

Immunofluorescence Localization of the Unconventional Myosin, Myo2p, and the Putative Kinesin-related Protein, Smy1p, to the Same Regions of Polarized Growth in *Saccharomyces cerevisiae*

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Abstract. Myo2 protein (Myo2p), an unconventional myosin in the budding yeast *Saccharomyces cerevisiae*, has been implicated in polarized growth and secretion by studies of the temperature-sensitive *myo2-66* mutant. Overexpression of Smy1p, which by sequence is a kinesin-related protein, can partially compensate for defects in the *myo2* mutant (Lillie, S. H., and S. S. Brown. 1992. *Nature (Lond.)*. 356:358–361). We have now immunolocalized Smy1p and Myo2p. Both are concentrated in regions of active growth, as caps at incipient bud sites and on small buds, at the mother-bud neck just before cell separation, and in mating cells as caps on shmoo tips and at the fusion bridge of zygotes. Double labeling of cells with either Myo2p or Smy1p antibody plus phalloidin was used to compare the localization of Smy1p and Myo2p to actin, and by extrapolation, to each other. These studies confirmed that Myo2p and Smy1p colocalize, and are concentrated in the same general regions of the cell as actin spots. However, neither colocalizes with actin. We noted a correlation in the behavior of Myo2p, Smy1p, and actin, but not microtubules, under a number of circumstances. In *cdc4* and *cdc11* mutants, which

produce multiple buds, Myo2p and Smy1p caps were found only in the subset of buds that had accumulations of actin. Mutations in actin or secretory genes perturb actin, Smy1p and Myo2p localization. The rearrangements of Myo2p and Smy1p correlate temporally with those of actin spots during the cell cycle, and upon temperature and osmotic shift. In contrast, microtubules are not grossly affected by these perturbations. Although wild-type Myo2p localization does not require Smy1p, Myo2p staining is brighter when *SMY1* is overexpressed. The *myo2* mutant, when shifted to restrictive temperature, shows a permanent loss in Myo2p localization and actin polarization, both of which can be restored by *SMY1* overexpression. However, the lethality of *MYO2* deletion is not overcome by *SMY1* overexpression. We noted that the *myo2* mutant can recover from osmotic shift (unlike actin mutants; Novick, P., and D. Botstein. 1985. *Cell*. 40:405–416). We have also determined that the *myo2-66* allele encodes a Lys instead of a Glu at position 511, which lies at an actin-binding face in the motor domain.

MYO2 protein (Myo2p) is an unconventional myosin in yeast (*Saccharomyces cerevisiae*), which has been classified as a class V myosin on the basis of the sequence of the putative motor domain (Cheney et al., 1993). Although Myo2p has not yet been tested for actin-based motor activity, such activity has been demonstrated for a chicken class V myosin (Espindola et al., 1992). Studies of the temperature-sensitive *myo2-66* mutant have implicated Myo2p in polarized growth (Johnston et al., 1991). Yeast grow by budding, a process in which virtually all growth is directed to the bud. This process is disturbed in the *myo2* mutant; cells at restrictive temperature continue

to increase in mass at a normal rate, but fail to produce buds, giving rise to a population of abnormally large round cells. In addition, there is a class of abnormally small cells derived from buds that ceased growth upon temperature shift and yet successfully completed cytokinesis and septation. Chitin deposition, normally restricted to the mother-bud neck, also becomes delocalized. Secretion continues, although the concentration of cytoplasmic vesicles increases. Actin loses its normal polarized localization, and, in addition, abnormal actin "bars" are formed. Other aspects of cell structure and function appear relatively normal, including nuclear division, and RNA and protein synthesis.

Actin has also been implicated in polarized secretion, not only by the phenotype of actin mutants (Novick and Botstein, 1985), which is similar to that of the *myo2* mutant, but also by the changing localization of actin during the cell cycle

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(Adams and Pringle, 1984; Kilmartin and Adams, 1984). Filamentous actin is found in both cortical spots and cables in wild-type cells. The actin cables are oriented roughly longitudinally in the mother cell, and extend into the bud. A cluster of actin spots forms at the incipient bud site, and spots are almost entirely restricted to the growing bud. This polarity is lost late in the cell cycle, when spots redistribute to the mother cell as well as the large bud. Just before cytokinesis, actin spots cluster at the mother-bud neck, where a cell-wall septum is being laid down. Taken together, available evidence suggests that Myo2p is involved in polarized growth by virtue of its interaction with actin.

To learn more about the role of Myo2p, we searched for multicopy suppressors (heterologous genes that, when overexpressed, can correct the temperature sensitivity of the *myo2* mutant). This search has yielded two genes so far, *SMY1* and *SMY2* (suppressors of myosin; Lillie and Brown, 1992). We have found that *SMY2* (accession #M90654) encodes a novel protein and is not an essential gene. Interestingly, overexpression of *SMY2* can also suppress mutations in several other apparently unrelated genes (e.g., *sec22* and *bet1*, Susan Ferro-Novick, personal communication; *secl6-3*, Neil Green, personal communication; *spt15*, David Poon and Tony Weil, personal communication).

Surprisingly, the sequence of *SMY1* indicates that Smylp is a member of the kinesin superfamily (Lillie and Brown, 1992). Although deletion of *SMY1* has no obvious phenotypic effect in wild-type cells, it is lethal in the *myo2* mutant (Lillie and Brown, 1992). If Smylp is really a microtubule-based motor protein, as its sequence suggests, it is unclear how it would compensate for the *myo2* defect. Cytoplasmic microtubules are in a position to play a role in polarized growth, in that they emanate from the spindle pole body (the equivalent of the centriole), and become oriented toward and extend into the bud (Adams and Pringle, 1984; Kilmartin and Adams, 1984). However, unlike actin, microtubules do not appear to be required for polarized secretion. That is, in cells lacking microtubules, buds both form and grow normally; such cells instead show defects in nuclear positioning and mitosis (Jacobs et al., 1988; Huffaker et al., 1988).

To further explore the function of Myo2p and the nature of its interaction with Smylp, we have immunolocalized Myo2p and Smylp. We compare their localizations to that of actin and microtubules during the cell cycle and during perturbation by temperature or osmotic shift. We examine how Myo2p and Smylp localizations are affected in mutants defective in actin, Myo2p, or secretory proteins, and by *SMY1* overexpression. In addition, we report the sequencing of the *myo2-66* mutation.

Materials and Methods

Reagents

Yeast lytic enzyme was obtained from ICN Biochemicals (Cleveland, OH). CHAPS was obtained from Polysciences Inc. (Warrington, PA). Rabbit anti-goat and goat anti-rabbit IgG were from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). FITC-conjugated goat anti-rat IgG (affinity purified), and FITC-conjugated goat anti-rabbit IgG (affinity purified) were from Sigma Chem. Co. (St. Louis, MO). YOL1/34 anti-yeast tubulin monoclonal antibody was from Accurate Chemical and Scientific Corp. (Westbury, NY). Rhodamine-conjugated phalloidin, as a 3.3- μ M stock in methanol, was from Molecular Probes Inc. (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit IgG (affinity purified) was

from Bio-Rad Labs. (Richmond, CA). The ECL kit was from Amersham Corp. (Arlington Heights, IL).

Yeast Strains and Media

All yeast strains used are listed in Table I. Liquid media used were rich medium YM-P (Lillie and Pringle, 1980) and synthetic complete medium SC lacking the appropriate supplement to select for plasmid (Sherman et al., 1986). Standard (Sherman et al., 1986) solid media were used, rich medium YPD and minimal medium SD with the required nutritional supplements. For all media, glucose was autoclaved separately and added to 2%.

DNA Manipulations, Yeast Genetic Techniques, and Plasmids

Standard procedures were used for DNA manipulations and *Escherichia coli* transformation (Sambrook et al., 1991), and for yeast transformation (by the lithium acetate method) and genetic manipulations (Sherman et al., 1986).

Plasmid pJP10-2B (which we refer to as YCpMYO2) was kindly provided by G. Johnston (Johnston et al., 1991). This low-copy-number plasmid contains the full-length *MYO2* gene. The full-length *SMY1* gene was introduced into the high-copy-number vectors YEp351 (2 μ *LEU2*) and YEp352 (2 μ *URA3*) (Hill et al., 1986) as follows: To construct plasmid YEpSMY1-26, the \sim 4.5-kbp Eco RV *SMY1*-containing fragment from the genomic library clone (Lillie and Brown, 1992) was inserted at the Sma I site of YEp352. YEpSMY1-38 was constructed by inserting the \sim 3.5-kbp Pst I *SMY1*-containing fragment from YEpSMY1-26 into the Pst I site of YEp351. (One end of this insert is derived from vector sequence.) To truncate *SMY1*, YEpSMY1-40 was created by digesting YEpSMY1-26 with Sst I and religating to remove the COOH-terminal 79 amino acids of *SMY1* product. This truncated gene encodes the complete head domain and 206 amino acids of the tail.

MYO2 Deletion

Plasmid p Δ M::TRP1, which contains the *MYO2* deletion allele (see Fig. 10 c), was constructed by inserting sequentially the NH₂-terminal *MYO2*-containing fragment, the COOH-terminal *MYO2*-containing fragment, and finally the yeast *TRP1* gene into the multiple-cloning-site region of Bluescript (Stratagene, LaJolla, CA) vector pSK⁺ as follows: The \sim 1-kbp Eco RV-Pvu II fragment from YCpMYO2-10 (derived from YCpMYO2) was inserted at the Xba I site in pSK⁺ after treatment with Klenow fragment to fill the Xba I ends. (The Eco RV site of this fragment lies in vector sequence as indicated in Fig. 10, a and c and was destroyed by insertion into the filled Xba I site.) Next, the \sim 500-bp Eco RV-Cla I fragment was inserted at the Eco RV/Cla I sites of pSK⁺. Finally, the Bam HI-Eco RI *TRP1*-containing fragment was inserted into the Bam HI/Eco RI sites of pSK⁺ to yield plasmid p Δ M::TRP1.

A *trp1/trp1* diploid was transformed with the gel-purified Bgl II-Cla I fragment (Fig. 10) from p Δ M::TRP1 to delete the *MYO2* gene by one-step gene replacement (Rothstein, 1991). Two Trp⁺ transformants were picked and found to be stably Trp⁺. Southern blot analysis of these two diploids confirmed that the expected deletion had occurred, and tetrad analysis of these strains gave the expected results (Johnston et al., 1991; see Results).

Mapping and Sequencing the *myo2-66* Mutation

The mutated region of the *MYO2* gene was identified by marker-rescue experiments as follows: The temperature-sensitive *myo2 ura3* mutants SLY1 and SLY33 were transformed at room temperature (RT)¹ with autonomously replicating YEp or YCp *URA3*-based plasmids containing fragments of the wild-type *MYO2* gene. Ura⁺ transformants were plated at 36°C. If, and only if, the *MYO2* fragment included DNA corresponding to the mutated region in the chromosome, gene conversion gave rise to papillae of growth on a background of temperature-sensitive (non-growing) cells. In this way the mutation was found to reside in a 1-kbp Xba I segment (Fig. 10 e). Conversely, a plasmid that included all of the *MYO2* gene except this 1-kbp Xba I fragment failed to provide marker rescue.

To recover this segment by "gap repair" (Rothstein, 1991), plasmid YCpMYO2 was cut with Xba I and religated to yield YCpMYO2-12 (Fig. 10 d), which lacks the 1-kbp Xba I segment. When (*URA3*-based)

1. Abbreviation used in this paper: RT, room temperature (19–22°C).

Table I. Yeast Strains Used in This Study

Strain	Relevant genotype*	Source
SLY1	<i>MATα myo2-66[‡] ura3-52 trp1-Δ1 his6 ade2</i>	This study
SLY33	<i>MATα myo2-66 ura3-52 leu2-3,112</i>	This study
SLY34	<i>MATα myo2-66 ura3-52 leu2-3,112</i>	This study
SLY54	<i>MATα ura3-52 leu2-3,112 trp1-289 his4</i>	This study
SLY56	<i>MATα ura3-52 leu2-3,112 his4</i>	This study
SLY86	<i>MATα smy1-Δ2::LEU2 ura3-52 leu2-3,112 trp1-289 his4</i>	This study
	<i>MATα smy1-Δ2::LEU2 ura3-52 leu2-3,112 TRP1 his4</i>	
SLY87	<i>MATα ura3-52 leu2-3,112 trp1-289 his4</i>	This study
	<i>MATα ura3-52 leu2-3,112 TRP1 his4</i>	
SLY88	<i>MATα myo2-66 ura3-52 leu2-3,112 trp1-Δ1 his3 his6</i>	This study
	<i>MATα myo2-66 ura3-52 leu2-3,112 TRP1 HIS3 HIS6</i>	
SLY91	<i>MATα myo2-66 leu2-3,112 his3</i>	This study
	<i>MATα myo2-66 leu2-3,112 HIS3</i>	
SLY107	<i>MATα myo2Δ::TRP1 trp1-Δ1 ura3-52 leu2-3,112</i>	This study
	<i>MATα MYO2 trp1-289 ura3-52 leu2-3,112</i>	
SLY175	<i>MATα ura3-52</i>	This study
SLY176	<i>MATα ura3-52</i>	This study
DBY1999	<i>MATα act1-1[§] ura3-52</i>	D. Botstein
DBY2003	<i>MATα ura3-52</i>	D. Botstein
DBY2018	<i>MATα act1-2 his4-619</i>	D. Botstein
C276	<i>MATα prototrophic</i>	J. Pringle
	<i>MATα</i>	
JPT194-HO1	<i>MATα cdc11-6 prototrophic</i>	J. Pringle
	<i>MATα cdc11-6</i>	
314D5	<i>MATα cdc4-1 ade1 ade2 ura1 tyr1</i>	J. Pringle
	<i>MATα cdc4-1 ade1 ade2 ura1 tyr1</i>	
NY3 \times NY4	<i>MATα sec1-1 ura3-52 his4</i>	This study
	<i>MATα sec1-1 URA3 HIS4</i>	
NY16 \times NY17	<i>MATα sec6-4 ura3-52 his4</i>	This study
	<i>MATα sec6-4 URA3 HIS4</i>	
NY757 \times NY760	<i>MATα sec7-1 ura3-52 his4</i>	This study
	<i>MATα sec7-1 URA3 HIS4</i>	

* Some strains may carry additional mutations; most or all are S288C derivatives and therefore *gal2*.

[‡] The *myo2-66* allele was obtained from Gerry Johnston in strains GR663-7 and GR663-13, and crossed into our strain background.

[§] Formerly known as *act1-3* (Novick et al., 1989).

^{||} These diploids were made by mating the indicated haploids, NY3 etc., obtained from P. Novick.

YCpMYO2-12, linearized with Xba I, was introduced into *myo2 ura3* yeast, some of these plasmid molecules are repaired from chromosomal information, giving rise to Ura⁺ transformants. Such gap repair plasmids were recovered from one transformant each of *myo2 ura3* mutants SLY1 and SLY33 and were named YCpGap1 and YCpGap4, respectively. The 1-kbp Xba I segment from each, as well as the corresponding wild-type segment from plasmid YCpMYO2, were shuttled to Bluescript sequencing vectors. Single-strand sequence of each Xba I fragment was obtained by the dideoxy-chain termination method (Sanger et al., 1977). As our sequencing did not extend beyond this Xba I fragment, we cannot rule out the possibility that an additional mutation outside of this region was recovered by "chew-back" in the gap repair plasmid.

Preparation of Total *E. coli* and Yeast Proteins, Gel Electrophoresis, and Protein Blotting

Total *E. coli* proteins were prepared from cells grown under the appropriate inducing conditions (see below) as follows: Cells were collected by centrifugation and resuspended in SDS-solubilizing buffer (Laemmli, 1970) plus 6 M urea, heated 3 min in a boiling-water bath, and loaded onto gels (see below).

Total yeast proteins were prepared from log-phase cells growing in the appropriate medium following suggestions by D. Drubin (UC Berkeley) as follows: Working as quickly as possible, cells were collected by centrifugation and resuspended by sonication into prewarmed SDS-solubilizing buffer plus protease inhibitors (Lillie and Brown, 1987). Samples were placed in a boiling-water bath for 3 min, glass beads (0.5 mm) were added, the mix was vortexed 60 s, and loaded onto standard SDS-polyacrylamide gels, which were either stained with Coomassie blue or blotted to nitrocellulose as de-

scribed previously (Lillie and Brown, 1987). Blots were stained with Pon-ceau S (Lillie and Brown, 1987) to assess relative amounts of proteins transferred.

Constructions of Gene Fusions

The 2.35-kbp Hpa I-Cla I fragment of *MYO2* encodes the tail portion of Myo2p from amino acid 833 through the end of the coding region (Fig. 10 b). To create a *trpE:MYO2* gene fusion, this fragment was inserted into Sma I/Cla I - cut fusion vector pATH2 (Koerner et al., 1991). To create a *lacZ:MYO2* gene fusion, the same fragment was inserted into the Hind III site of fusion vector pUR290 (Rüther and Müller-Hill, 1983) after treating both fragment and vector with Klenow fragment to produce blunt ends.

The 715-bp Dra I-Sac I fragment of *SMY1* encodes the tail portion of Smylp from amino acid 410 through 647 (this fragment lacks the last 9 amino acids of Smylp). To create *trpE:SMY1* and *lacZ:SMY1* gene fusions, the fragment was inserted into the Cla I site of pATH1 (Koerner et al., 1991) and the Bam HI site of pUR291 (Rüther and Müller-Hill, 1983), respectively, after treating each vector with Klenow fragment to produce blunt ends.

Preparation of Antigens and Antibodies

The *lacZ:MYO2* and *lacZ:SMY1* fusion proteins were induced in *E. coli* strain BMH 71-18 as described by Kim et al. (1991), except that induction was at 30°C rather than 37°C. The *trpE:MYO2* and *trpE:SMY1* fusion proteins were induced in *E. coli* strain RRI as described by Haarer and Pringle (1987), except that induction time ranged from 1 to 4 h. Proteins were separated on gels (see above); gel slices containing fusion protein were excised

and used to immunize rabbits as described previously (Lillie and Brown, 1987).

Antibody Affinity Purification and Immunoblots

Myo2p- and Smylp-specific antibodies were affinity purified from crude antisera using nitrocellulose strips containing the heterologous fusion protein as affinity matrix (e.g., *trpE:MYO2* antiserum on *lacZ:MYO2* affinity matrix, etc.). In some cases, a second round of affinity purification was performed; this used the homologous fusion protein as affinity matrix. Affinity purifications were carried out as described by Lillie and Brown (1987) and Pringle et al. (1991), except that after elution in low pH buffer, the purified antibody was neutralized with an equal volume of 100 mM Tris base containing 1% BSA. Affinity-purified Smylp antibody was adsorbed against fixed *SMY7* deletion cells as an additional purification step (Roberts et al., 1991). However, as this step did not alter the staining pattern of cells, it was not included routinely.

To assess antibody specificity and the success of affinity purifications, blots of total *E. coli* or yeast proteins were probed with affinity-purified antibody and fractions generated during affinity purification as described previously (Lillie and Brown, 1987). For detection of bound antibody, we used either 4-chloro-1-naphthol and hydrogen peroxide as described previously (Lillie and Brown, 1987), or ECL staining (performed according to the manufacturer's instructions).

Growth Conditions for Immunofluorescence

Cells were grown at RT (19–22°C), unless otherwise indicated, in the appropriate liquid medium with rotary shaking for at least 16 h, subculturing if necessary to maintain log phase cells at $\leq 10^7$ cells/ml. Cells were then fixed for immunofluorescence (see below) or shifted to experimental conditions.

To obtain cells responding to mating pheromone (shmoos) and zygotes, haploid strains of opposite mating types (SLY175 and SLY176) were grown separately in YM-P at RT to $\sim 10^7$ cells/ml. Five ml of each culture were then mixed in a 50-ml flask and incubated with shaking for 1 h. Shaking then was discontinued for the duration of the experiment to allow cells to mate, and aliquots were processed for immunofluorescence microscopy at intervals over 5 1/2 h. The expected transient rearrangements of actin, Myo2p, and Smylp were observed in response to discontinuation of shaking (see Discussion), but the cells had recovered by the time period of interest. Many shmoos and zygotes had formed by 3 1/4 h after mixing, while at 4 1/4–5 1/2 h the majority of zygotes had produced a first bud.

Temperature-sensitive *cdc4*, *cdc11*, *secl*, *sec6*, *sec7*, *act1-1*, and *act1-2* mutants, and the appropriate parent (control) strains were grown at permissive temperature (RT for most strains) in YM-P to $\leq 10^7$ cells/ml. (For the *act1* mutants, which are somewhat abnormal even at RT, 18–20°C was used as permissive temperature.) To shift cells to restrictive temperature, aliquots were diluted fivefold into prewarmed YM-P at 36°C.

The *myo2 (ura3)* mutant plus plasmid, or control vector, was grown at $\leq 20^\circ\text{C}$ in SC-uracil to select for plasmid. To shift cells to restrictive temperature, aliquots of the culture were diluted fivefold into prewarmed SC-uracil at 30, 32, or 36°C. (Mock-shifted control cultures were diluted into medium at $\leq 20^\circ\text{C}$.)

To examine the effects of temperature shift itself, wild-type strain SLY87 was grown in YM-P; strain SLY87 carrying plasmid YEpSMY1-26 or control vector was grown in SC-uracil, and *SMY7* deletion strain, SLY86, was grown in SC. Cells were grown to $\leq 10^7$ cells/ml at RT. For each time point of the temperature shift, 1 ml was removed rapidly from the RT culture and added to 9 ml of medium which was shaking and prewarmed to 36°C (or at RT for mock-shift control cultures).

For osmotic-stress experiments, strains (SLY87, SLY86, and SLY88) were grown in YM-P at 20°C to a density of $\sim 5 \times 10^6$ cells/ml. Osmolarity of the cultures was increased by addition of 0.4 M NaCl (using 5 M NaCl stock). A comparable volume of water was added to control cultures.

Immunofluorescence Microscopy

For most experiments, cells were fixed for immunofluorescence microscopy by adding 37% formaldehyde directly to the shaking culture to give a final concentration of 3.7% and shaking was continued for the duration of fixation (1–1 1/2 h). For some experiments with several time points, aliquots of culture were pipetted quickly to formaldehyde-containing tubes which were placed on a roller drum during fixation. We found that this latter procedure did not detectably perturb the cellular actin arrangement, a sensitive indicator of rapid fixation (see Results). Immunofluorescence results were indis-

tinguishable using cells grown in either rich medium (YM-P) or minimal medium (SC), and no differences were noted when using parental (wild-type) strains from several different laboratories (Table I).

For immunofluorescence localization of Myo2p and Smylp, we used procedures based on those of Pringle et al. (1991), with the following modifications: Yeast cells were fixed in 3.7% formaldehyde for 1–1 1/2 h. (Longer fixations [≥ 3 h] gave poor Myo2p staining and eliminated detectable Smylp staining; limited attempts to find an alternative to formaldehyde fixation failed.) After washes in 1.2 M sorbitol/phosphate buffer (solution A of Pringle et al., 1991), cell walls were removed from $\sim 10^8$ cells in 1 ml solution A containing 5 μl β -mercaptoethanol and 30 μl of yeast lytic enzyme stock (10 mg/ml in solution A). Incubation was for 30 min at 36°C with gentle agitation. For Smylp staining, best results were obtained if washed spheroplasts (stored at 4°C in solution A) were used within 12 h. Spheroplasts were attached to polylysine-coated multiwell slides and incubated for 5–30 min in phosphate buffer/BSA (solution F of Pringle et al., 1991) before addition of affinity-purified primary antibody or additional treatments as appropriate. Solution F was used for all washes and antibody dilutions. For Myo2p staining, either of two procedures was used. Cells were incubated with primary antibody (at 1:10–1:20 dilution) for 10–12 h at RT, or cells were pretreated with methanol at -20°C for 6 min, followed by 30 s in acetone at -20°C , and then incubated with primary antibody for 2–4 h. For Smylp staining, cells were treated with detergent (either 0.5% SDS or 0.5% CHAPS) for 3–4 min, washed five times, and incubated with primary antibody (at 1:5–1:10 dilution) for 4–8 h at RT. After incubation with primary antibody, antibody amplification (Roberts et al., 1991), which was essential for Smylp and helpful for Myo2p visualization, was accomplished by treatment with goat anti-rabbit IgG (at 1 $\mu\text{g}/\text{ml}$) for 1 h, and then rabbit anti-goat IgG (at 1 $\mu\text{g}/\text{ml}$) for 1 h, and finally FITC-conjugated goat anti-rabbit IgG (at 1:80 dilution) for 1–2 h. Wells were washed four times between secondary antibodies.

Microtubules were visualized using the anti-yeast tubulin monoclonal antibody YOL1/34 (Kilmartin and Adams, 1984) and actin was visualized using anti-yeast polyclonal antibodies as described previously (Haarer et al., 1990).

For double labeling of Myo2p or Smylp and actin, rhodamine-conjugated phalloidin was used to visualize actin as described by Adams and Pringle (1991). As treatment of cells with methanol/acetone or SDS destroys the ability of phalloidin to interact with actin, we used the treatments described above that avoided those reagents.

Nuclei were stained by adding 4',6'-diamidino-2-phenyl-indole (DAPI) to the mounting medium (Kilmartin and Adams, 1984).

Stained cells were viewed with an Orthoplan fluorescence microscope and photographed using TMAX 400 film (Eastman Kodak Co., Rochester, NY). Negatives were printed using different exposure times to optimize detail, except as noted.

Quantitation of Filamentous Actin upon Temperature Shift

Quantitation of filamentous actin and fluorescence microscopy were carried out on temperature-shifted cells, using rhodamine-phalloidin. Cells (C276) were grown in YM-P at RT to a density of $\sim 10^7$ cells/ml. For each time point, 1 ml was removed rapidly from the RT culture and added to 9 ml of YM-P medium which was shaking and prewarmed to 37°C (or at RT for mock-shift control cultures). One ml 37% formaldehyde, also prewarmed, was added while the above culture was still shaking. After 1/2 h, cultures were removed from the shaker and fixation continued overnight at RT. The cells were collected by centrifugation, incubated in 100 μl of 0.33 μM rhodamine-phalloidin, 0.2% Triton X-100 in PBS for 1/2 h in the dark on a roller drum at RT, and then washed twice with PBS. A 1/20 volume was reserved for fluorescence microscopy, and the rest of the cells were collected by centrifugation and incubated for 1 1/2 h in 700 μl methanol on a roller drum in the dark. Extracted fluor was quantitated by fluorimetry (Howard and Oresajo, 1985). We confirmed by diluting aliquots of fixed cells two- or fourfold before phalloidin staining that fluorescence was linear in this range. Cells before temperature shift bound $\sim 7\%$ of the input phalloidin.

Results

Myo2p Antibody Specificity

Members of the myosin superfamily are similar to one another in their motor domains, but have divergent tails.

Therefore, polyclonal antibodies were raised against the tail of Myo2p, to reduce the possibility of crossreactivity with other yeast myosins. As antigens we used fusion proteins that included amino acids 833-1574 (i.e., the COOH-terminal half) of Myo2p. Both *trpE:MYO2* and *lacZ:MYO2* gene fusions were made, so that antibody raised against one fusion protein could be affinity purified against the heterologous fusion protein (Haarer and Pringle, 1987). Affinity-purified antibodies were tested for specificity on blots of total yeast protein (Fig. 1 *a*). Whereas the crude antiserum (lane 1) stained multiple bands, affinity-purified antibody (lane 2) stained only one major band, at the expected molecular weight. (We sometimes saw minor bands; as these varied from sample to sample, we believe they are due to proteolysis.) A second round of affinity purification demonstrated that all of the antibody affinity purified against the heterologous fusion protein (lane 2) could be subsequently affinity purified against the homologous fusion protein (compare lanes 3 and 4). This confirmed that the antibody specifically recognizes the Myo2p portion of the fusion proteins. Thus, immunoblotting demonstrates the efficacy of the affinity purification, and confirms that the staining is not simply spillover or contamination from the depleted fraction. Additional immunoblotting evidence that the antibodies specifically recognized Myo2p include the following (data not shown): (*a*) Antibody produced against each of the two different fusion proteins stained the same molecular weight band when affinity-purified antibody but not when depleted serum was used. (*b*) Introduction of *MYO2* on a high-copy number plasmid resulted in a low level of overexpression of Myo2p on immunoblots. (We did not expect a high level of overexpression, because the plasmid appears to be selected against and copy number is most likely reduced in these cells; Haarer et al., 1994). (*c*) Introduction of a truncated *MYO2* (see below) results in the appearance of a new band of the expected molecular weight on immunoblots. (*d*) We have also confirmed that the stained band corresponds to Myo2p, using a strain that carried only HA-tagged Myo2p (generously provided by Govindan, B., and P. Novick, Yale University). Proteins were separated on a 5% gel to maximize resolution in the vicinity of Myo2p. When adjacent strips of the immunoblot were treated with anti-HA antibody and the Myo2p antibody, a band of the same molecular weight was stained.

Antibody specificity was also examined upon immunofluorescence labeling of yeast. We found that affinity-purified antibody from both the first and second round of purification gave the localization patterns described in the next section, whereas neither depleted fraction generated during affinity purification gave this localization. Thus, the localization we report is specific, and not due to spillover during affinity purification. Similar immunofluorescence results were obtained with antibody against either of the two fusion proteins described above. Furthermore, the alterations in localization we describe below, including fainter staining of the *myo2* mutant at permissive temperature and abolition of localization at restrictive temperature, are consistent with the evidence that the staining is specific for Myo2p.

Cells stained with the depleted fraction from the first round of affinity purification gave a speckled appearance as well as a bright overall glow; this staining pattern was much fainter when affinity-purified fractions or the depleted frac-

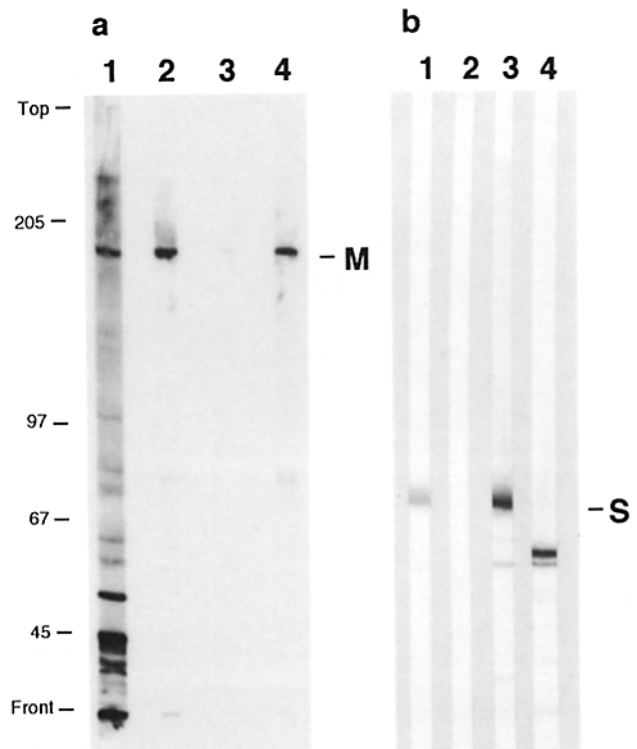


Figure 1. Demonstration of antibody specificity on immunoblots. (*a*) Anti-Myo2p antibody labeling of a blot of total yeast protein (strain SLY87). Crude serum against *trpE:MYO2* fusion protein (lane 1) was affinity purified against *lacZ:MYO2* fusion protein. The affinity-purified antibody (lane 2) was taken through a second round of affinity purification against *trpE:MYO2* fusion protein, to generate depleted (lane 3) and twice-affinity-purified (lane 4) fractions. (*b*) Anti-Smy1p antibody labeling. Affinity-purified antibody against *trpE:SMY1* fusion protein was used to label blots of total protein from wild-type cells (SLY87) (lane 1), or from *SMY1* deletion cells (SLY86) either without added plasmid (lane 2), with plasmid encoding full-length *SMY1* (YE_pSMY1-26) (lane 3), or encoding truncated *SMY1* (YE_pSMY1-40) (lane 4). Proteins were separated on 7% SDS-polyacrylamide gels and visualized on Western blots with ECL reagent (*a*) or α -chloro-naphthol (*b*). Molecular masses of protein standards are indicated in kDa, and the positions of Myo2p (*M*) and Smy1p (*S*) are labeled.

tion from the second round of affinity purification were used. Therefore, at least some of this staining pattern is non-specific.

We have identified a second class V myosin in yeast (Myo4p; Haarer et al., 1994). Even though the level of amino-acid similarity to Myo2p is low in the tail, we looked for evidence of antibody crossreactivity. None was found; antibodies against Myo2p and Myo4p each label a band of different molecular weight on Western blots, with no detectable crossreactivity. Furthermore, immunofluorescence microscopy showed the same Myo2p staining pattern in a *MYO4* deletion strain as in wild-type cells (data not shown).

Immunolocalization of Myo2p

In both haploid and diploid wild-type strains (SLY54, SLY56, SLY87), Myo2p was concentrated as a cap at or near the membrane on unbudded cells and small buds (Fig. 2, *a*, *b*, and *c*). These caps were usually at the tip of the bud, but

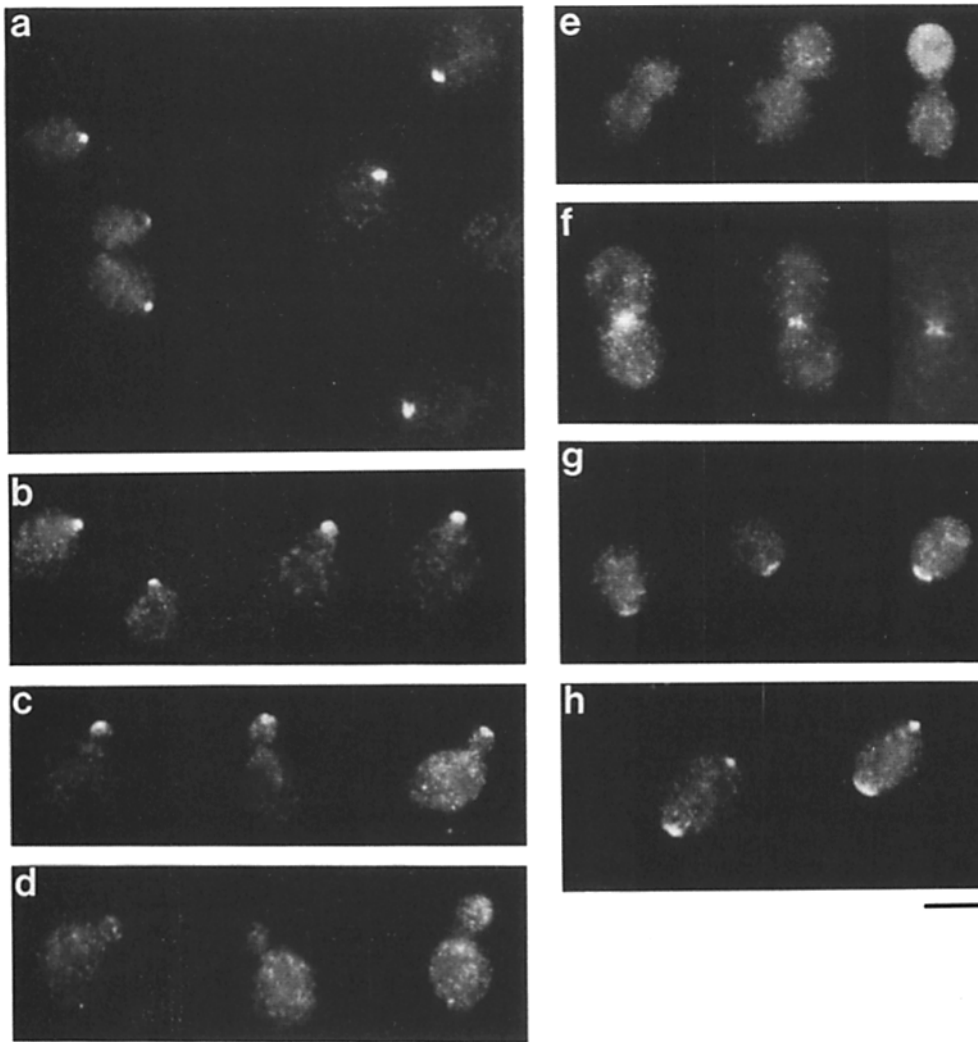


Figure 2. Immunolocalization of Myo2p in wild-type cells. Cells (SLY87) were labeled with anti-Myo2p antibody. Shown is a sequence of cells at various stages of the cell cycle: (a) unbudded cells with a Myo2p cap, (b) small-budded cells with a Myo2p cap, (c) cells with slightly larger buds (note that the Myo2p cap can be off center, e.g., in the cell to the left), (d) medium-budded cells without a cap, (e) large-budded cells without a cap (note that the bud may have a brighter glow than the mother cell, e.g., in the cell to the right), (f) large-budded cells with staining at the mother-bud neck, (g) unbudded cells with a flat patch of Myo2p, (h) unbudded cells with both a cap and flat patch of Myo2p staining. Bar, 5 μ m.

were sometimes a little off-center (Fig. 2 c, cell on the left). Discrete cap staining disappeared as buds enlarged (Fig. 2, d and e), although a glow or multiple spots of Myo2p persisted in some medium-sized buds (Fig. 2 e, cell on the right). Myo2p appeared at the neck between mother and daughter cells (Fig. 2 f) shortly before cytokinesis and cell septation (i.e., in cells that had completed nuclear division, as judged by DAPI staining). Some unbudded cells had a flat patch instead of a rounded cap of Myo2p (Fig. 2 g). In diploid strains, which display a bipolar budding pattern (Chant and Pringle, 1991), an occasional unbudded cell had both a cap and at the opposite end a flat patch of Myo2p (Fig. 2 h). (As expected, this was not observed in haploids, which display a unipolar budding pattern.) Most likely the cap marks the new bud site and the flat patch is a "leftover" from the neck staining, marking the site of the previous cell division. (Further support for this interpretation will be provided below.) The morphological distinction between caps and patches presumably reflects the cell shape where the septum is laid down between mother and bud at the neck. Cells at all stages of the cell cycle also displayed a background glow and speckles. This staining was variable, and some or all may be nonspecific, as mentioned above.

It is striking that Myo2p was concentrated at sites of active

growth in these vegetative yeast cells. We therefore asked if Myo2p were also localized to sites of active growth in mating cells. Such cells form projections ("shmoo") which fuse with a partner of the opposite mating type. Exponentially growing haploid strains (SLY175 and SLY176) were mixed to induce shmoo formation and mating. Myo2p was indeed localized in caps at shmoo tips, at the fusion bridge of zygotes, and as a pronounced cap on the zygote's first bud, all regions of polarized growth.

Smylp Antibody Specificity

We have used the same strategy as described for Myo2p antibody to obtain affinity-purified antibody against the tail portion of Smylp (amino acids 410–647). Antibodies against either of the two fusion proteins containing the Smylp tail gave the same immunoblotting and immunofluorescence results. As an additional purification step, we sometimes adsorbed the affinity-purified antibody against cells of a *SMY1* deletion (*smy1* Δ) strain (SLY86). However, as this treatment did not alter the localization we report, it was not routinely included. Affinity-purified antibody stained a fuzzy band of the expected molecular weight on immunoblots (Fig. 1 b, lane 1). This band was not seen in a *smy1* Δ strain (lane 2), and was

overexpressed in a strain containing *SMY1* on a high-copy-number plasmid (lane 3). Furthermore, a *smy1Δ* strain carrying truncated *SMY1* on a plasmid gave a lower molecular weight band of the expected size (lane 4). (The minor bands seen in lanes 3 and 4 are presumably proteolytic fragments, as they are not seen in lanes 1 and 2.) Thus, it is clear that the affinity-purified antibody specifically recognizes Smylp on immunoblots. Results consistent with the immunoblots were obtained when these various strains were examined by immunofluorescence (see below).

Immunolocalization of Smylp

Strikingly, the localization of Smylp (Fig. 3) was indistinguishable from that of Myo2p in both haploid and diploid wild-type cells (SLY54, SLY56, SLY87), although the intensity of Smylp staining was variable in different experiments. Smylp formed caps in unbudded and small-budded cells, and, occasionally, staining of the mother-bud neck was detectable (in the two cells at the bottom of Fig. 3 *a*). Smylp also formed flat patches (distinct from caps) in unbudded cells (Fig. 3 *b*, arrows). In mating cells, Smylp was found in the same locations as reported above for Myo2p. In all cells stained with Smylp antibody, background glow and speckles were also seen. The *smy1Δ* strain (SLY86) did not show staining of caps, necks, or flat patches, and the background glow and speckles were greatly reduced as well. These observations demonstrate the specificity of the staining patterns we see, including at least some of the background glow and speckles. Cells overexpressing *SMY1* (SLY87 + YEpSMY1-26) displayed the same Smylp localizations as wild type (Fig. 3 *b*), although the intensity of staining increased significantly, and therefore the frequency of cells with detectable Smylp also increased. Most cells overexpressing Smylp also displayed a big increase in the background glow. It appears that cells can localize part of the excess Smylp, but that some may remain diffusely distributed.

We have begun to ask what parts of the Smylp molecule are required for localization, by examining a *smy1Δ* strain with plasmid-borne truncated *SMY1* (SLY86 + YEpSMY1-40). It can be seen in Fig. 1 *b* (lane 4) that the truncated Smylp is (over)expressed, has the expected molecular weight, and is recognized by antibody. However, the truncated Smylp did not localize properly. All that was seen by immunofluorescence was an increased cytoplasmic glow, as compared to the *smy1Δ* strain carrying a control vector (SLY86 + YEp352). As expected, strain SLY86 carrying full-length *SMY1* (YEpSMY1-26) did display normal Smylp localization. We conclude that the COOH-terminal portion of the tail is required for proper localization of Smylp. The truncation would not be expected to inactivate the putative motor domain, so we looked for possible deleterious effects of overexpressing this construct in either wild-type (SLY87) or *smy1Δ* (SLY86) cells, but did not observe any; truncated Smylp had no effect on actin, Myo2p, or tubulin localization.

Double Labeling of Actin and Myo2p or Smylp

To compare the localization of Myo2p, Smylp, and actin, we have labeled cells (strain SLY87 ± YEpSMY1-26) with rhodamine-phalloidin and either anti-Myo2p or anti-Smylp antibody. Smylp and Myo2p are concentrated in the same

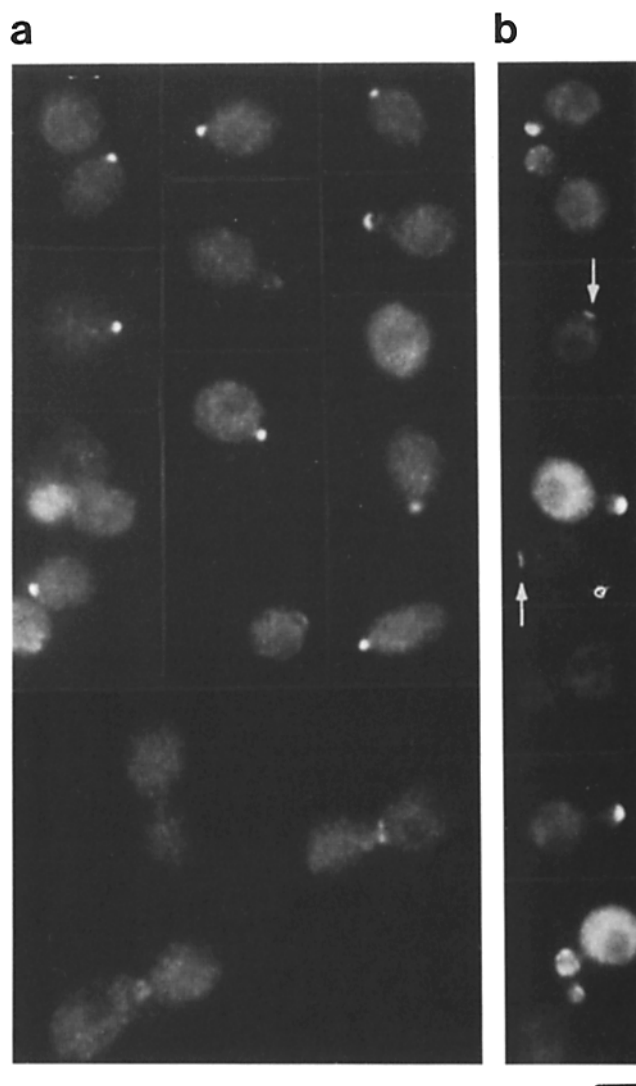


Figure 3. Immunolocalization of Smylp. (a) Wild-type cells (SLY87) labeled with anti-Smylp antibody. Labeling of the mother-bud neck can be seen on the two large-budded cells at the bottom of the panel. (b) Wild-type cells carrying *SMY1* on a high-copy-number plasmid (SLY87 + YEpSMY1-26), labeled with anti-Smylp antibody. Arrows indicate unbudded cells with flat patches of Smylp, which are morphologically distinct from the caps of Smylp seen elsewhere in the figure. The staining of cells overexpressing *SMY1* is actually much brighter than is apparent in this figure; they were underexposed relative to the cells in panel *a* to achieve an optimum appearance. Bar, 5 μ m.

general regions of the cell as actin spots, at the same times during the cell cycle and during mating (Fig. 4). Cells with Myo2p or Smylp at the mother-bud neck also had actin clustered at the neck, confirming that the timing of this localization was similar. Unbudded cells with flat patches of Smylp or Myo2p also had in the same region large faint clusters of actin that are characteristic of residual neck staining (Haarer, B.K., unpublished results; Kim et al., cited in Ford and Pringle, 1991; Lew and Reed, 1993). Unbudded cells with caps of Smylp or Myo2p also had in the same region smaller brighter clusters of actin spots that are characteristic of incipient bud sites (Haarer, B.K., unpublished

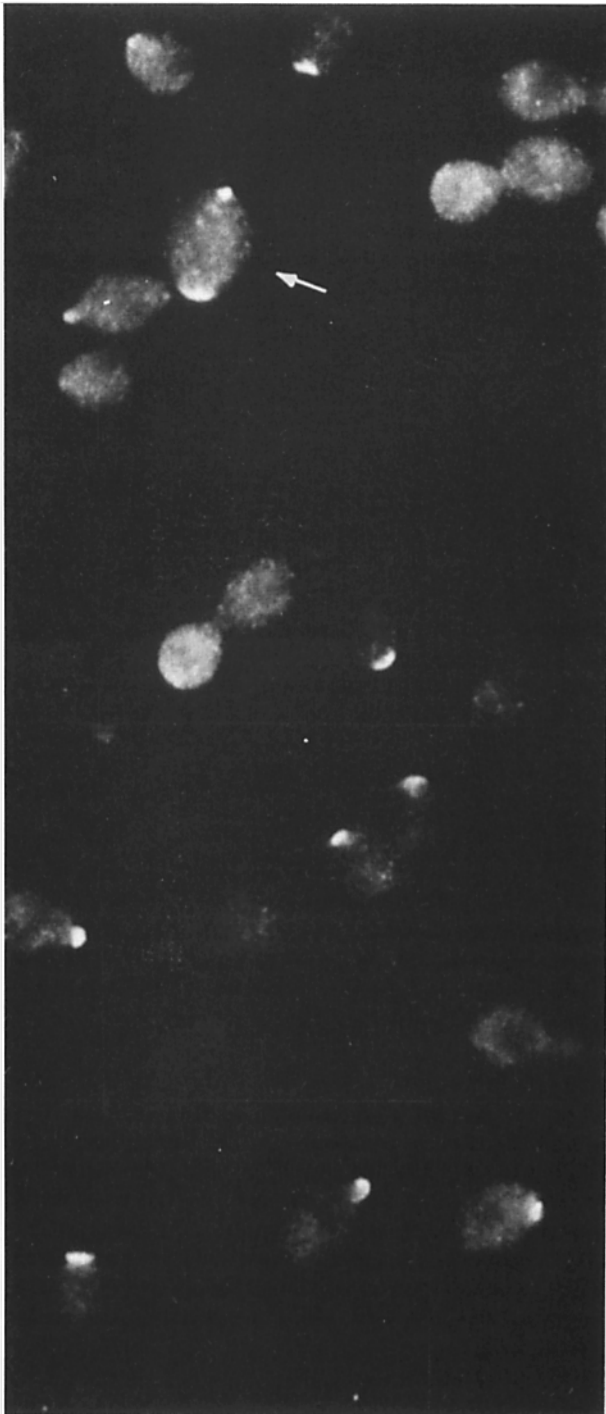
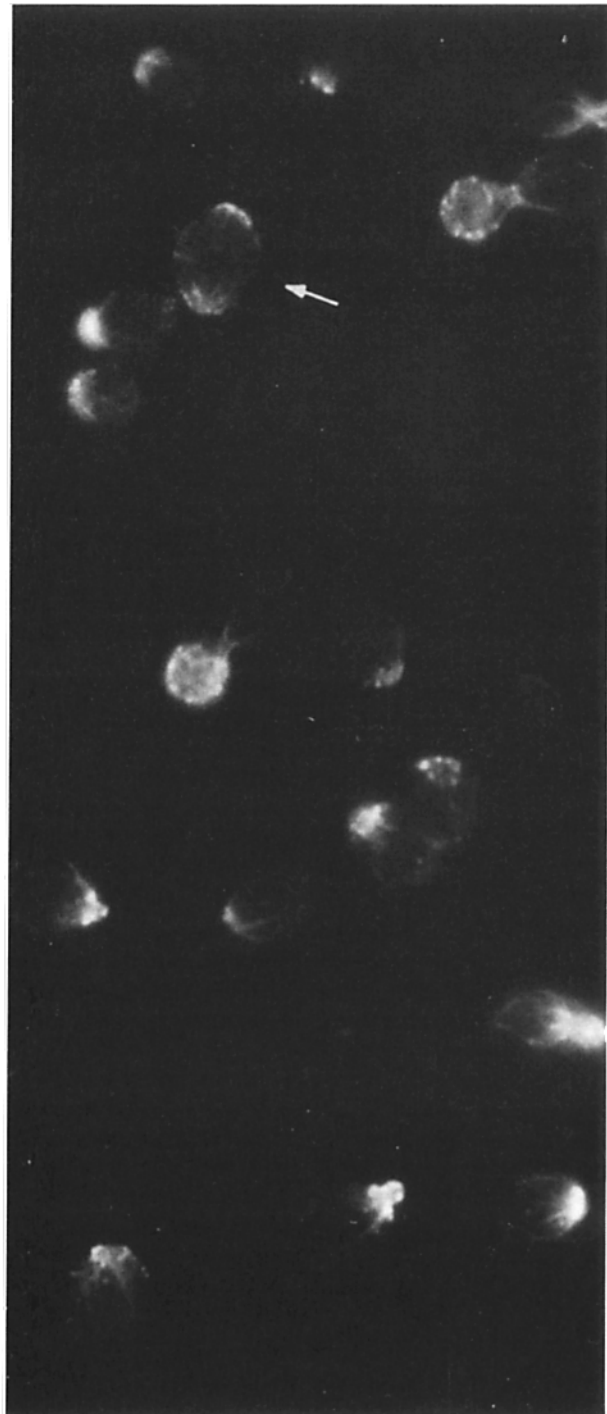
a**b**

Figure 4. Double labeling of Myo2p and actin in wild-type cells. Cells (SLY87) were double labeled with anti-Myo2 antibody (*a*) and rhodamine-phalloidin (*b*). Arrows indicate a cell displaying both a cap and a flat patch of Myo2p, with actin spots clustered in the same regions. Bar, 5 μm .

results; Adams and Pringle, 1984; Lew and Reed, 1993). These comparisons, which are most readily made in cells having both a cap and a patch (see cell indicated by the arrows in Fig. 4, *a* and *b*), constitute a second piece of evidence that the cap marks the incipient bud site, whereas the patch marks the site of the previous mother-bud neck.

Although we cannot readily do Smy1p/Myo2p double labeling, comparisons to the actin staining indicate that Myo2p and Smy1p effectively colocalize; they are found in the same regions of the cell and their localization patterns are indistinguishable. In contrast, although actin spots are also concentrated in the same general regions of the cell,

they do not colocalize with Smylp/Myo2p. In unbudded cells, the Smylp/Myo2p caps or flat patches overlie clusters of actin spots, but do not appear to be made up of a cluster of spots themselves. In some cells, the cluster of actin spots can be seen to form a ring; in this case, the Smylp/Myo2p cap or patch often lies within the ring (Fig. 4). In small buds, the Smylp/Myo2p cap often appears to lie in a region devoid of actin spots. We looked for, but did not find any evidence that Smylp/Myo2p might be associated with actin cables. However, we cannot rule out such a localization, especially given the background glow.

We have asked whether actin or Myo2p arrives first at the incipient bud site, as this might tell us something about the mechanism by which they localize. All unbudded cells with Myo2p (or Smylp) caps had a cluster of actin spots in the same region, but some cells with a cluster of actin spots had no detectable Myo2p or Smylp cap. It may be that actin arrives first, but due to the variability and fainter staining of Myo2p, we cannot rule out the possibility that Smylp and/or Myo2p arrives at about the same time.

Mutants with Multiple Buds

The temperature-sensitive mutants, *cdc4* and *cdc11*, both produce multiple elongate buds at restrictive temperature, with striking actin cables and polarized cortical spots in a subset of the buds (Adams and Pringle, 1984). Using Con A pulse labeling, Adams and Pringle (1984) have demonstrated that only those buds containing polarized actin are actively growing, at the bud tips. Therefore, these cells are especially well suited to comparisons of actin and Smylp or Myo2p distributions, as we can ask which buds have Smylp or Myo2p caps. Double labeling of *cdc4* (314D5) or *cdc11* (JPT194-HO1) cells after 4 or 6 h at restrictive temperature (36°C) all yielded identical results. We confirmed that actin was concentrated in a subset of the elongate buds, both as spots clustered at the tips, and as prominent cables (Fig. 5, *b* and *d*). This same subset of buds displayed Myo2p caps (Fig. 5 *a*) and (in experiments where staining was optimal) Smylp caps (Fig. 5 *c*). Conversely, no Myo2p or Smylp caps were detected at bud tips that lacked actin. Interestingly, while the Smylp/Myo2p staining was brighter than the actin staining at a number of bud tips, the opposite was true for small projections, which we take to be new buds (data not shown). (Examples of such small projections can be seen in Fig. 5 *e*.) Since actin cables are more prominent in these mutants than in wild-type cells, we again looked for but were still unable to detect any obvious association of Myo2p or Smylp with the cables.

In contrast to the distribution of Smylp, Myo2p, and actin (Fig. 5 *f*), staining of the *cdc4* mutant for tubulin (Fig. 5 *g*) confirmed that virtually every bud contains a prominent bundle of cytoplasmic microtubules (Adams and Pringle, 1984). By extrapolation, some buds have microtubules but not Smylp or Myo2p caps. Thus, there is a correlation between Smylp/Myo2p localization, actin localization, and regions of cell growth in these mutants, but there is not a correlation between buds with Smylp/Myo2p caps and those with microtubules.

Temperature Shift

Shifting wild-type cells from RT to 36°C (cells grow well at both temperatures) causes a dramatic, although transient,

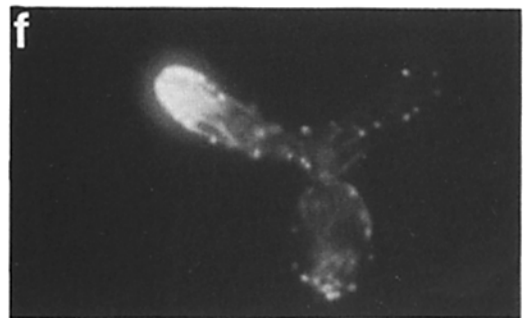
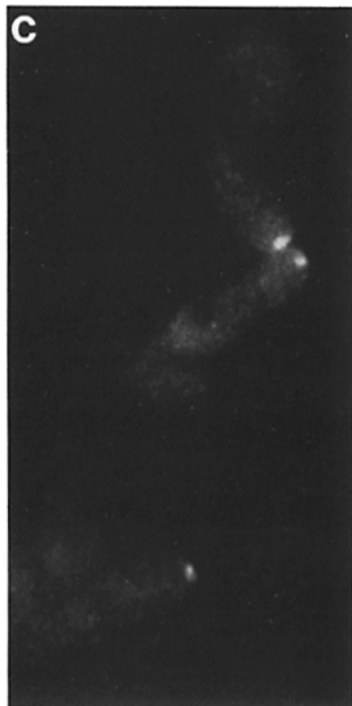
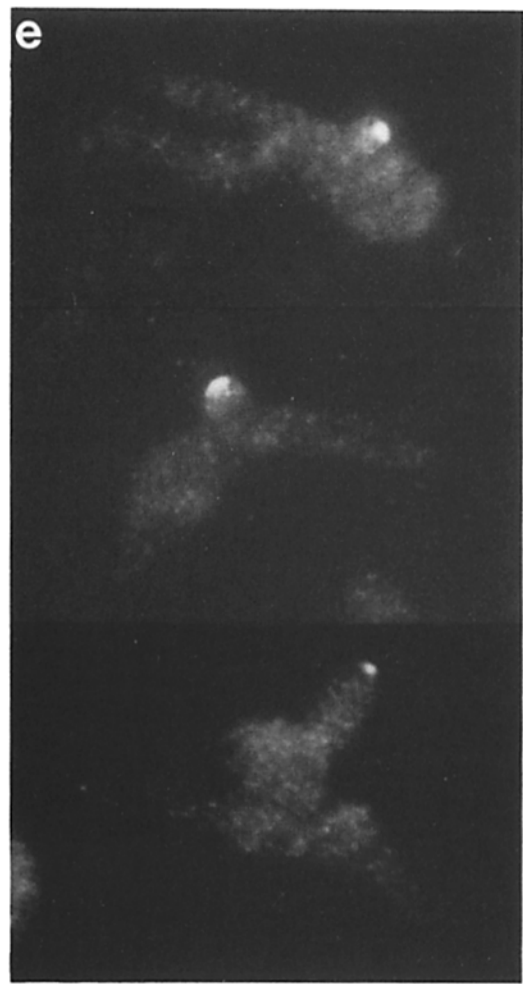
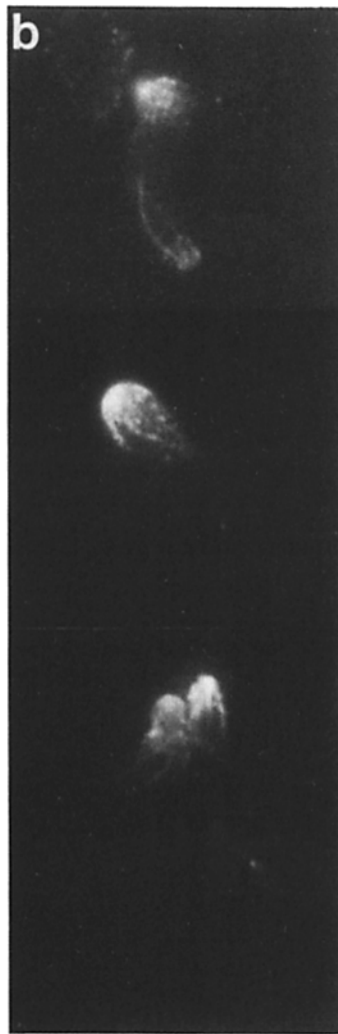
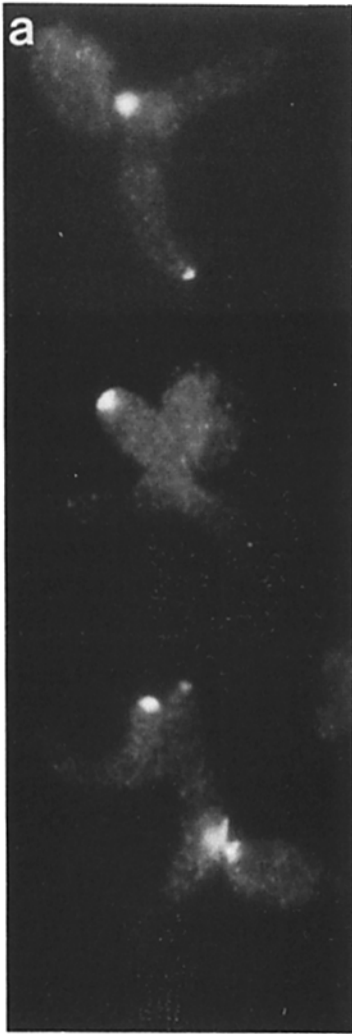
rearrangement of the actin cytoskeleton (Palmer et al., 1992; Lew and Reed, 1993). We would like to understand as much as possible about the mechanics of this rearrangement, for two reasons. First, we would like to study the localization of Myo2p and Smylp in various temperature-sensitive mutants, and perturbation of actin upon temperature shift is a complicating factor. Second, we would like to look at what happens to Myo2p and Smylp localization in wild-type cells when actin is perturbed (recognizing that temperature shift also has many other effects on the cell).

Therefore, we have looked closely at the timing of several changes in the actin cytoskeleton upon temperature shift of wild-type cells (C276). The earliest event that we observed was a rapid but transient reduction in filamentous actin (Fig. 6), quantitated fluorometrically using extracts of cells labeled with rhodamine-phalloidin (Howard and Oresajo, 1985). The amount of filamentous actin decreased 25–50% within 30 s, and returned to initial levels within 3 min of the shift. Fluorescence microscopy of cells stained with rhodamine-phalloidin revealed that actin cables became fainter or disappeared at about the same rate as filamentous actin was lost. However, the cables did not reappear as rapidly, taking 2 h or more to return to their original intensity. Actin spots (which are also composed of filamentous actin) lost their localization to the bud or incipient bud site more slowly; maximal loss of polarization was seen at 1/2 h. Recovery of spot polarization appeared complete within 2 h. We also confirmed an earlier observation that there is a transient increase in the frequency of unbudded cells upon temperature shift (Plesset et al., 1987); this was maximal at 1 h.

Next, we asked whether Myo2p or Smylp localization were also affected by temperature shift. Wild-type cells (SLY87) were shifted from RT to 36°C, fixed at intervals from 5 min to 2 1/2 h, and parallel aliquots were stained with antibody to Myo2p, Smylp, actin, or tubulin. (Mock-shifted cells were indistinguishable from untreated cells.) Actin changes were as described above, except cables were detectable longer (for 10 min) and returned earlier (45 min) after temperature shift (Fig. 7 *a*). This difference was not unexpected, as we (and others; Adams and Pringle, 1991) have found that actin antibody is more sensitive in detecting cables than is the rhodamine-phalloidin used in the previous experiments. As expected, the arrangement of microtubules was not grossly altered by temperature shift (Palmer et al., 1992 have previously shown that although spindles become misoriented upon temperature shift, there are no gross changes in microtubule morphology).

We found that Myo2p localization was also perturbed by temperature shift (Fig. 7 *b*). Furthermore, Myo2p caps disappeared and reappeared with a time course similar to that of actin spot depolarization and repolarization (compare Fig. 7, *a* to *b*). We noted that Myo2p caps were significantly brighter upon recovery from temperature shift (75 min) than in mock-shifted or untreated cells (Fig. 7 *b*). In addition, medium to large buds often displayed several brightly staining regions upon recovery, a pattern that was seldom seen in unperturbed cells (although this same pattern was also seen in cells overexpressing *SMY1*; Fig. 8 *c*). In contrast, no increase in the intensity of actin staining was noted (although this staining was brighter to begin with).

Examination of Smylp staining revealed that it, too, was sensitive to temperature shift (Fig. 7 *c*). Smylp caps, which were initially fainter than Myo2p caps, appeared to be lost



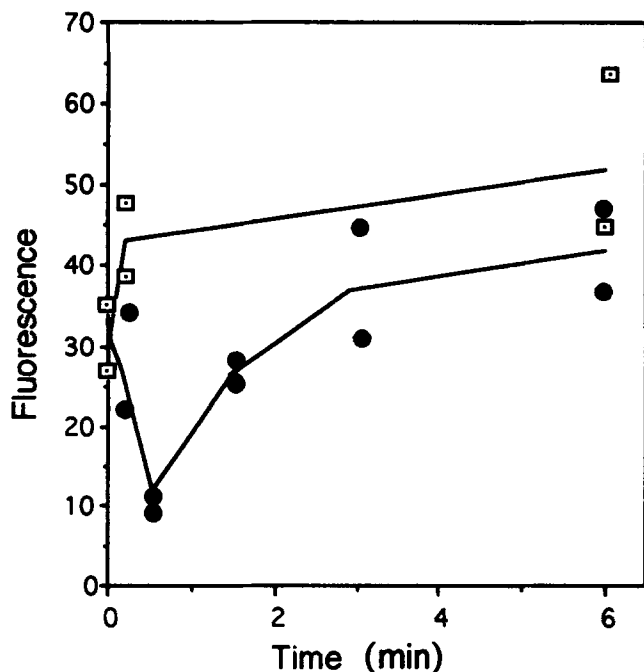


Figure 6. Effect of temperature shift on the amount of filamentous actin. Duplicate aliquots of wild-type cells (C276) were shifted to 36°C (closed circles) or mock-shifted to RT (open squares). The amount of rhodamine-phalloidin that bound to cells fixed at various timepoints after temperature shift is expressed in arbitrary fluorescence units.

earlier. However, they recovered with a similar time course as Myo2p caps, and also appeared brighter upon recovery. Most likely Myo2p and Smylp respond identically to temperature shift, with the apparent differences being due to a detection problem with Smylp. The similarity to the time course of actin rearrangement suggests but by no means proves that Myo2p and Smylp localizations may both be dependent on actin.

In contrast to the behavior of Myo2p caps, Myo2p staining at mother-bud necks and in flat patches on unbudded cells was insensitive to temperature shift (Fig. 7 b). This observation further supports the notion that the flat patches are leftovers from the neck staining of the previous cell cycle, and raises the interesting question of why staining in this region behaves differently. It was difficult to determine whether Smylp necks and flat patches were also temperature-insensitive, as they were difficult to detect at RT or at early times after temperature shift. (They did stain brightly at 75 min and 2 h after temperature shift, the times at which Myo2p staining had also become brighter.) Therefore, we examined cells overexpressing *SMY1* (SLY87 + YEpSMY1-26), in which the Smylp staining should be more detectable. In these cells, necks and flat patches stained as well for Smylp as for Myo2p at 15 and 45 min after temperature shift,

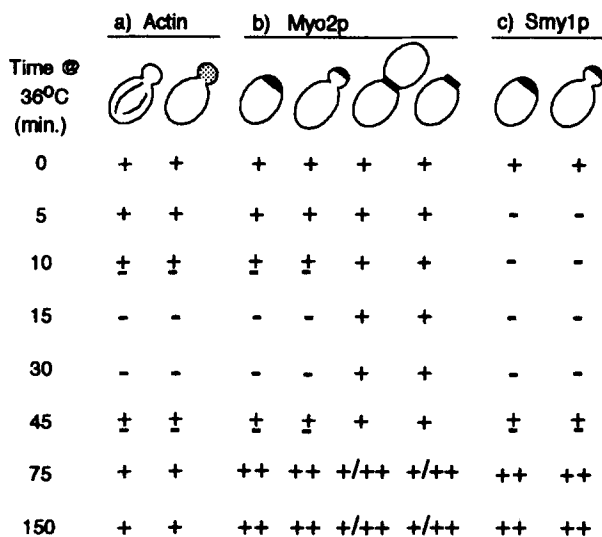


Figure 7. Effects of temperature shift on Myo2p, Smylp, and actin localization. Wild-type cells (SLY87) were shifted from RT to 36°C for various times, and stained with (a) anti-actin antibody, (b) anti-Myo2 antibody, or (c) anti-Smylp antibody. Cells were scored for (a) the presence of actin cables or actin spot polarization, (b) Myo2p staining at caps (on unbudded or small-budded cells), necks, or flat patches, and (c) staining of Smylp caps. Relative staining intensities of these structures (or degree of polarization of actin spots) at various times after temperature shift are represented qualitatively by pluses and minuses; note that these intensities should be compared only within a column, and not between columns.

whereas Smylp/Myo2p caps were lost (at 15 min). Thus, Smylp staining, like Myo2p staining, at necks and flat patches was stable to temperature shift. We also note that overexpressed Smylp does not prevent cap loss by stabilizing either Myo2p or Smylp.

Actin Mutants

To further explore the possible role of actin in Myo2p and Smylp localization, we looked at Myo2p, Smylp, and actin in temperature-sensitive actin mutants. Wild-type (DBY2003), *act1-1* (DBY1999), and *act1-2* (DBY2018) cells were examined at 20°C (permissive temperature) and after 75 min or 2 1/2 h at 36°C (times when wild-type cells have recovered from temperature shift, and the majority of *act1* mutant cells are still viable; Novick and Botstein, 1985). As expected (Novick and Botstein, 1985), actin spots were not polarized in most *act1* cells, even at 20°C. Furthermore, cells were very heterogeneous in size, including many that were very large and round. We observed Myo2p caps in both *act1* mutants at 20°C, although at a much lower frequency than in wild-type cells. (These caps, when seen, were found in morphologically normal small buds.) At 36°C, caps were rare;

Figure 5. Labeling of a multiply budded mutant. Mutant *cdc4* cells (314D5) grown at restrictive temperature (36°C) were double labeled with anti-Myo2p antibody (a) and rhodamine-phalloidin (b); with anti-Smylp antibody (c) and rhodamine-phalloidin (d); or single labeled with anti-Smylp antibody (e); anti-actin antibody (f); or anti-tubulin antibody (g). The actin staining was less intense in double-labeling experiments (b and d) than it was in single-labeling experiments using either anti-actin antibody (f) or rhodamine-phalloidin, making it more difficult to record the actin staining photographically. For example, we could observe actin as well as Myo2p staining at the neck of the cell shown at the bottom of a and b. Bar, 5 μm.

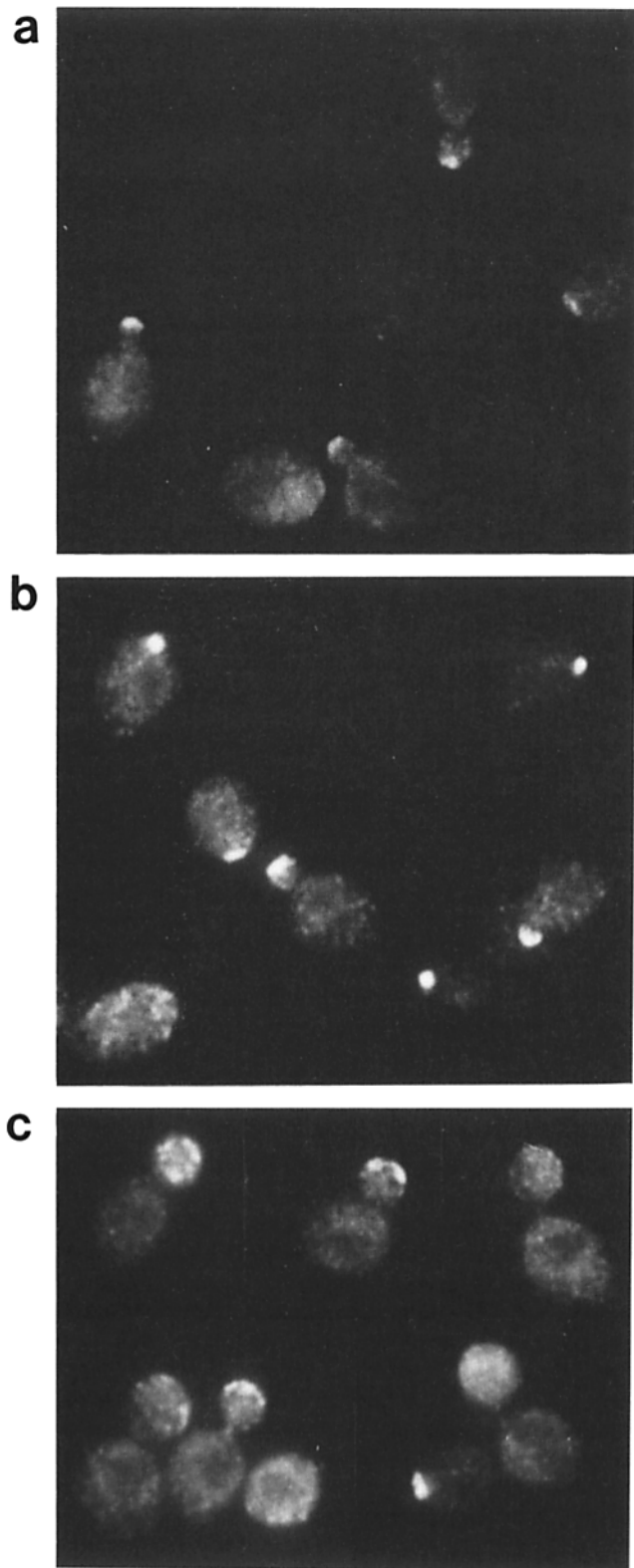


Figure 8. Myo2p localization in wild-type cells upon *SMY1* overexpression. Wild-type cells (SLY87) plus control vector (+ YEp352, *a*) or plus *SMY1* on a high-copy-number plasmid (+ YEpSMY1-26, *b*) were labeled with anti-Myo2p antibody, and printed at the same exposure so that relative intensity of staining can be compared. *c* shows the staining of medium-sized buds, which becomes more pronounced in cells overexpressing *SMY1*. Bar, 5 μ m.

the loss was greater in the *act1-1* than the *act1-2* mutant. We saw no indication that Myo2p localized to the abnormal actin "bars" present in *act1-2* mutants (Novick and Botstein, 1985). Similar results were obtained for Smy1p. Thus, mutations in actin interfere directly or indirectly with Myo2p and Smy1p localization. We hypothesize that the occasional cell at 36°C which has a normal small bud and Smy1p/Myo2p cap may be due to residual actin function. (It seems likely that mutant actin can have residual function in the absence of detectable localization, as the mutants bud and grow relatively normally at permissive temperature even though actin localization appears disrupted; Novick and Botstein, 1985.)

Effects of *SMY1* Dose on Myo2p Localization in Wild-Type and *myo2* Mutant Cells

The colocalization of Smy1p and Myo2p suggested that they might in some way be interdependent. Therefore, we examined the effects of *SMY1* deletion and overexpression on Myo2p localization. Interestingly, overexpression of *SMY1* in wild-type cells (SLY87 + YEpSMY1-26) increased the intensity of not only the Smy1p cap (see above), but also the Myo2p cap, as compared to the wild-type control (SLY87 \pm control vector) (compare Fig. 8, *a* and *b*). Although *SMY1* overexpression enhances Myo2p localization, it did not stabilize Myo2p caps against the effects of temperature shift described above. Deletion of *SMY1*, on the other hand, had little effect; Myo2p caps appeared normal or perhaps slightly fainter in a *smy1* Δ strain (SLY86), both at 20°C and after recovery from temperature shift. We conclude that Smy1p can enhance but is not required for Myo2p localization in wild-type *MYO2* cells.

Given these results, it was clearly of interest to ask if *SMY1* overexpression also enhances localization of mutant Myo2p, as this could tell us something about the mechanism for *SMY1* suppression of the *myo2* mutant defects. First, we examined Myo2p localization in temperature-sensitive *myo2* strains (SLY34, SLY88, SLY91, \pm control vector). At 20°C (Fig. 9 *a*), a temperature at which these mutants grow well, Myo2p localization was reduced significantly as compared to that of either wild-type strains (SLY54, SLY87) or a *myo2* mutant with plasmid-borne wild-type *MYO2* (SLY88 + YCpMYO2). After shifting cells to 30, 32, or 36°C, Myo2p localization disappeared as expected in response to the temperature shift, but did not recover in the *myo2* mutant (after 75 min, 4 3/4 h, 6 h, 12 h, or 18 h) (Fig. 9, *b* and *c*), in contrast to wild-type cells or *myo2* cells carrying YCpMYO2. Strikingly, *SMY1* overexpression, which suppresses the temperature sensitivity of the *myo2* mutant at 30 or 32°C (Lillie and Brown, 1992), also restored the ability of Myo2p to recover its localization. That is, *myo2* mutants carrying *SMY1* on a high-copy-number plasmid (SLY34 or SLY88, + YEpSMY1-26) localized Myo2p at about wild-type levels both at 20°C (Fig. 9 *d*), and at times after shift to 30 or 32°C (75 min, 4 3/4 h, 6 h, 12 h, 18 h) (Fig. 9, *e* and *f*) when wild-type cells (SLY87 \pm control vector) had recovered, but *myo2* mutants with control vector had failed to recover Myo2p localization (Fig. 9, *b* and *c*). Thus, overexpression of Smy1p may suppress the *myo2* defect by participating either in delivery of Myo2p, or in maintenance of its localization. In contrast, overexpression of *SMY2* (a second multi-copy suppressor of *myo2*; Lillie and Brown, 1992; see Introduction) did not restore detectable Myo2p localization

in these mutants, even though at 30°C it suppresses temperature sensitivity nearly as well as does *SMY1*.

While it would be interesting to know whether overexpression of Myo2p can enhance Smy1p localization, such experiments are hard to interpret because of the deleterious effects of Myo2p overexpression (Haarer et al., 1994). More informative was our examination of the effects of the *myo2* mutation on Smy1p localization. Smy1p caps appeared to be slightly fainter in the *myo2* mutant than in wild type at RT. Furthermore, upon temperature shift, Smy1p localization showed the same behavior as did Myo2p localization. That is, the *myo2* mutant (SLY88 + control vector) failed to recover Smy1p localization after shift to 32°C (for 75 min, 4 3/4 h, or 12 h). We conclude not only that Smy1p plays a role in localization of Myo2p, but also that Myo2p plays a role in localization of Smy1p.

Secretory Mutants

We have examined the localization of Myo2p and Smy1p in temperature-sensitive *secl-1* (NY3 × NY4) and *sec6-4* (NY16 × NY17) mutants. These mutants are blocked at a late stage in secretion, and accumulate secretory vesicles in the bud within 15 min of shift to restrictive temperature (Govindan et al., 1994). We reasoned that if Myo2p were associated with secretory vesicles, as proposed by Johnston et al. (1991), we might see an increase in the intensity of Myo2p staining in these mutants. However, this was not the case. What we observed instead was a permanent loss of actin, Myo2p, and Smy1p localizations upon shift to restrictive temperature. Although these localizations were all normal in the mutants at RT, and were lost as expected upon shift to 36°C, neither mutant strain recovered from the temperature shift; after 75 min and 2 h at 36°C, actin spots remained unpolarized and no Myo2p or Smy1p caps were found. We also looked carefully at earlier timepoints (15, 30, and 50 min) for any indication of Myo2p or Smy1p accumulation, but found none. Similar results were obtained with the *sec7-1* mutant (NY757 × NY760), which is blocked at an earlier stage of secretion (Schekman, 1992). We conclude that mutations in these secretory genes interfere directly or indirectly with actin, Myo2p, and Smy1p localizations. The fact that an (putative) increase in secretory vesicles at the bud tip in *secl* or *sec6* mutants does not result in an increase in Smy1p/Myo2p cap intensity indicates either that the mutations interfere with a hypothetical association between Smy1p/Myo2p and the secretory vesicle, or that cap staining does not in fact correspond to groups of secretory vesicles at the bud tip.

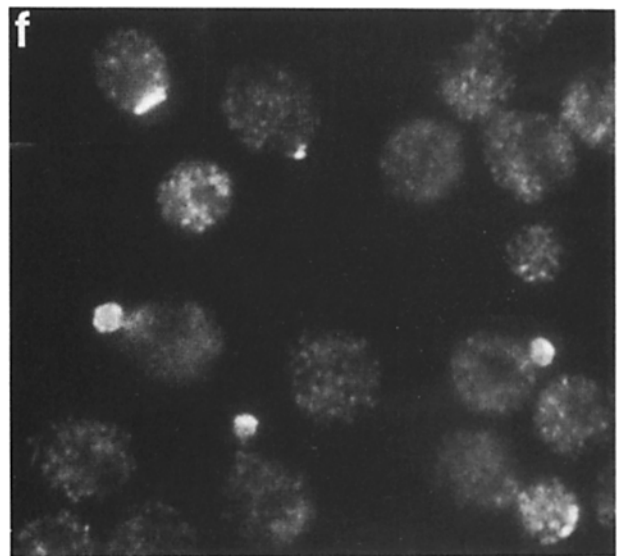
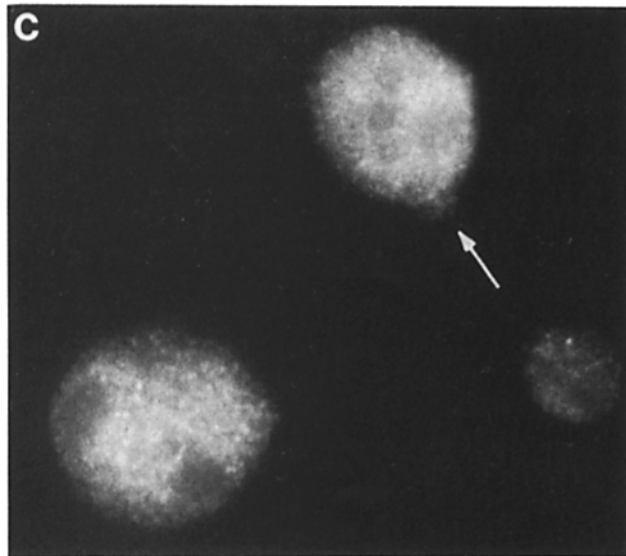
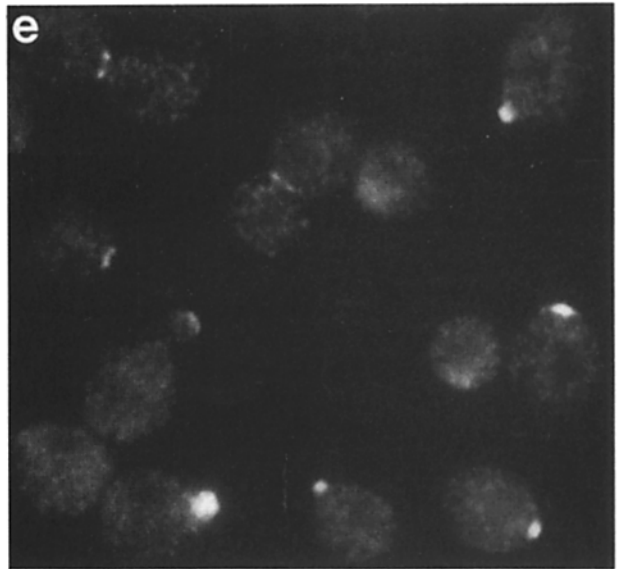
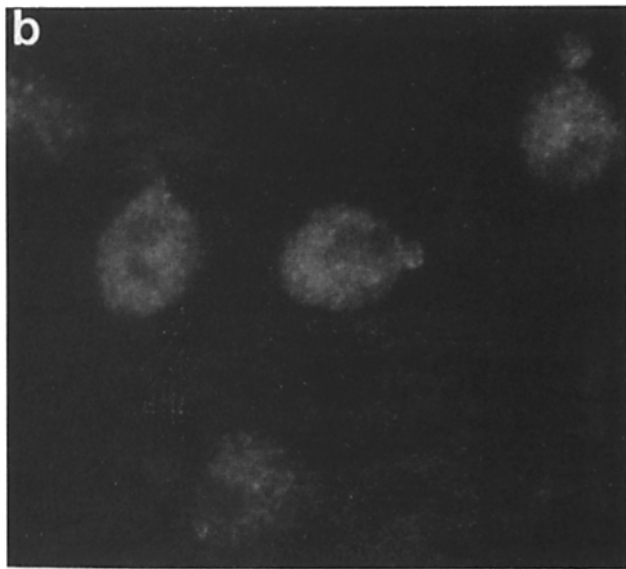
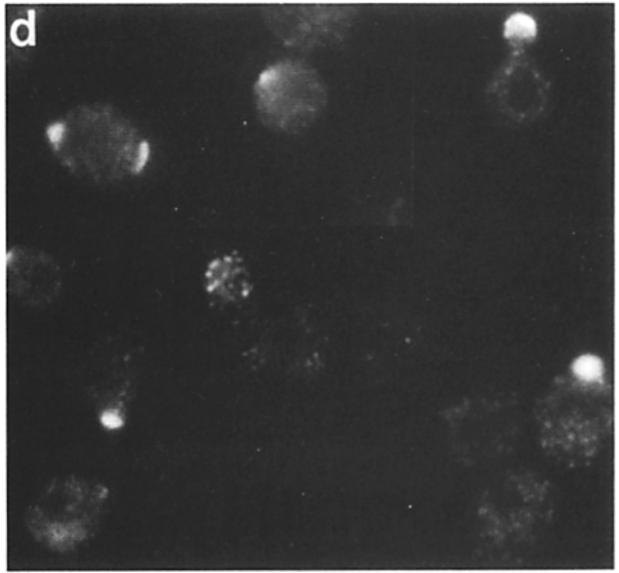
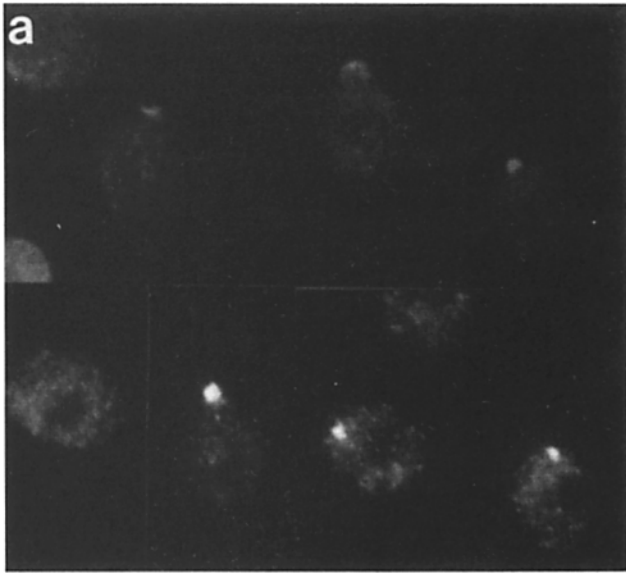
Osmotic Stress

Chowdhury et al. (1992) have shown that osmotic stress induces the same dramatic and transient changes in the actin cytoskeleton that are observed upon temperature shift. We examined the effects of osmotic stress on Myo2p and Smy1p localization for two reasons. First, it was of interest to know whether altering actin localization using a different stress would have the same effects on Myo2p and Smy1p. Second, we wanted to know whether the *myo2* mutant, like strains with actin defects (Chowdhury et al., 1992), might be osmosensitive as well as temperature-sensitive. We examined actin, Myo2p, and Smy1p localizations after addition of 0.4 M NaCl to wild-type (SLY87), *smy1Δ* (SLY86), and *myo2* mu-

tant (SLY88) strains growing at 20°C. Rearrangements of actin, Myo2p, and Smy1p in wild-type cells occurred with the same time course as during temperature shift: at 5 min after addition of salt there were no detectable changes, but by 30 min actin cables had disappeared, actin spot polarization was lost, and Smy1p/Myo2p caps had disappeared. By 2 h the original localizations were restored and Smy1p/Myo2p staining was brighter than before the shift, as observed upon temperature shift. Mock-treated cells of each strain were indistinguishable from untreated cells. Thus, osmotic stress, like temperature shift, promotes changes in Myo2p and Smy1p localization that show a temporal correlation with changes in actin arrangement. Cells lacking Smy1p behaved similarly to wild type, except, of course, for the fact that Smy1p was absent. The *myo2* mutant strain also behaved similarly to wild type, except for the fact that Myo2p (and possibly Smy1p) staining was less intense throughout. The fact that *myo2* cells recovered from osmotic shift but not temperature shift suggests that the defect is not an inability to reform caps after any stress-induced delocalization. The *myo2* mutant strain gave no other indication of being osmosensitive; salt did not affect the growth rate or the wild-type morphology of cells followed for ~ten generations ± 0.4 M NaCl at 20°C. As a control, we confirmed that a parallel aliquot of cells at 30°C developed the morphology expected at restrictive temperature.

Overexpression of *SMY1* Did Not Rescue a *MYO2* Deletion Strain

To learn whether Smy1p can bypass the need for Myo2p, we asked whether *SMY1* overexpression could rescue a *MYO2* deletion (*myo2Δ*) strain, as it does the *myo2* mutant. Johnston et al. (1991) have shown that disruption of *MYO2* is lethal. However, since we have found that the portion of *MYO2* that remains intact in that disruption gives deleterious results when introduced into yeast on a high-copy-number plasmid (Haarer et al., 1994), we have constructed a more complete deletion. We deleted one copy of the gene in a diploid strain by a one-step gene replacement that left only the NH₂-terminal 83 and COOH-terminal 116 codons of *MYO2* marked with the *TRP1* gene (Fig. 10 c). Southern blot analysis confirmed that the expected gene replacement had occurred in the *MYO2/myo2Δ::TRP1* diploid SLY107 (data not shown). Tetrad analysis confirmed that *myo2Δ::TRP1* segregants are unable to grow. Of 12 tetrads, 10 gave 2 live (Trp⁻): 2 dead segregants and 2 tetrads gave 1 live (Trp⁻): 3 dead segregants; most of the "dead" segregants germinated and produced two to four cells. When plasmids were introduced into SLY107, subsequent tetrad analysis showed that *MYO2* on a low-copy-number plasmid (YCpMYO2) rescued *myo2Δ* segregants as expected. However, *SMY1* on high-copy-number plasmid YEpSMY1-38 (like control vector) did not rescue *myo2Δ* segregants: of 31 tetrads, 29 gave 2 live (Trp⁻): 2 dead segregants and the other 2 gave 1 live (Trp⁻): 3 dead segregants. These dead segregants, like those above, germinated but failed to produce more than two to four cells. While we cannot demonstrate directly that the dead *myo2Δ* segregants retained YEpSMY1-38, it seems likely that many did, since both live segregants of 17 tetrads retained plasmid. We also found that *myo2Δ* segregants carrying both YCpMYO2 and YEpSMY1-38 were unable to lose YCpMYO2 even after long periods of growth in medium selecting only



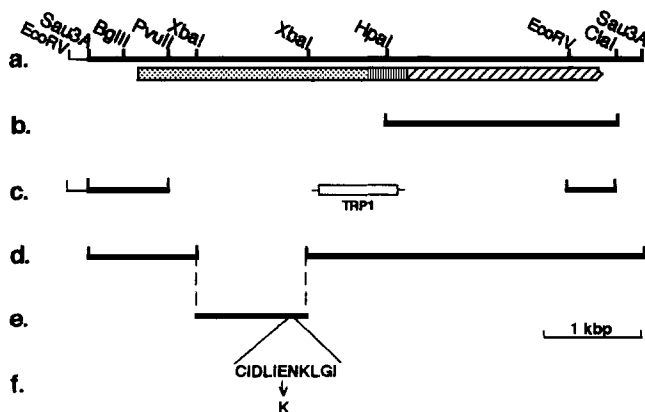


Figure 10. Physical map of the *MYO2* region and constructs derived from it. (a) Restriction map of the *MYO2*-containing insert plus adjacent vector sequence of YCpMYO2. The box indicates the position of the *MYO2* open reading frame, which encodes the head (stippled), neck (vertical stripes), and tail (diagonal stripes) of Myo2p. (b) The fragment of *MYO2* incorporated into gene fusions for production of anti-Myo2p antibodies. (c) The construct used in making the *MYO2* deletion (insert of plasmid pΔM::TRP1). (d) The insert of the gap-repair plasmid, YCpMYO2-12, used to recover the fragment containing the *myo2-66* mutation, shown in e. (e) The fragment containing the *myo2-66* mutation, which was sequenced. (f) The amino-acid sequence alteration caused by the *myo2-66* mutation. Thick lines represent yeast sequences; thin lines represent vector sequences. Not all Bgl II, Eco RV, Hpa I, and Sau3A sites are shown.

for YEpSMY1-38 (~30 generations). (Most wild-type segregants have lost YCpMYO2 under these conditions.) Thus, overexpression of *SMY1* cannot rescue *myo2Δ* spores, and almost certainly cannot replace *MYO2* in vegetatively growing cells.

Mapping and Sequencing the *myo2* Mutation

Since Myo2p has several functional domains, deduced from comparisons to other myosins (Cheney et al., 1993), determining where the defect lies in mutant Myo2p might suggest how it is malfunctioning. Using recombination-mediated marker rescue, we determined that the mutation responsible for temperature sensitivity of the mutant (allele *myo2-66*) lay within a 1-kbp Xba I fragment (Fig. 10 e). This segment was recovered by "gap repair" (Rothstein, 1991) from two different strains (SLY1, SLY33) carrying the *myo2* mutation. Both DNA segments, as well as the corresponding wild-type segment from plasmid YCpMYO2, were sequenced and the only change (in both mutant DNA's) was a G to A transition at the third position of the codon for Glu511, altering it to a Lys (Fig. 10 f). While the marker-rescue experiment showed that this mutation is necessary for temperature sensitivity, it does not rule out the possibility that there are muta-

tion(s) in other regions of *myo2* that are also required. We addressed this point using the low-copy-number plasmids generated by gap repair, which contain the above Xba I segment of either *myo2* or *MYO2* in an otherwise wild-type copy of the gene. These gap repair plasmids were introduced into a *MYO2/myo2Δ* diploid (SLY107) and tetrad analysis was carried out. As expected, the control plasmid carrying the *MYO2* segment rescued *myo2Δ* segregants not only at RT, but also at 36°C, and the cells displayed wild-type morphology. The plasmids carrying the *myo2* segment also rescued *myo2Δ* segregants at RT, but at >30°C these segregants arrested with characteristic *myo2* mutant morphology. Thus, it seems likely that the single mutation is both necessary and sufficient for the temperature sensitivity of the *myo2* mutant.

Rayment et al. (1993) have recently published the crystal structure of the motor domain of a chicken skeletal myosin. We can unambiguously align Glu511 in Myo2p with Glu527 in the chicken myosin, a residue at the actin binding face of the "lower 50K domain." Glu527 forms a salt bridge with Lys 486 (Rayment, I., University of Wisconsin, personal communication), a residue that can be unambiguously aligned with Lys471 in Myo2p. Thus, the alteration of Glu511 to a Lys is likely to perturb the actin binding of Myo2p.

Discussion

Myo2p and *Smy1p* Colocalize to Regions of Polarized Growth

Our finding that overexpression of *Smy1p* can overcome defects in the *myo2* mutant (Lillie and Brown, 1992) raises the question of how a protein with sequence similarity to kinesin can partially compensate for a defect in a myosin. Because this finding is so unexpected, we originally suspected that the mechanism might be rather indirect. However, immunolocalization results strongly argue for a direct relationship between *Smy1p* and Myo2p, as these proteins appear to colocalize under all conditions examined. They show the same rearrangements during the vegetative cell cycle and in response to mating. They also show the same response to either temperature or osmotic shift; caps of *Smy1p* and Myo2p are perturbed and recover with the same time course, while neck and flat-patch staining of both are unperturbed. Mutations in actin or secretory genes have the same effect on *Smy1p* and Myo2p. Furthermore, overexpression of *Smy1p* enhances Myo2p localization in wild-type cells, and restores Myo2p localization in the *myo2* mutant at restrictive temperature. These findings suggest that *Smy1p* and Myo2p may be physically associated, although we know little about the nature of the association. *Smy1p* may bind directly to Myo2p, or be indirectly associated in a supramolecular complex. Perhaps *SMY1* overexpression suppresses the *myo2* mutation but not *MYO2* deletion because *Smy1p* is binding to and

Figure 9. Myo2p localization in the *myo2* mutant. Mutant cells (SLY88) carrying (a-c) control vector (YEp352) or (d-f) *SMY1* on a high-copy-number plasmid (YEpSMY1-26) were incubated at 20°C (a and d) or at 32°C for 75 min (b and e) or 4 3/4 h (c and f), and labeled with anti-Myo2p antibody. Exposure times of all panels were matched so that the intensity of staining can be compared. The cells shown in the montage in a were chosen to show the range of staining intensities seen at permissive temperature. After 4 3/4 h at restrictive temperature, the increase in cell size originally reported by Johnston et al. (1991) is obvious (c), and is suppressed by *SMY1* overexpression (f). Although buds are rare on *myo2* mutant cells at this time point, we have shown an example in c (arrow), in order to make the point that Myo2p staining is not seen in these buds. Bar, 5 μm.

stabilizing the mutant Myo2p. However, it is not necessary to invoke direct binding of Smylp to Myo2p to explain the suppression results. An alternative explanation is that *MYO2* deletion causes a more severe defect than the *myo2* mutation, so that more Smylp function is needed to compensate. This fits with our observations that no Smylp is required when wild-type Myo2p is present (in *smylΔ* strains), the normal level of Smylp is required to compensate for the defect in mutant Myo2p at room temperature (as *smylΔ myo2* double mutants are inviable; Lillie and Brown, 1992), overexpression of Smylp is required at 30–32°C when there may be a more severe defect in mutant Myo2p, and even overexpression of Smylp is insufficient at 36°C (or at any temperature when *MYO2* is deleted).

We conclude not only that Smylp and Myo2p are likely to be associated, but also that they probably participate in the same function of polarized growth. This conclusion is based on the phenotype of the *myo2* mutant (Johnston et al., 1991 and see Introduction), its partial suppression by overexpression of *SMY1* (Lillie and Brown, 1992), and immunolocalization of both Smylp and Myo2p to sites of polarized growth. Similar localizations have been seen for Bem1p, Spa2p, and Cdc42p, all of which are also implicated in this process. Bem1p has been implicated in bud site assembly (Chant et al., 1991; Chenevert et al., 1992), and its immunolocalization pattern (Corrado, K., University of Texas, and J. Pringle, University of North Carolina, personal communication) is very similar to that of Smylp/Myo2p. Cdc42p is a Rho-like GTPase which participates in a step of bud-site assembly that is upstream of actin (Johnston and Pringle, 1990; Ziman et al., 1991). It immunolocalizes to the same regions of the cell as Myo2p (Ziman et al., 1993). Spa2p, on the other hand, is not required for bud formation and growth, but does appear to be involved in the polarized growth that occurs in mating cells (Gehring and Snyder, 1990). The published Spa2p localization (Snyder et al., 1991) differs from Smylp/Myo2p localization in that Spa2p persists as a cap in medium and large buds. However, Spa2p is concentrated in all of the same regions as Smylp/Myo2p, and many aspects of its behavior are similar; it is found only in the subset of buds that contain clusters of actin in multiply budded mutants, and it is not completely delocalized in *act1-1* and *act1-2* mutants (Snyder et al., 1991). Another protein with a similar localization pattern (Novick and Brennwald, 1993) is Sec4p, a Rab GTPase implicated in fusion of secretory vesicles with the plasma membrane. Finally, calmodulin localization is indistinguishable from that of Myo2p/Smylp, presumably because calmodulin binds to Myo2p (Brockerhoff et al., 1994).

Alteration of Smylp and Myo2p Domains

We investigated which domains of Smylp and Myo2p might be required for localization. Both of these proteins are predicted to have the organization typical of motor proteins, including a head with putative motor activity, and a tail which may attach to a "cargo." Truncation of Smylp indicates that the COOH-terminal portion of the tail is required for localization (in a *smylΔ* background), and for suppression of the growth defect of the *myo2* mutant. We do not know currently whether the head of Smylp is also required. (We do know that overexpression of truncated Smylp, which presumably still has an active head, does not interfere with wild-type Myo2p localization.) In the case of Myo2p, mapping of

the *myo2* mutation to the head, together with the observation that mutant Myo2p does not localize well, suggests that the head is involved in localization. It is more difficult to assess whether the tail is also required, because we can examine the behavior only of functional Myo2p's in the (lethal) *MYO2* deletion strain. However, Myo2p caps do appear brighter when a truncated Myo2p is overexpressed in otherwise wild-type cells (unpublished observations). Thus, either (part of) the tail is not required for localization, or wild-type Myo2p can help localize the truncated Myo2p, perhaps by dimerization.

The Role of Actin Filaments and Microtubules

Since the sequences of Smylp and Myo2p suggest that they could be motor proteins that move along microtubules and actin, respectively, an important question is what role these filaments play in Smylp and Myo2p function. Several pieces of evidence implicate actin but not microtubules. First, the *myo2* mutation is predicted to alter an actin contact site, based on the structure of chick myosin (Rayment et al., 1993). Second, the similarity in the phenotypes of actin and *myo2* mutants (Johnston et al., 1991) indicates that Myo2p, like actin, functions in polarized cell growth. In contrast, microtubules are not normally required for polarized growth (see Introduction). Third, actin, Smylp, and Myo2p all localize to regions of polarized growth, and undergo the same rearrangements at each stage in the cell cycle. Lew and Reed (1993) present evidence that actin rearrangements are under the control of cell-cycle-specific cyclins. G1 cyclins appear to be involved in bud initiation, triggering the assembly of a cluster of actin spots at the incipient bud site. Smylp/Myo2p arrive at the incipient bud site either simultaneously with or immediately after actin. Early on, buds grow at their tips, but later on are induced to switch to isotropic growth by a subset of G2 cyclins. Smylp/Myo2p caps are localized at the growing tips of small buds, but can not be visualized in older buds; perhaps discrete caps disappear coincident with the switch to isotropic growth. A failure to make this switch can result in elongate buds, similar to those seen in multiply budded mutants. The fact that a number of the buds in such mutants retain Smylp/Myo2p caps, even though the buds are much larger than normal, argues that the normal loss of caps is not simply due to dilution during bud growth, but may instead result from the switch. Later in the cycle, inactivation of the cyclin-kinase complex appears to be responsible for the rearrangement of actin to the mother-bud neck. Smylp and Myo2p are also concentrated at the neck at this time. Actin persists at the neck for a while after completion of cell separation or septation, as a large faint cluster of actin at the site of the former neck sometimes can be seen in unbudded cells (Kim et al., cited in Ford and Pringle, 1991; Lew and Reed, 1993). Smylp/Myo2p appear to persist in like manner, as flat patches associated with the clusters of actin.

Actin and Smylp/Myo2p behave similarly under other conditions. First, in the multiply budded *cdc4* mutant, only the subset of buds that have a concentration of actin spots and prominent cables also have Smylp/Myo2p caps. Second, although many changes occur upon temperature or osmotic shift, there is a striking temporal correlation between the dramatic rearrangements of actin spots and those of Smylp/Myo2p in both situations. Finally, all three are affected in actin and secretory mutants. In contrast, microtubules are not

grossly affected under these various conditions (although spindles are transiently disoriented upon temperature shift; Palmer et al., 1992). The subset of buds in a *cdc4* cell that have microtubules but lack concentrations of actin also lack Smylp/Myo2p caps. Furthermore, using the *cdc4* mutant, we have observed that nocodazole does not abolish Smylp/Myo2p caps, during a period of hours after microtubules have disappeared (Lillie, S. H., and S. S. Brown, manuscript in preparation). We conclude that while Myo2p is likely to interact with actin, there is no evidence to date that Smylp interacts with microtubules. Instead, there is evidence that Smylp interacts with Myo2p and/or actin in some way. Since neither microtubules (Jacobs et al., 1988; Huffaker et al., 1988) nor Smylp (Lillie and Brown, 1992) are required for polarized growth in wild-type cells, but Smylp is required in the *myo2* mutant, it will be interesting to determine whether microtubules are also required in the mutant.

Temperature and Osmotic Shift

The earliest change we have detected upon temperature shift is a reduction in polymerized actin. Actin cables seem to be affected first, followed by concomitant effects on the arrangement of Myo2p, Smylp, and actin spots. Possibly, actin cables might cause/direct Myo2p/Smylp localization. There are several aspects of the response to temperature shift that are unexplained. We do not know why actin depolymerizes, but suspect that depolymerization is not specific to temperature shift and may occur in response to other stresses that result in actin rearrangement (see below). We do not know why it takes so much longer to restore the proper actin, Myo2p, and Smylp localization than it takes for the actin to repolymerize. Perhaps actin can only reorganize properly at certain times during the cell cycle (see the discussion of the cell-cycle control of actin by different cyclins, above). We also do not know why the Myo2p/Smylp staining at the mother-bud neck is resistant to temperature shift (Fig. 7). Perhaps it is again a matter of different cell cycle controls.

The effects of temperature shift can confound interpretation of the phenotype of a temperature-sensitive mutant. For example, in the first hour after shift to restrictive temperature, the loss in actin and Smylp/Myo2p organization is superimposed on the effects of the mutation. Failure to recover this organization at later times (e.g., by *myo2* and secretory mutants) is hard to interpret. Even though these mutants do not show a large loss of viability in an hour, they nonetheless may be too sick to reorganize their actin properly. Alternatively, we may be seeing a direct effect of *myo2* or secretory mutations on actin. Perhaps Myo2p participates in actin organization (by anchoring it to the bud tip?), or perhaps Myo2p and secretory vesicles are needed to replenish a polarized distribution of membrane components that organize actin.

Other perturbations may also complicate interpretation of phenotype. Rearrangements in actin have been documented not only upon temperature shift (Palmer et al., 1992; Lew and Reed, 1993), but also in response to a variety of other stresses. Chowdhury et al. (1992) have demonstrated that osmotic shock gives rise to the same changes that are seen with temperature. Novick et al. (1989) found that cycloheximide, azide, or removal of glucose could also cause a loss of actin polarity. The stresses can be subtle; Pringle et al. (1989) have observed better preservation of actin polarity when fixative

is added directly to cells shaking in medium than when cells are first washed. Thus, actin rearrangement is easily induced, and may be a somewhat nonspecific mutant phenotype that can be caused by a variety of metabolic or other changes in the cell. For this reason, actin rearrangement should be interpreted with caution.

Given these caveats, we wondered about the specificity of the *myo2* mutant phenotype. The actin mutants, *act1-1* and *act1-2*, cannot recover from either temperature or osmotic shift (Novick and Botstein, 1985; Chowdhury et al., 1992), and may simply be incapable of recovering from any stress-induced loss of actin polarity. In contrast, we find that the *myo2* mutant can recover from osmotic (although not temperature) shift. Two interpretations of this specificity are possible. Either the mutant Myo2p molecule is itself temperature sensitive but not osmotically sensitive, or "dosage" (the reduction in Myo2p function) is more crucial at elevated temperature than at high osmolarity. Dosage is a plausible explanation for the osmosensitivity of actin mutants, as a diploid lacking one of its copies of the wild-type actin gene (*ACT1/act1Δ*) is also osmosensitive (see reference to Shortle in Chowdhury et al. [1992]). However, as a *MYO2/myo2Δ* diploid is not thermosensitive (Johnston et al., 1991), this level of Myo2p reduction is not sufficient to cause temperature sensitivity, and the dosage hypothesis may not obtain.

Possible Interpretations

How might Smylp and Myo2p function in polarized growth and secretion? Myo2p appears to be required for delivery of vesicles to the bud. If vesicle fusion with the plasma membrane is blocked (in *secl* or *sec6* mutants), vesicles build up in the bud; however, they build up in the mother cell of the *myo2* mutant or of double mutants (Govindan et al., 1994). Perhaps Myo2p is a secretory vesicle motor, as originally proposed by Johnston et al. (1991) based on their characterization of the *myo2* mutant. In this case, Smylp might enhance Myo2p localization either by acting as a second vesicle motor or by somehow enhancing Myo2p activity. Another possibility is that Smylp/Myo2p are part of a complex of proteins (perhaps including Bemlp, Cdc42p, and/or Spa2p) that sit at the incipient bud site and are involved in bud site assembly and/or bud growth. If this is the case, we speculate that Myo2p may anchor actin filaments, which may in turn act as railroad tracks for the polarized delivery of vesicles to the bud. In an attempt to distinguish between these possibilities, we asked whether Smylp/Myo2p staining in the bud increased in *secl* or *sec6* mutants when vesicles reportedly build up. Since the staining did not increase (and in fact was lost under these conditions), we suspect that Smylp/Myo2p caps do not correspond to accumulations of vesicles at the site of fusion in the bud tip. However, we cannot rule out the possibility that these mutations somehow interfere with the hypothetical binding of Smylp/Myo2p to vesicles. An alternative possibility is that Smylp/Myo2p are not abundant enough on vesicles to be visualized, but become concentrated at the plasma membrane at the site where the vesicles fuse.

A puzzling aspect of the *myo2* mutant phenotype is that although vesicles build up in the mutant, several labs have been unable to demonstrate any dramatic build-up of secretory product within the cell (Govindan et al., 1994; Liu and Bretscher, 1992). Based on studies of a-factor receptor, the explanation may be that Myo2p functions in recycling vesi-

cles, rather than in the delivery of secretory vesicles (Govindan et al., 1994).

Although it seems likely that Myo2p is an actin-based motor, we have not yet tested Myo2p for motor activity in *in vitro* motility assays. Furthermore, there is no evidence that Smylp is a microtubule-based motor. To the contrary, our results suggest that microtubules may not be required for Smylp localization, at least in cells that are wild type for *MYO2*. It will be interesting to ask whether microtubules are required under conditions where Smylp function is required, that is, in the *myo2* mutant.

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