Myo3p (Goodson and Spudich, 1995). We conducted a synthetic lethality screen to identify such proteins. This genetic screen is based on the principle that yeast bearing mutations of either of two genes with overlapping function will remain viable. However, yeast mutated at both genes should have a defect in a basic biological function and are expected to die (Huffaker et al., 1987). Yeast carrying a chromosomal deletion of MYO3 and a plasmid which contains a functional MYO3 gene were mutagenized. Mutants which die if forced to lose the plasmid-borne MYO3 were then identified by replica plating mutagenized colonies onto solid media containing 5-FOA. 5-FOA kills cells expressing the URA3 gene, a nutritional marker present on the MYO3-containing plasmid. Therefore, cells that cannot lose the MYO3-containing plasmid will not grow on 5-FOA.

Three mutant strains (C11, 37, and D2) which were dependent on the MYO3 plasmid were produced using this synthetic lethal screen. Dependence of these strains on MYO3 was demonstrated in three ways. First, the mutants were transformed with a second plasmid (p314SRMYO3) containing the MYO3 gene and a different selectable marker (TRP1). These strains were now able to grow on 5-FOA. In addition, plasmid loss assays indicate that these transformants could lose either MYO3-bearing plasmid, but not both. Second, mutants containing the MYO3 gene under control of the galactose promoter displayed a galactose dependence for growth. Finally, a mutant form of p314SRMYO3 containing a MYO3 gene with a frame shift in the tail of MYO3 (Fig. 1) will not support growth of the mutant. Backcrossing of the synthetic lethal mutants to the parent strain indicated that the mutations were recessive. Complementation tests indicated that all three synthetic lethal strains were in the same complementation group.

A new yeast myosin gene, MYO5, was cloned by complementation of the synthetic lethal phenotype. In complementation studies, this gene was also identified by PCR using degenerate, myosin I-specific oligonucleotides. The sequence of Myo5p (Fig. 2) shows that MYO5 encodes a classic myosin I protein, like MYO3. It is 76.6% identical to MYO3 over the whole protein, 87 and 62% identical in the head and tail, respectively. All of the motor and tail domains expected from a classic myosin I and identified in the tail of MYO3 are also found in MYO5, including a basic (putative membrane-binding) region, an SH3 domain (Src Homology 3; see Hammer, 1991 for review), and a hyper-proline-rich region 10 amino acids before the SH3 domain (Goodson and Spudich, 1995). The region 100 amino acids amino terminal to the SH3 domain contains a higher proline content than that of the corresponding region in MYO3 (25 vs 17% proline, respectively). In this respect, MYO5 is more similar to other classical myosin I proteins than MYO3. Like MYO3, MYO5 contains two IQ motifs, which are putative light chain binding sites (Cheney and Mooseker, 1992). The stretch of acidic residues followed

![Figure 1. Plasmid construction and deletion of the MYO5 gene. (a) MYO3 expression plasmid p316SRMYO3; (b) map of chromosomal locus of MYO5; (c) deletion plasmid p304KO3; (d) schematic illustrating replacement of MYO5 sequences with plasmid DNA; and (e) a mutant form of p314SRMYO3 containing a MYO3 gene with a frame shift in the tail of MYO3 (p314SRMYO3FS). Restriction sites marked by "*" were introduced into MYO5 by PCR subcloning and do not exist in the genomic version. The CiaI site in p304KO3 was placed at the stop codon. Nonmyosin regions of the plasmid are not drawn to scale. | MYO5 5' noncoding; ■ MYO5 coding region; | MYO5 3' noncoding; ———, = 1 kb.](image-url)
Figure 2. Derived amino acid sequence of Myo5p. This sequence is based on the DNA sequence of the MY05 gene. The MY05 DNA sequence shows 99% identity to sequence SC9718.08 obtained by the yeast genome sequencing project. The ATP binding site (underlined); IQ motifs (■); and SH3 motif (□).

by a tryptophan which is found at the end of both MY03 and Aspergillus nidulans MYOA (McGoldrick et al., 1995) is also found in MY05. The functional significance of this sequence is not known. Sequence analysis of complementing genomic yeast DNA in regions adjacent to the MY05 gene indicate that this gene is located adjacent to ILV2, a gene required for amino acid biosynthesis previously mapped to chromosome XIII by classic genetic techniques (Falco and Dumas, 1985; Falco et al., 1985).

Mutants Bearing Deletions in MY03 and MY05 Display Slow Growth

MY05 mutants obtained from the synthetic lethal screen were found after backcrossing to be viable under standard growth conditions (YPAD, pH 5.5, 30°C), but display severe growth defects. Therefore, the role of myosin I proteins in yeast cell function was studied in mutant yeast strains bearing deletions in one or both of the myosin I genes. Deletion of one myosin I gene has no significant effect on growth rates: myo3Δ and myo5Δ single mutants display doubling times equivalent to isogenic wild-type cells (Table II; Goodson and Spudich, 1995). In contrast, cells bearing deletions in MY03 and MY05 show greatly lengthened doubling times (Table II). Approximately 10% of the cells in an exponentially growing myo3Δmyo5Δ mutant culture are inviable, as determined using the fungal viability dye Fun-1. However, mathematical modeling of cell doubling indicates that this loss of cell viability contributes to but is not solely responsible for low growth rates (data not shown).

Double mutants are sensitive to cold (15°C) and elevated (37°C) temperature (Fig. 3). Double mutants did not accumulate at a particular point in the cell cycle after shift to cold (data not shown). During exponential growth at 30°C, they displayed a similar proportion of unbudded, small, medium, and large budded cells as the wild-type parental strain. Double mutants were defective for growth on solid rich media with high osmotic strength (0.75 M NaCl) or low pH (3.5). Tetrad analysis revealed that sensitivity to cold, elevated temperature, high osmotic strength, and low pH showed 2:2 segregation and segregated with deletion of both MY03 and MY05 (Fig. 3). Transformation of myo3Δmyo5Δ mutants with a centromeric plasmid bearing MY05 under its own promoter restores the wild-type growth rates at 30°C (Table II), as well as growth at low and high temperatures, low pH, and high osmotic strength (data not shown). Thus, the low growth rate, sensitivity to cold, acidic pH, and high osmolarity observed in the myo3Δmyo5Δ double mutants are due to the loss of both type I myosins.

To evaluate the localization of Myo5p in yeast (see below), we constructed epitope-tagged Myo5p by insertion of three copies of the myc tag (Evan et al., 1985) at the extreme COOH terminus of the protein. myc-tagged Myo5p was expressed in the myo3Δmyo5Δ mutant using a low copy plasmid under control of the endogenous MYO5 promoter. myc-tagged Myo5p restored normal growth rates in the myosin I double deletion mutant (Table II). This observation indicates that myc-tagged Myo5p is functional in living yeast.

Actin Patches and Cables Are Depolarized in the Double Mutant, myo3Δmyo5Δ

The actin cytoskeleton of exponentially growing cultures of a wild-type haploid strain, a myo5Δ single mutant, and a myo3Δmyo5Δ double deletion strain were visualized using rhodamine-phalloidin. The wild-type isogenic strain (HA10-1b) exhibits a typical polarized arrangement of actin patches (Fig. 4 B). 95% of wild-type cells with small- and medium-sized buds have actin patches exclusively in the buds. These actin patches appear to be invaginations in the plasma membrane which are invested with F-actin (Mulholland et al., 1994) and are thought to be involved in

Table II. Deletion of MY03 and MY05 Results in Slow Growth

| Strain          | Doubling time | SEM |
|-----------------|---------------|-----|
| HA10-1b (wt)    | 1.41          | 0.07|
| HA31-3 (myo3Δ)  | 1.34          | 0.05|
| HA31-1a (myo5Δ)| 1.40          | 0.11|
| HA31-9c (myo3Δmyo5Δ) | 4.45          | 0.29|
| HA31-9c-prs-Y2-MY05 | 1.34          | 0.09|
| HA31-9c-prs-Y2-myc-MY05 | 1.35          | 0.09|

Cultures of wild-type cells, mutants bearing single or double mutations in MY03 and MY05, and a myo3Δmyo5Δ mutant transformed with MY05 or myc-tagged MY05 on a centromeric plasmid (prs-Y2) were grown to early log phase in rich medium (YPD) at 30°C. Aliquots were removed periodically from all cultures and the cells were sonicated briefly to disrupt cell clumps. Cell densities were determined by OD660 measurement and apparent doubling times were calculated.
Mitochondrial and Nuclear DNA Inheritance Occurs in the Double Mutant, myo3Δ,myo5Δ

Previous studies indicate that mutations in the actin-encoding ACT1 gene result in defects in nuclear migration during cell division, aggregation of mitochondria, as well as defects in mitochondrial motility and inheritance (Novick and Botstein, 1985; Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1995). The arrangement of nuclear and mitochondrial DNA in wild-type cells and in the myo3Δ,myo5Δ mutant was examined using the fluorescent DNA binding dye DAPI. In wild-type cells, DAPI staining reveals threads of mitochondria in both the mother cell and bud and accumulation of mitochondria in the bud tip (Fig. 4 A). Nuclear staining is quite prominent. In some cases, nuclei were observed to extend from a mother cell to the bud and were therefore fixed during transport of nuclei into a bud (Fig. 4 A).

In myo3Δ,myo5Δ mutant cells, mitochondria show low levels of aggregation but extended tubular mitochondrial structures are clearly visible (Fig. 4 C). Transformation of the myo3Δ,myo5Δ mutant strain with a centromeric plasmid containing MYO5 fully rescues the wild-type mitochondrial phenotype (Fig. 4 E). Thus, deletion of both of these yeast myosin I genes causes some defects in mitochondrial spatial arrangement. However, transport of mitochondria into buds is not significantly affected in the double mutant. In mutant and wild-type strains, mitochondrial tubules were observed traversing the bud neck and accumulating at the bud tip. In addition, mitochondrial DNA was observed to be transferred to buds in greater than 90% of cells bearing small- to medium-sized buds. Nuclear inheritance also appears to be normal in myo3Δ, myo5Δ mutants: fewer than 3% of cells examined displayed multinucleation (data not shown).

The myo3Δ,myo5Δ Mutant Displays Defects in Chitin Deposition and Cell Shape

Shortly before bud emergence, a ring of chitin is formed in the yeast cell wall. The bud then emerges within the confines of this chitin ring. After cell–cell separation, a chitin-containing ring is left on the surface of mother cells. Thus, chitin localization is an indicator of the polarity of bud site selection: haploid cells typically show an axial budding pattern and produce buds to previous bud sites. In contrast, diploid cells display a bipolar budding pattern and produce buds either adjacent to or at the pole opposite from previous bud sites (Roberts et al., 1983). Since polarization of actin cables and patches is disrupted in myo3Δ,myo5Δ double mutants, it was of interest to determine whether the polarized bud site selection and chitin deposition might also be affected.
Figure 4. Actin, mitochondrial, and nuclear structure in wild-type cells, myo3Δ,myo5Δ double mutants and in double deletion mutants rescued with MYO5 gene. Wild-type HA10-1b (A and B), myo3Δ,myo5Δ double deletion strain (HA31-9c; C and D), and myo3Δ, myo5Δ mutants rescued with MYO5 on a low copy plasmid (E and F) were grown to mid-log phase at 30°C, fixed with paraformaldehyde, converted to spheroplasts, and stained with DAPI (A, C, and E) and rhodamine phalloidin (B, D, and F). DAPI staining reveals threads of mitochondria are inherited in all three strains (A, C, and E), but only the double mutant strain shows aggregation of mitochondria (C). Wild-type parental strain and MYO5 rescued myo3Δ,myo5Δ double mutants show cortical actin patches polarized to the bud and actin cables prevalent in mother cells (B and F). Actin patches are randomly distributed over the surface of mother and bud in a large majority of myo3Δ,myo5Δ double mutant cells (D). b, bud; m, mitochondria; n, nucleus. Bar, 2 μm.
Defects in size and shape of myo3Δ,myo5Δ mutants are also revealed by Calcofluor white stain. In wild-type yeast, early stages of bud growth are polarized and directed to the bud tip. Thereafter, growth is directed over the entire bud surface (Farkas et al., 1974). This pattern of bud growth produces oval shaped cells (Fig. 6A). The length to width ratio of wild-type HA10-1b haploid yeast is 1.137 ± 0.109 (n = 179). Enlarged cells (>7 µm) are rare in wild-type cultures, accounting for <7% of the population during mid-log growth. Single mutations in either MYO3 or MYO5 lead to slightly rounder cells with a length to width ratio of 1.085 ± 0.087 (n = 144) and 1.065 ± 0.101 (n = 158), respectively, but there is no apparent accumulation of enlarged cells (Fig. 6, B and C). In contrast, >80% of double mutant cells are spherical or nearly spherical (Fig. 6 D), and a small but significant minority of cells (15%) are enlarged in comparison to wild-type cells (>7 µm in length).

**Electron Microscopy Reveals Asymmetrically Thickened Cell Walls and Intracellular Membrane Accumulation in the myo3Δ,myo5Δ Mutant**

The ultrastructure of myo3Δ mutant cells and myo5Δ mutant cells is similar to that of wild-type yeast cells (Fig. 7, A–C). A single large vacuole occupies a considerable portion of the cytoplasm, and a nucleus, mitochondria, and tubules of endoplasmic reticulum can be distinguished in most cells. When a mother–daughter pair is sectioned such that both cells and their bud neck are visible, all identifiable organelle structures are detected in transit through the bud neck into the daughter cell. Cell walls are resolved as an electron translucent inner portion 100–300-nm-thick surrounded by an electron dense shell of mannanproteins (reviewed in Klis, 1994).

The ultrastructure of vacuoles, mitochondria, ER, and nucleus is not significantly affected by deletion of MYO3 and MYO5. However, 65% of budded myo3Δ,myo5Δ mutant cells accumulate multilamellar structures (Figs. 7 D and 8 B, arrow). These multilamellar organelles are ~200–500 nm in diameter and are often enriched in the bud. These structures resemble the “Berkeley Bodies” which have been previously described in late secretory mutants (Novick et al., 1981). Similar structures are not seen in wild-type cells or either single type I myosin mutant. Smaller vesicles (50–80-nm diam) also appear to accumulate in myo3Δ,myo5Δ mutants. These smaller vesicles are much less abundant than the multilamellar structures (Fig. 8 B, arrowhead).

myo3Δ,myo5Δ also display thickened cell walls. The cell walls of HA10-1b, the wild-type cell, and of myo3Δ and myo5Δ single mutant cells are 210 ± 38, 202 ± 34, and 212 ± 48 nm thick, respectively, and never exceeded a thickness of 500 nm. In contrast, 34% of myo3Δ,myo5Δ cells examined displayed cell walls which exceeded 500 nm; in some cells, wall thicknesses greater than 1 µm were observed. A pattern of concentric rings can be seen in many of the cells with extremely thick cell walls, suggesting that they are built through repeated deposition of cell wall material. In addition, virtually all cell wall thickening was detected in the mother cell: most buds display normal cell wall thickness.

To address whether defects in cell wall deposition and...
membrane accumulation observed in the myo3Δmyo5Δ double mutant are consequences of long term perturbation of the actin cytoskeleton, we studied a mutant bearing defects in the actin-encoding ACT1 gene. The temperature sensitive act1-3 mutant used undergoes loss of actin cables at all conditions and temperature-dependent depolarization of actin patches (Novick and Botstein, 1985). At permissive temperature (22°C), act1-3 cells display cell wall thickness similar to that of wild-type cells (Fig. 8 C). After short-term shift of the act1-3 cells to 37°C, we observe modest cell wall thickening (Fig. 8 D). Moreover, we observe extensive cell wall thickening in act1-3 mutants propagated at semi-permissive temperatures (30°C) for multiple generations (data not shown). These observations are consistent with a previous report that cell wall thickening occurs in actin mutants incubated at restrictive temperatures for prolonged time periods (Gabriel and Kopecký, 1995).

The actin mutant also displays abnormal intracellular membrane accumulation. At 22°C, act1-3 cells display some small vesicle accumulation (Fig. 8 C). After shift to restrictive temperature (37°C) for 45 min, these mutants show accumulation of both small vesicles and multilamellar vesicles (Fig. 8 D). The levels of accumulated membranes differ in the actin mutant compared to the myosin I double deletion mutant. In the myo3Δmyo5Δ double mutant multilamellar structures are abundant and small vesicles rare. In contrast, small vesicles are abundant and multilamellar structures are rare in act1-3 cells after short term shift to 37°C. However, upon propagation of the actin mutant at a semi-restrictive temperature (30°C), the amount of multilamellar structures and 50–80 nm vesicles in the act1-3 strain is similar to that in the myosin I double deletion mutant (data not shown). These findings suggest that defects in cell wall deposition and membrane accumulation observed in the myo3Δmyo5Δ double mutant are consequences of long-term perturbation of actin cytoskeletal organization.

**myo3Δmyo5Δ Mutants Display Defects in Invertase Secretion and Fluid Phase Endocytosis**

Accumulation of intracellular membranes in the myo3Δ, myo5Δ double mutants suggests possible defects in membrane trafficking. Therefore, we examined the effect of myosin I gene deletion on invertase secretion and fluid phase endocytosis of Lucifer yellow. Invertase is a highly glycosylated, secretory protein which catalyzes cleavage of α-glycosidic
linkages and is required for growth using many di- and trisaccharides as a carbon source. The \textit{myo3A,myo5A} double mutant displays low but detectable growth on medium containing the trisaccharide raffinose. While poor growth on raffinose is consistent with a defect in invertase secretion, this result is difficult to interpret because the double mutant also displays poor growth on galactose and glycerol (data not shown). However, defects in the rate of invertase secretion can also be shown biochemically (Fig. 9). Wild-type cells secrete invertase immediately after induction of invertase expression and display a maximal level of external invertase within 90 min. In contrast, \textit{myo3A,myo5A} mutants display a delay in the onset of invertase secretion after induction. Analysis of intracellular invertase revealed accumulation of partially processed glycosylation intermediates during this initial lag phase (data not shown). Thus, the double mutant is capable of responding to induction, but displays defects in the invertase secretion pathway. Invertase secretion is detected within 60 min after induction of the myosin I double mutant and occurs at rates similar to that of isogenic wild-type strains up to 180 min after induction. The maximum level of invertase secretion in the mutant is 25% greater than that of the wild-type cell. It is possible that the thickened cell wall of the myosin double deletion mutant retains greater levels of secreted invertase.

Lucifer yellow is a hydrophilic fluorescent dye which is excluded from cells by their plasma membrane. Yeast take up Lucifer yellow by fluid phase endocytosis and target the dye to their vacuole (Riezman, 1985). Mutations affecting actin or the actin binding protein Sac6p perturb this process (Kübler and Riezman, 1993). Lucifer yellow uptake into the vacuole is observed in >90% of wild-type HA10-1b cells after 30 min of incubation. In contrast, Lucifer yellow uptake and accumulation in the vacuole was observed in only 10% of \textit{myo3A,myo5A} mutants under the same experimental conditions (Fig. 10). After 120 min of incubation with Lucifer yellow, the percentage of double mutant cells with labeled vacuoles was unchanged. Thus, deletion of myosin I genes compromises fluid phase endocytosis in yeast.

**Myo5p Co-localizes with Actin Patches**

To determine the subcellular localization of Myo5p, the wild-type \textit{MYO5} gene was tagged with three copies of the myc epitope, and expressed in the \textit{myo3A,myo5A} mutant. To insure that myc-tagged Myo5p was expressed at levels

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*Figure 7. The myo3A,myo5A mutant displays cell wall thickening and intracellular accumulation of membranes. Wild-type (HA10-1b; A), myo3A mutant (HA31-9a; B), myo5A mutant (HA51-1a; C), and myo3A,myo5A mutant (HA31-9c; D) cells were grown to mid-log phase at 30°C. Cells were fixed with glutaraldehyde, stained with KMnO4, embedded, and sectioned. Arrows indicate large multilamellar vesicles; Arrowheads indicate small vesicles; n, nucleus; v, vacuole. Bar, 1 μm.*
similar to that of wild-type Myo5p, expression was carried out using a low copy, centromere-based plasmid under control of the endogenous MYO5 upstream sequences. As described above, expression of the epitope-tagged Myo5p in the myo3A, myo5A mutant restores normal growth rates (Table II). Therefore, addition of the myc tag to the COOH terminus of Myo5p does not appear to have a deleterious effect on Myo5p function.

The expression and detection of the tagged protein was evaluated using Western blot analysis (Fig. 11 a) and indirect immunofluorescence (Fig. 11 b). We find that the anti-myc monoclonal antibody 9E10 (Evan et al., 1985) recognizes a single band in whole cell extracts of myc-Myo5p-expressing cells (Fig. 11 A). The antibody does not recognize any protein in the myo3A,myo5A mutant or in a myo3A,myo5A mutant expressing untagged Myo5p. The electrophoretic mobility of the band detected in myc-Myo5p-expressing cells, 135 kD, is in good agreement with the size predicted from the DNA sequence of the MYO5 gene. Double label indirect immunofluorescence experiments indicate that myc-tagged Myo5p localizes with actin patches (Fig. 11 B). Within the bud, myc-tagged Myo5p is detected in some, but not all, of the actin patches. In addition, we observe co-localization of myc-tagged Myo5p with actin patches that accumulate at the site of bud emergence. myc-tagged Myo5p was not detected in actin cables.

**Discussion**

Myosins I are ubiquitous proteins expected to play an important role in cytoskeletal function. In a previous study, the first yeast myosin I was identified (Goodson and Spudich, 1995). This classic myosin I protein is encoded by the MYO3 gene. Deletion of this gene has no obvious effect. This raised the possibility that yeast contain other protein(s) with redundant function(s). Our approach to study myosin I function was to use a synthetic lethality screen to identify yeast proteins which are functionally redundant with Myo3p. This screen revealed a new classic myosin I gene, MYO5. MYO5 is 75.4% identical to MYO3. Both genes contain coding regions for motor and tail sub-domains typical of a classic myosin I including a basic (possible membrane-binding) region, an SH3 domain, and a proline-rich
myo5A sensitivity to high osmotic strength, defects in chitin depo-
vesicle and membrane accumulation, cell wall thickening,
larization, rounded cell shape, random bud site selection,

30°C and transferred to growth media containing 0.05% glucose
the indicated times and assayed for external invertase.

netic demonstration that myosin I proteins are required
to derepress the expression of invertase. Aliquots were taken at
the indicated times and assayed for external invertase.

region (for review see Hammer, 1991). Deletion of either
MY03 or MY05 has no obvious phenotype. However, we
show that a yeast strain bearing deletions of both MY03
and MY05 displays severe growth defects, enlarged cell
size, delocalized and disorganized chitin deposition, asym-
metrically thickened cell walls, disruption in the normal
polar distribution of actin, accumulation of abnormal
membrane bound structures and growth defects under
conditions of osmotic stress, low pH, low temperature and
elevated temperature. Our findings indicate that yeast
MY03 and MY05 genes encode classic myosin I proteins
with overlapping function. These results are the first ge-
netic demonstration that myosin I proteins are required
for normal cell function in yeast.

The phenotype of the myosin I double deletion mutant
is similar to that of yeast strains bearing mutations that
perturb the actin cytoskeleton. For example, mutation of
myosin V (MY02) leads to most or all of the following phenotypes:
partial or complete loss of actin cables, actin patch depo-
larization, rounded cell shape, random bud site selection,
vesicle and membrane accumulation, cell wall thickening,
sensitivity to high osmotic strength, defects in chitin depo-
sition, invertase secretion, endocytosis, and mitochondrial
organization (Novick and Botstein, 1985; Haarer et al.,
1990; Rodriguez and Patterson 1990; Adams et al., 1991;
Johnston et al., 1991; Amatruda et al., 1992; Chowdhury et
al., 1992; Liu and Bretscher, 1992; Kübler and Riezman,
1993; Drubin et al., 1993; Lazzarino et al., 1994; Simon et
al., 1995). One distinguishing feature of the myosin I double
deletion mutant is that the depolarization of actin structures
occurs without a significant reduction in the
amount of either actin cables or patches. This, coupled
with the observation that the phenotype of the myosin I
double deletion mutant is similar to that of strains with
mutations in actin or actin-binding proteins, supports a
role for Myo3p and Myo5p in control of actin cable and
patch polarization. At present, this myosin I double dele-
tion mutant and cells bearing mutations in the SLA1,
RVS161, or RVS167 genes are the only yeast mutants de-
fective specifically in actin organization (Bauer et al., 1993;
Holtzman et al., 1993; and Silvadon et al., 1995). However,
since actin cables are difficult to detect in myo3Δ,myo5Δ
mutants by epifluorescence microscopy, it is possible that
other mutants thought to have a significant reduction in
actin cables may instead have a similar loss of actin organi-
ization.

Since myosin I proteins have been implicated in control
of membrane–actin interactions, it is possible that Myo3p
and Myo5p serve as organelle motors for vesicle move-
ment during endocytosis and secretion. This interpretation
is consistent with the postulated function of classic myosin
I proteins in Aspergillus (McGoldrick et al., 1995). How-
ever, actin organization is altered upon deletion of yeast
myosin I genes. In addition, actin is known to be necessary
for secretion and endocytosis in yeast. Therefore, we favor
the interpretation that the primary function of yeast myo-
sin I proteins is control of actin organization, and we pro-
pose that the observed defects in secretion, endocytosis,
and osmotic sensitivity are secondary effects due to loss of
actin organization.

This interpretation is supported by the finding that
Myo5p is present in actin patches. Indirect immuno-
fluorescence and epitope-tagging were used to determine the
localization of Myo5p. We find that the myc-tagged pro-
tein is fully functional and observe co-localization of myc-
tagged Myo5p with actin patches under conditions where
the tagged protein was expressed at levels similar to wild-
type Myo5p. Previous studies suggest that (a) actin patches
are invaginations in the plasma membrane that are associ-
ated with F-actin (Mullholland et al., 1994), and (b) actin
nucleation and assembly occur at actin patches in the bud
of small-budded cells. This nucleation activity requires the
SLA1 and SLA2 gene products and may be regulated by
Cdc42p, a Rho-like GTP binding protein (Li et al., 1995).
Thus, the finding that Myo5p is localized at actin patches is
consistent with a model whereby myosin I proteins are re-
quired for polarization of the actin cytoskeleton. In princi-

Figure 9. Invertase secretion is defective in mutants bearing dele-
tions of MY03 and MY05. Wild-type (HA10-1b) and myo3Δ,
myo5Δ mutant (HA31-9c) cells were grown to mid-log phase at
30°C and transferred to growth media containing 0.05% glucose
to derepress the expression of invertase. Aliquots were taken at
the indicated times and assayed for external invertase.

Figure 11. Localization of myc-tagged Myo5p at actin patches. (a) Whole cell extracts from the myo3Δ,myo5Δ mutant (lane 1),
myo3Δ,myo5Δ mutant expressing untagged MYO5 (lane 2), and myo3Δ,myo5Δ mutant expressing myc-tagged MYO5 (lane 3) were an-
alyzed by Western blot analysis using a monoclonal anti-myc antibody. The antibody recognizes a band with the predicted molecular
weight of myc-tagged Myo5p only in cells expressing epitope-tagged MYO5. (b) Mid-log phase myo3Δ,myo5Δ mutants transformed
with plasmid bearing MYO5 (A and C) or myc-tagged MYO5 (B and D) were fixed and converted to spheroplasts, as described above.
Figure 10. Uptake of Lucifer yellow is defective in the myo3Δmyo5Δ mutants. Mid-log phase wild-type (HA10-1b; A and B) and myo3Δmyo5Δ mutant (HA31-9c; C and D) cultures were incubated with Lucifer yellow (4 mg/ml) for 120 min at 30°C. (A and C) phase contrast images; (B and D) fluorescence images showing Lucifer yellow uptake into the vacuole; v, vacuole. Bar, 2 µm.

In this double label experiment, actin (A and B) and myc-tagged Myo5p (C and D) were visualized using a polyclonal antibody raised against yeast actin and a mouse anti-myc monoclonal antibody. Arrows indicate cortical actin patches which co-localize with myc-tagged Myo5p. Bar, 1 µm.
ple, targeting of myosin I proteins to membrane–cytoskeletal junctions may be controlled by SH3 and possible membrane binding subdomains found in the tail domain. In addition, translocation of F-actin along microfilament tracks, and/or ATP-sensitive actin cross-linking during actin reorganization and polarization may be mediated by the ATP-insensitive and sensitive actin binding sites in the tail and motor domains.

Although myo3A/myo5A double mutants share phenotypes with other mutants which perturb actin structure, the severity of specific phenotypes differs among mutants. For example, the temperature-sensitive act1-3 mutant displays severe defects in mitochondrial organization and motility, accumulation of high levels of 50-nm vesicles, and only limited accumulation of multilamellar structures which resemble Berkeley bodies (Novick and Botstein, 1985; Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1995). In contrast, deletion of both yeast myosin I genes results in accumulation of high levels of multilamellar structures and only minor defects in mitochondrial organization and 50-nm vesicle accumulation. We observe that some of these differences are due to chronic versus acute actin disorganization. However, it is also possible that the variation in phenotype severity reflects differential dependence of different processes on actin cables and actin patches. As described above, deletion of MYO3 and MYO5 results in defects in polarization of actin cable and patch structures without significantly affecting the number of actin patches and cables. In contrast, act1-3 mutants show complete loss of actin cables under all conditions and depolarized actin patches only at restrictive temperature. Therefore, it is possible that mitochondria, which co-localize with actin cables (Drubin et al., 1993; Lazzarino et al., 1994), may be more severely compromised in mutants like act1-3 which do not contain these actin structures. In contrast, the relatively strong effect of the deletion of MYO3 and MYO5 on endocytosis may reflect a dependence of this process on the specific organization of cytoskeletal elements, possibly at a very local level.

The conservation of classic myosin I proteins in evolutionarily divergent organisms (Goodson and Spudich, 1993; Cheney et al., 1993) suggests that they have a significant role in many eukaryotic cell types. It has long been postulated that myosin I proteins play a role in the generation of cell motility (for review, see Pollard et al., 1991; Spudich and Warrick, 1991). Results from analysis of Dictyostelium myosin I mutants support this idea (Titus et al., 1993). Here, we show that two classic myosin I proteins are important for cell growth in an organism which does not move. Similar results were obtained recently in Aspergillus (McGoldrick et al., 1995). These results demonstrate that classic myosin I proteins are fundamentally important to the function of non-motile cells and suggest that they have a key role in cytoskeletal processes common to both motile and non-motile cells. More specifically, we provide evidence that (a) MYO3 and MYO5 encode myosin I proteins with overlapping function, and (b) these classic myosin I proteins are necessary for control of actin polarity in yeast. Our results in yeast, together with recent results in Dictyostelium (Novak et al., 1995), suggest that myosin I proteins play an important organizational role in the actin cytoskeleton in many eukaryotes.

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