Suppression of the Profilin-Deficient Phenotype by the RHO2 Signaling Pathway in *Saccharomyces cerevisiae*

Nathaly Marcoux, Simon Cloutier, Ewa Zakrzewska, Pierre-Mathieu Charest, Yves Bourbonnais and Dominick Pallotta

Pavillon Charles-Eugène Marchand, Laval University, Ste-Foy, Quebec G1K 7P4, Canada

Manuscript received December 20, 1999
Accepted for publication June 26, 2000

ABSTRACT

Profilin plays an important role in actin organization in all eukaryotic cells through mechanisms that are still poorly understood. We had previously shown that Mid2p, a transmembrane protein and a potential cell wall sensor, is an effective multicopy suppressor of the profilin-deficient phenotype in *Saccharomyces cerevisiae*. To better understand the role of Mid2p in the organization of the actin cytoskeleton, we isolated five additional multicopy suppressors of *pfy1*Δ cells that are Rom1p, Rom2p, Rho2p, Smy1p, and the previously uncharacterized protein Syp1p. The problems of caffeine and NaCl sensitivity, growth defects at 30° and 37°, the accumulation of intracellular vesicular structures, and a random budding pattern in *pfy1*Δ cells are corrected by all the suppressors tested. This is accompanied by a partial repolarization of the cortical actin patches without the formation of visible actin cables. The overexpression of Mid2p, Rom2p, and Syp1p, but not the overexpression of Rho2p and Smy1p, results in an abnormally thick cell wall in wild-type and *pfy1*Δ cells. Since none of the suppressors, except Rho2p, can correct the phenotype of the *pfy1-111/rho2*Δ strain, we propose a model in which the suppressors act through the Rho2p signaling pathway to repolarize cortical actin patches.

IN yeast, actin filaments form cortical patches and actin cables. Cortical patches are compact structures that relocalize during the cell cycle in accordance with changes in patterns of growth and secretion. During the isotropic growth in the G1 phase, the cortical patches are uniformly distributed within the cell. At the beginning of S phase they are located at the presumptive bud site, which becomes a site of active growth. As the cycle progresses, the cortical patches become localized almost exclusively in the growing bud. Electron microscopic observation showed that at this stage the cortical patches are associated with invaginations of the plasma membrane, presumably to maintain cell wall integrity during cell growth (Mulholland *et al.* 1994). Finally, during cytokinesis the patches are found at the neck region that separates the mother and daughter cells. Any mutation that affects the location of the cortical patches also affects polarized growth. Cortical patches are thought to be essential for viability, since a viable cell without cortical patches has never been observed (Karpova *et al.* 1998).

Actin cables are long filamentous structures that traverse the cell. They have an uneven thickness along their length, suggesting that they are formed of short overlapping filaments (Karpova *et al.* 1998). Cables and patches are both present throughout the cell cycle and their distribution is coordinated. When patches are polarized the cables are oriented toward the patches and when patches are dispersed cables are randomly oriented. The frequent localization of patches at the ends of cables is further evidence for the association of these two structures.

The distribution and formation of actin cables and patches is controlled by many different proteins. Some, like Cdc42p, are signaling proteins that control actin distribution throughout the cell cycle. Others are structural proteins that bind directly to either filamentous or monomeric actin. Profilin is a small actin monomer-binding protein that influences actin organization. It is clearly involved in actin polymerization, although there is still controversy over whether profilin is a sequestering agent or whether it facilitates polymerization. All known profilins bind to poly-L-proline residues and to proline-rich sequences in proteins. In *Saccharomyces cerevisiae*, Pfy1p binds to the proline-rich formin-homology (FH) domains of Bni1p and Bnr1p (Evangelista *et al.* 1997; Imamura *et al.* 1997). Bni1p is also a target of Rho1p, a Ras-like GTPase, involved in cell wall synthesis and organization of the actin cytoskeleton (Kohno *et al.* 1996). Finally, profilin binds to the membrane phospholipid phosphatidylinositol-biphosphate (PIP_2), which in mammalian cells at least, inhibits its hydrolysis by phospholipase C (Lassing and Lindberg 1985; Goldschmidt-Clermont *et al.* 1990). This suggests a role for profilin in cell signaling, possibly as an intermediate between signaling molecules and the organization of the actin cytoskeleton.

Profilin-deficient cells (*pfy1*Δ) are viable but have an

Corresponding author: Dominick Pallotta, Pavillon Marchand, Laval University, Ste-Foy, Quebec G1K 7P4, Canada.
E-mail: pallotta@rsvs.ulaval.ca

abnormal morphology. Actin cables are no longer visible, and cortical patches are present in the mother cell at all stages of the cell cycle (Haarer *et al.* 1990, 1993; Marcoux *et al.* 1998, 1999). This results in large, round cells that grow slowly and undergo cell lysis at high temperatures. The polarity of the cells is also lost, and the normal axial budding of haploid cells becomes random. In addition, polarized secretion is compromised so that cells accumulate vesicular structures in their cytoplasm and show a lag in the secretion of the α-factor. Maturation of the α-factor is also reduced, which results in a decreased mating efficiency of the *MAT*α strain, but not the *MAT***a** strain (Marcoux *et al.* 1998).

We have previously shown that *MID2* is an effective multicopy suppressor of the profilin-deficient phenotype (Marcoux *et al.* 1998). The acronym *MID* stands for "*m*ating pheromone-*i*nduced *d*eath" since *MID* mutants cannot resume the cell cycle after shmoo formation (Iida *et al.* 1994). Mid2p is a small plasma membrane protein containing a serine/threonine-rich glycosylated extracellular domain, a single transmembrane domain, and a charged intracellular domain rich in aspartic acid residues. In addition to the suppression of the profilin-deficient phenotype, the *MID2* gene was selected as a multicopy suppressor in several different genetic screens. Overexpression of *MID2* suppresses the growth defect of the *cik1*Δ and *kar3*Δ strains, mutants defective in microtubule-based processes (Manning *et al.* 1997), and it can correct the deleterious effects caused by overexpression of protein kinase A, Tpk1p (Daniel 1993). Hcs77p, also called Slg1p or Wsc1p, is a transmembrane protein without any sequence similarity to Mid2p but with domains similar to those found in Mid2p (Gray *et al.* 1997; Verna *et al.* 1997; Jacoby *et al.* 1998; Lodder *et al.* 1999). The *hcs77*Δ cells undergo cell lysis at high temperatures, a phenotype that is corrected by the expression of *MID2* (Ketela *et al.* 1999; Rajavel *et al.* 1999). The phenotypes of temperature-sensitive growth defects and the inability to recover from mating pheromone-induced G1 arrest of the *mpt5*Δ mutation are also corrected by the overexpression of *MID2*. Finally, *MID2* has also been selected as an activator of the Skn7p transcription factor (Ketela *et al.* 1999).

An explanation for these seemingly disparate results was provided by recent work identifying Mid2p as a potential cell wall stress sensor that activates the PKC1 pathway, probably through Rho1p, to ensure cell wall integrity (Ketela *et al.* 1999; Rajavel *et al.* 1999). These results support a role for Mid2p in cell wall synthesis, but they do not indicate how it influences actin cytoskeleton organization. To obtain more information on this question, we used the caffeine and NaCl sensitivities of the *pfy1*Δ cells to identify five additional multicopy suppressors of the profilin-deficient phenotype. We propose a model in which Mid2p affects the actin cytoskeleton in the absence of profilin through a pathway leading to Rom1/Rom2 and Rho2p.

MATERIALS AND METHODS

Strains, media, and transformations: Strains used in this study are presented in Table 1. Cells were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose) or SD (0.67% yeast nitrogen base w/o amino acids, 2% glucose) containing all amino acids except uracil (−Ura). Unless otherwise indicated, cells were transformed by a modified lithium acetate method (Kaiser *et al.* 1994).

Deletions of *MID2* and *SYP1* were performed using the PCR-based method described by Baudin *et al.* (1993). The coding sequence of *MID2* was replaced with the *HIS3* gene, amplified using oligos 5′-TTCGTTGAAGATTGGACATATAAAATACG CAAATCATAGTTTCCCTAGCATGTACGTGAG and 5′-GAA AAGTAGCCATAAGCACTAAATGATATGAATGGATATGGT GTCACTACATAAGAACAC. The PCR product containing the *HIS3* gene flanked by 50 nucleotides of the 5′ and 3′ noncoding sequences of *MID2* was used to transform the 22ABΔ1-6A and DJP100 haploid strains by the standard lithium acetate method (Kaiser *et al.* 1994). Deletion of *MID2* was confirmed by PCR analysis.

The coding sequence of *SYP1* was replaced with the kanamycin gene, amplified from the pFA6-kanMX4 plasmid (Wach *et al.* 1994) using oligos 5′-ATGACGGAACAAAGAACCAAA TATGCAGATAGCATATTGACTACTAAGAGTCAGCTGAAG CTTGTACGC and 5′-TCAAGCGAGACCATGGTAGTTACCA GTAGTCAAGTTCTTTTTGTGTTTACGCATAGGCCACTAG TGGATCTG. The resulting PCR product, containing the *kan* gene flanked by 51 bp homologous to the first and last nucleotides of the *SYP1* coding sequence, was used to transform the BY4743 diploid strain by the standard lithium acetate method (Kaiser *et al.* 1994). Transformants were selected on YPD medium containing 200 μg/ml geneticin, and the deletion of *SYP1* was confirmed by PCR analysis. The haploid mutant *syp1*Δ*::kan* strain was obtained by tetrad dissection from a heterozygous *syp1*Δ*::kan/SYP1* strain.

Plasmids: Overexpression of Rho2p, Rho3p, and Rho4p was obtained by inserting the genes and their promoters into the multicopy pRS426 plasmid (Christianson *et al.* 1992). The three genes were obtained by PCR amplification and ligated into the *Eco*RI-*Bam*HI sites of pRS426. Sequences of oligos used are the following: *RHO2*, 5′-AAGGATCCATACCTCCA CAAGG and 5′-AAGAATTCGTAGGACATTA-ACGA; *RHO3*, 5′-ACTCGAATTCAGGCCACTTACC and 5′-AAGGGTACCT ACAC-CTTCGACT; *RHO4*, 5′-GGAGAATTCTTTATACCCGT and 5′-GAGCGGTACCTCA-CACCAGATT. For overexpression of Mtl1p and Wsc11/Slg1/Hcs77, the plasmids pRS424 (MTL1) and pRS424 (HCS77) were used (Rajavel *et al.* 1999).

To construct the Syp1p-GFP fusion protein, the *Bss*HII-*Nar*I genomic fragment of *SYP1*, containing the complete coding sequence of *SYP1* plus 544 bp upstream of the initiation codon and 746 bp downstream of the STOP codon, was inserted into the *Hinc*II/*Cla*I sites of pBluescript KS (+). The resulting plasmid, pBS-*SYP1*, was digested by *Nco*I and *Bam*HI and the ends were filled in and religated, which eliminated the last three codons. The green fluorescent protein (GFP) sequence present in the plasmid p2312MAL (Cormack *et al.* 1997) was amplified with oligos 5′-GAACGGATCCATGTCTAAAGGT GAAGAATT and 5′-GAACACTAGTTTATTTGTACAATTCA TCCA. The PCR product was inserted into the *Bam*HI-*Spe*I sites of pBS-*SYP1*, which places the GFP coding sequence in frame with the sequence coding for Syp1p at its C terminus, with the addition of a serine and a methionine between the two sequences. Finally, the *Xho*I-*Spe*I fragment of pBS-*SYP1*-GFP, containing the promoter of *SYP1* plus the sequence coding for the Syp1p-GFP fusion protein was inserted into the centromeric plasmid pRS316 or the multicopy plasmid pRS416 (Sikorski and Hieter 1989).

TABLE 1

Yeast strains

Strain	Genotype	Reference
22ABΔ1-6A	*MAT*α *lys2-80 ura3-52 his3-*Δ*200 trp1-1 leu2-3*	1
22ABΔ1-6C	*MAT***a** *lys2-80 ura3-52 his3-*Δ*200 trp1-1 leu2-3 pfy1*Δ*::LEU2*	1
BY4743	*MAT***a***/MAT*α *his3 leu2 met15 ura3*	2
BHY46	*MAT***a** *ura3 his3 leu2 ade2 ade3 pfy1-111::LEU2*	3
DJP100	*MAT***a** *lys2-80 ura3-52 his3*Δ*200 trp1-1 leu2-3*	4
DJP102	*MAT*α *lys2-80 ura3-52 his3*Δ*200 trp1-1 leu2-3 pfy1*Δ*::LEU2*	4
DJP200	*MAT***a***/MAT*α *lys2-80 ura3-52 his3-*Δ*200trp1-1 leu2-3*	5
NHM101	*MAT*α *lys2-80 ura3-52 his3-*Δ*200 trp1-1 leu2-3 mid2*Δ*::HIS3*	5
SCY25	*MAT***a***/MAT*α *his3 leu2 met15 ura3 SYP1/syp1*Δ*::kan*	5
SCY26	*MAT***a** *his3 leu2 met15 ura3 syp1*Δ*::kan*	5
Y1242	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-*Δ*1 his3*Δ*200 rom2*Δ*::HIS3*	6
Y1695	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-*Δ*1 his3*Δ*200 rho2*Δ*::TRP1*	6

References: 1, Haarer *et al.* (1990); 2, H. Bussey; 3, Haarer *et al.* (1993); 4, Marcoux *et al.* (1998); 5, this study; 6, Manning *et al.* (1997).

Phalloidin staining, Calcofluor staining, and electron microscopy: Staining of actin filaments was carried out according to the modified protocol of Karpova *et al.* (1998). Cells from exponential phase cultures were fixed at room temperature for 30 min with 3.75% formaldehyde in the culture medium. Cells were washed with and resuspended in PBS. Staining was performed with 8.25 mm FITC-conjugated phalloidin for 90 min on ice in the dark. Cells were washed repeatedly with PBS before microscopic observation.

Bud scars were visualized by staining chitin rings with Calcofluor (Pringle 1991). Cells from overnight cultures were fixed for 30 min with 3.75% formaldehyde in the culture medium. Cells were pelleted and resuspended in Solution A (50 mm KPO_4 pH 6.5, 0.5 mm $MgCl_2$) containing 3.75% formaldehyde and further fixed for 1 hr (Pringle *et al.* 1991). Cells were then washed three times with and resuspended in Solution A. Staining was carried out by adding 1/500 volume of 1 mg/ml Calcofluor White and incubating for 1 hr at room temperature in the dark. Cells were finally washed repeatedly in PBS prior to microscopic observation. For electron microscopy, cells were fixed and prepared as previously described (Marcoux *et al.* 1998).

RESULTS

Identification of *pfy1*Δ suppressors: To screen for multicopy suppressors, the *pfy1*Δ strain DJP102 was transformed with the YEp24 multicopy library constructed with DNA of the S288C strain (Carlson and Botstein 1982). Approximately 15,000 transformants, representing about five genomes, were obtained and replicated onto −Ura plates containing 1.5 mg/ml caffeine or 1.5 m NaCl. The plasmids that allowed growth on both media were isolated and studied further. As expected from the number of genome equivalents screened, we obtained five plasmids containing *PFY1*. We also isolated five plasmids containing *MID2*. In addition, four other genes were isolated as suppressors: *SMY1*, *ROM1*, *ROM2*, and an open reading frame (ORF), *YCR030C* (Table 2). We named the ORF *SYP1* for suppressor of yeast profilin deletion.

Given the established role for the Rho-type GTPases in controlling the actin cytoskeleton and the identification in our screen of Rom1p and Rom2p, two exchange factors for Rho proteins, we tested directly the effect of the five Rho-type GTPases, *RHO1–RHO4* and *CDC42*, in the *pfy1*Δ strain DJP102. Among the RHO family members, only Rho2p is a suppressor of the growth defects of the *pfy1*Δ strain (Table 2). The *pfy1*Δ strain overexpressing Rho2p grows well in the presence of 1.25 mg/ml caffeine but grows slowly on 1.5 mg/ml caffeine, which may explain why it was not selected in the screen. A detailed analysis was carried out on Rho2p and the five suppressors isolated in the original screen.

Suppressors restore normal growth and an axial budding pattern to the *pfy1*Δ cells: The suppressors were selected for their ability to confer growth in the presence of caffeine and NaCl. We also tested their ability to correct other phenotypic defects. The *pfy1*Δ cells are viable but grow slowly at 30°, with a doubling time of ~6 hr in minimal medium, and do not grow at 37°. The *pfy1*Δ cells overexpressing any of the suppressors have a normal doubling time of ~2 hr in minimal media and grow at 37°.

TABLE 2

Suppressors and nonsuppressors of the *pfy1*Δ phenotype

Suppressors	Nonsuppressors
MID2 (5)	*CDC42*
ROM1 (1)	*RHO1*
ROM2 (1)	*RHO3*
SMY1 (2)	*RHO4*
SYP1 (3)	
RHO2	
WSC1	

The number in parentheses indicates the number of times the gene was isolated in the screen.

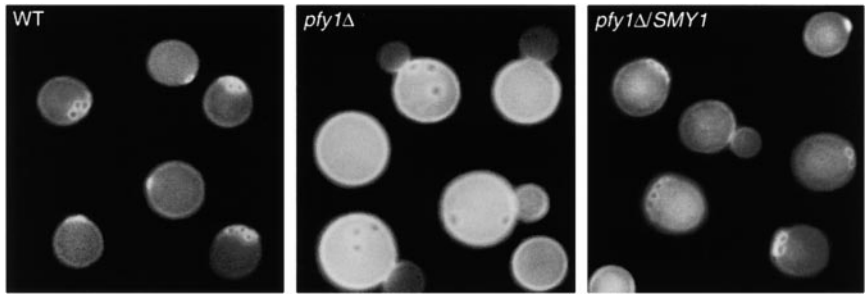

FIGURE 1.—The normal axial budding pattern is restored in haploid *pfy1*Δ cells overexpressing Smy1p. Cells from exponential phase cultures were stained with Calcofluor and observed by fluorescence microscopy. Wild-type (WT) cells and *pfy1*Δ cells overexpressing Smy1p (*pfy1*Δ/*SMY1*) have an axial distribution while *pfy1*Δ cells have a random distribution of bud scars.

Normal haploid yeast cells have an axial budding pattern, meaning that the bud scars are formed on the same side of the cell and adjacent to the previous bud scar. Haploid *pfy1*Δ cells have a random distribution of bud scars and also a delocalized chitin deposition. We tested the bud site location in *pfy1*Δ cells expressing the different suppressors by staining for chitin with the fluorescent dye Calcofluor white. In mutant cells overexpressing either Smy1p (Figure 1) or Rho2p, Rom1p, Rom2p, and Syp1p (results not shown), the axial budding pattern is restored and chitin deposition is concentrated mainly in the bud scars, as it is in wild-type cells. In mutant cells overexpressing Mid2p, the Calcofluor white staining was delocalized and very intense, even more so than in the *pfy1*Δ cells. We were not able to identify the bud scars in these cells and thus could not determine whether they have an axial distribution. Similar results were obtained when Mid2p was overexpressed in wild-type cells. An explanation for this intense staining is found in the recent work showing that cells overexpressing Mid2p produce ~250% more chitin than wild-type cells (KETELA *et al.* 1999). Since Calcofluor white intercalates into nascent chitin chains, cells overexpressing Mid2p are expected to show an intense staining reaction with this fluorescent dye. Thus, we were able to identify the bud scars in four of the suppressors, and they all showed the normal axial distribution in haploid cells.

Only some suppressors cause the formation of a thick cell wall: We showed previously that *pfy1*Δ cells are large and accumulate numerous intracellular vesicular structures due to problems with intracellular trafficking. The overexpression of Mid2p decreases cell size to near normal, eliminates the intracellular vesicles, and results in an abnormally thick cell wall (MARCOUX *et al.*1998). We reasoned that the phenotype of a thick cell wall could be informative for determining the signaling pathway involved in the suppression of the profilin-deficient phenotype. We therefore carried out electron microscopic examinations of *pfy1*Δ cells carrying different suppressors (Figure 2 and Table 3). It should be noted that the *pfy1*Δ cells have a wide range of size and morphology, and some of them from late log phase are much larger and contain more intracellular vesicular structures than the cell shown in Figure 2. The *pfy1*Δ cells overexpressing the suppressors are somewhat variable in size, but are always smaller than the *pfy1*Δ cells and are often about the same size as wild-type cells. No intracellular vesicles are seen in any of these cells.

The thickness of the cell wall in *pfy1*Δ and wild-type cells is similar. However, when Mid2p, Syp1p, Rom1p, or Rom2p are overexpressed in *pfy1*Δ cells, the cell walls in the mother cell are noticeably thicker. This is not accompanied by evident changes in the cell wall diameter of the bud. We also determined that the overexpression of Mid2p in wild-type cells leads to an increase in cell wall diameter of the mother cell, indicating that it is the suppressors alone that are responsible for the cell wall changes. In contrast, the cell walls in strains overexpressing Rho2p or Smy1p have normal dimensions. Therefore, not all suppressors cause the formation of a thick cell wall, indicating that a thick wall is not a prerequisite for the suppression of the profilin-deficient phenotype.

Nonetheless, we exploited this phenotype to determine whether Mid2p and Syp1p are in the same or in parallel signaling pathways. We reasoned that Mid2p, a plasma membrane and a putative sensor for cell wall integrity, should be the first step in a signaling pathway. If Syp1p is required to relay the signal from Mid2p to downstream components of the pathway, then overexpression of Mid2p in *syp1*Δ cells should give a normal cell wall. However, we observed that the *syp1*Δ cells overexpressing Mid2p have thick cell walls, suggesting that Mid2p does not signal cell wall biosynthesis through Syp1p (Table 3).

Rom2p is an exchange factor for Rho1p (OZAKI *et al.* 1996), and Mid2p activates the Rho1p signaling pathway (KETELA *et al.* 1999; RAJAVEL *et al.* 1999). To investigate whether the increase in cell wall diameter implicates activation of the Rho1p signaling pathway by the multicopy suppressors, we overexpressed Rho1p in *pfy1*Δ and wild-type cells. Indeed, we found that an increased dosage of *RHO1*, in either *pfy1*Δ or wild-type cells, leads to a thick cell wall (Figure 2 and Table 3). We therefore conclude from these data that some of the *pfy1*Δ multicopy suppressors increase cell wall biosynthesis, at least in part, through the activation of the Rho1p signaling pathway. The facts that Rho1p is not a suppressor of the *pfy1*Δ mutant and that not all multicopy suppressors influence cell wall synthesis suggest that correction of the *pfy1*Δ phenotype implicates another cellular

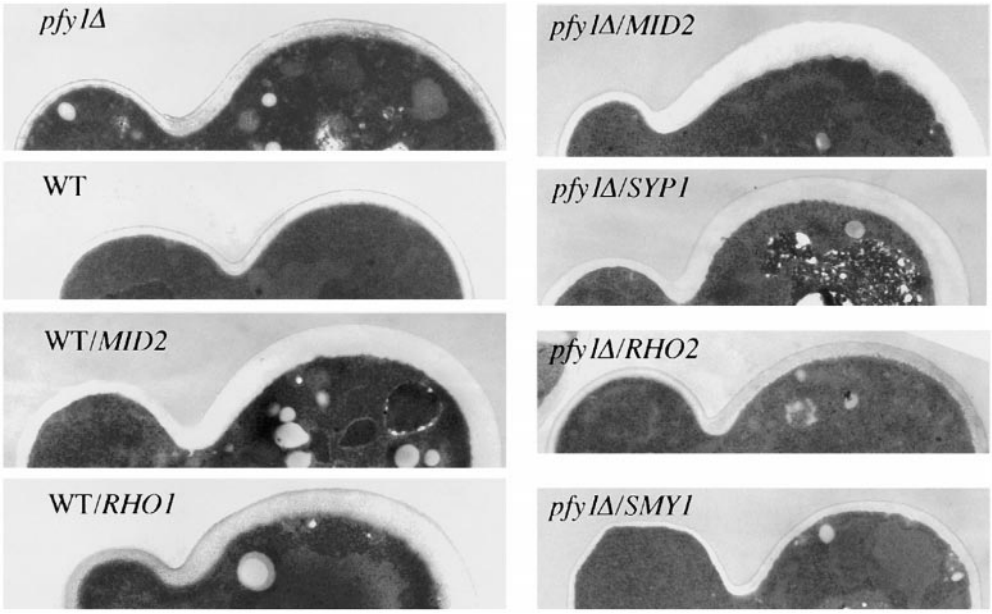

FIGURE 2.—Electron microscopy of cell walls. Thick cell walls are seen in wild-type strains overexpressing Rho1p and Mid2p and *pfy1*Δ cells overexpressing Mid2p and Syp1p. All the other strains have normal cell walls.

function. We thus turned our attention to the actin cytoskeleton.

Suppressors restore cortical patch polarity in the absence of visible actin cables: Deletion of *PFY1* results in large round cells that no longer have visible actin cables. These cells do have numerous, well-formed cortical patches, but their distribution is not normal. Although there is a concentration of cortical patches in small buds, numerous cortical patches are always found in the mother cell at all stages of the cell cycle (HAARER *et al.* 1990). Since the suppressors restored cell size to near normal in *pfy1*Δ cells, we verified whether this was accompanied by the formation of actin cables and by the normal, polarized distribution of cortical patches. We were able to visualize cables and patches by using the staining method of KARPOVA *et al.* (1998). We made most of our observations on cells with small buds, since at this stage the polarized distribution of cortical patches is most evident (Figure 3). In wild-type cells, actin cables are easily visible in the mother cells and are oriented toward the small buds, where the cortical patches are concentrated; few cortical patches are seen in the mother cell at this stage. In *pfy1*Δ cells, actin cables are not visible and numerous patches are distributed throughout the mother cell, even when small buds are present. When Mid2p is overexpressed in these cells, actin patches become mostly concentrated in the small buds (Figure 3 and MARCOUX *et al.* 1998). The redistribution of cortical patches is not complete, since some cells still contain a few patches in the mother cell. In spite of the near-normal distribution of cortical patches, none of the *pfy1*Δ cells overexpressing Mid2p contain visible cables. Staining of actin filaments in *pfy1*Δ cells overexpressing any of the other suppressors gives similar results: cortical patches are localized almost exclusively in small buds and the cells have no visible actin cables. The results with Syp1p, Rho2p, and Smy1p are shown (Figure 3).

TABLE 3

Production of a thick cell wall by the overexpression of Rho1p or the *pfy1*Δ suppressors in different strains as viewed by electron microscopy

Strain	Protein	Thick cell wall
WT		−
WT	Mid2p	+
WT	Rho1p	+
*pfy1*Δ		−
*pfy1*Δ	Mid2p	+
*pfy1*Δ	Rho1p	+
*pfy1*Δ	Rho2p	−
*pfy1*Δ	Rom1p	+
*pfy1*Δ	Rom2p	+
*pfy1*Δ	Smy1p	−
*pfy1*Δ	Syp1p	+
*syp1*Δ	Mid2p	+

+, thick cell wall; −, cell wall diameter as in WT cells.

In wild-type cells, cables are clearly visible in the mother cell. Although there are cables in the bud, the numerous cortical patches tend to mask their appearance. We do not believe, however, that the absence of cables in the *pfy1*Δ cells overexpressing the suppressors is due to a masking effect by the cortical patches. In most of these cells, few cortical patches are seen in the mother cell and in some instances none are present. However, we cannot rule out the presence of fewer, thinner, or much shorter actin cables. If present, however, they do not have the same organization as in wild-type cells.

Overexpression of Syp1p results in an abnormal phenotype: Five of the proteins we identified as suppressors,

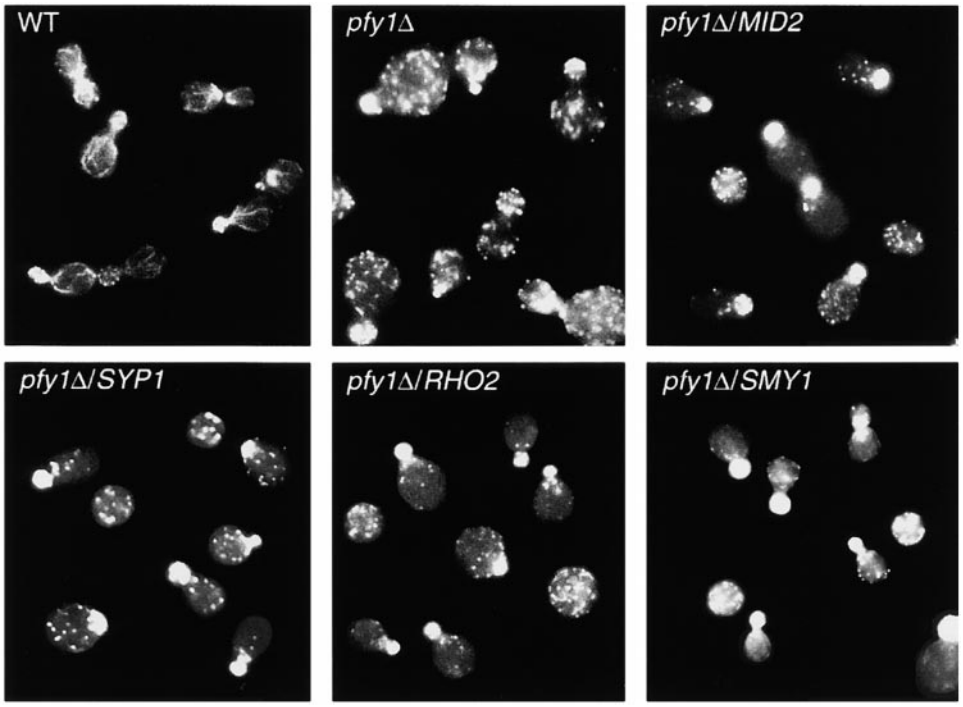

FIGURE 3.—Overexpression of the different suppressors in *pfy1*Δ cells repolarizes cortical patches without restoring visible actin cables. Cells from exponential phase cultures were stained with FITC-phalloidin to visualize actin filament distribution. Wild-type (WT), *pfy1*Δ, and *pfy1*Δ cells overexpressing Mid2p (*pfy1*Δ/*MID2*), Syp1p (*pfy1*Δ/*SYP1*), Rho2p (*pfy1*Δ/*RHO2*),and Smy1p (*pfy1*Δ/*SMY1*) are shown.

Mid2p, Rom1p, Rom2p, Rho2p, and Smy1p, have been studied previously. They all play a role in cell wall biosynthesis or in the organization of the actin cytoskeleton. The other suppressor, Syp1p, is a previously uncharacterized protein. In an attempt to obtain more information about the role of *SYP1*, its entire coding sequence was deleted in a diploid strain by gene replacement using the kanamycin gene. Following sporulation, dissection of tetrads resulted in four haploid spores giving rise to colonies of approximately the same size. Haploid *syp1*Δ strains were identified by growth on geneticin-containing medium and confirmed by PCR analysis. Morphology of the haploid Syp1p-deficient cells is normal, as is actin distribution (results not shown). These cells have normal growth rates at 30° and 37° and in the presence of caffeine, NaCl, and benomyl, a microtubule depolymerizing drug. The budding patterns of haploid *syp1*Δ and diploid *syp1*Δ/*syp1*Δ cells are also normal. Therefore, deletion of *SYP1* does not result in an obvious morphological or growth defect, suggesting that Syp1p has a redundant function with at least one other protein. Sequence alignment with the *S. cerevisiae* and all other available databases does not reveal proteins with significant sequence homologies, identifying Syp1p as a novel protein.

Although we have not observed a phenotype associated with the deletion of *SYP1*, overproduction of Syp1p alters morphology of wild-type haploid and diploid cells. Overexpression of Syp1p causes the formation of abnormally long projections in ~10% of exponentially growing haploid cells at 30° (Figure 4). Cortical patches are present in these projections, consistent with their localization in regions of cell growth. The cortical patches, however, are not located exclusively in the projections as they are also present in the mother cell. Staining of chitin rings with Calcofluor reveals that the budding pattern of these cells is normal. The long projection, however, does not respect the axial budding pattern as it always forms in a different area of the cell, separate from the bud scars (Figure 4). Occasionally, a projection and a bud form on the same cell, but the two are never contiguous. Thus, in these cells, the formation of the projection is not subject to the same spatial controls as bud formation. Chitin, in addition to its localization in bud scars, is also found throughout the projection, except at the rounded tip. The projections do not contain nuclei (results not shown). These projections are never formed by the overexpression of Syp1p in *pfy1*Δ cells.

The effect of overproduction of Syp1p in diploid cells is less dramatic; no long projections are seen. In ~10% of cells, however, a protuberance is seen. In cells with buds, the protuberance is found at the opposite end of the cell from the bud. This protuberance does not seem to be a site of active growth, since cortical patches are not present in this area. Chitin staining shows that the normal bipolar budding pattern of diploid cells is respected (Figure 4). This staining also shows that the small protuberance contains bud scars, suggesting that it may be formed by problems in chitin deposition or cytokinesis.

Syp1p localizes to the mother-bud neck and sites of active growth: A cellular location has been established for five of the suppressor proteins we isolated. Mid2p is a plasma membrane protein and the four other proteins are localized in the cortical patch region of small

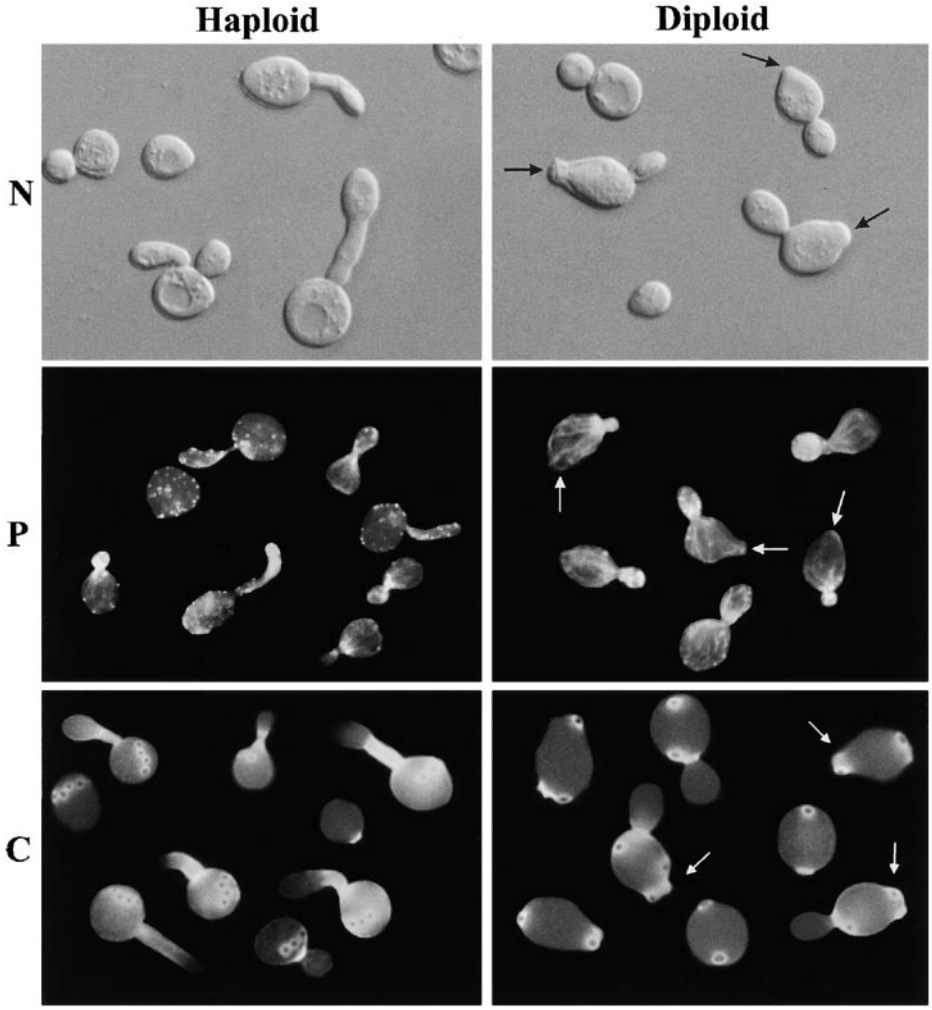

FIGURE 4.—Effect of overexpression of Syp1p in wild-type cells. Morphology of haploid and diploid cells were visualized by Nomarski (N). Actin filament distribution was viewed by staining cells with FITC-conjugated phalloidin (P). Calcofluor staining of chitin rings was performed to observe budding patterns (C). The arrows indicate the position of protuberances formed in the diploid cells.

buds. We reasoned that the cellular location of Syp1p could be informative for determining its role in the suppression of the profilin-deficient phenotype. It was localized using a Syp1p-GFP fusion protein. This fusion protein was expressed using the *SYP1* promoter in a centromeric plasmid. Since *syp1*Δ cells have no observable phenotype, we could not test whether the protein was functional by suppression of the mutant phenotype. However, overexpression of the fusion protein in wild-type haploid cells resulted in the formation of long projections in ~10% of the cells. In addition, overexpression of the fusion protein in *pfy1*Δ cells restored normal growth at 30° and 37° and eliminated the caffeine sensitivity phenotype. The *pfy1*Δ cells overexpressing the Syp1-GFP fusion protein or Syp1p are morphologically indistinguishable in the light microscope (results not shown). Therefore, Syp1p-GFP is functional, and its localization likely respects the normal distribution of Syp1p in cells.

The intracellular location of Syp1p was determined by fluorescence microscopy observations of exponentially growing cells expressing the Syp1p-GFP fusion protein from a centromeric plasmid. In cells without a bud, there is a low level of diffuse fluorescence staining as well as the presence of small dots located at the cell periphery (Figure 5a). The diffuse staining and the peripheral dots remain during the entire cell cycle, but an intense fluorescence develops in specific locations during budding. As the bud emerges, Syp1p becomes concentrated at the mother-bud neck and at the tip of the forming bud (Figure 5b). As the bud grows, the Syp1p fluorescence is abundant in the mother-bud neck region and in the bud (Figure 5c). At cytokinesis, the fusion protein is found predominantly at the junction between the mother cell and the bud (Figure 5d). In shmoos, Syp1p is present mainly at the base of the projection (Figure 5e). The distribution of Syp1p in growing buds, in the septum of dividing cells, and at the base of shmoo projections is consistent with a role for this protein in polarity establishment and/or cytokinesis.

The expression of the Syp1p-GFP fusion protein from a centromeric plasmid does not suppress the *pfy1*Δ phenotype. These cells, which are large and irregularly shaped, have such low levels of fluorescence that the fusion protein could not be localized. The expression of the Syp1p-GFP fusion protein from a multicopy plas-

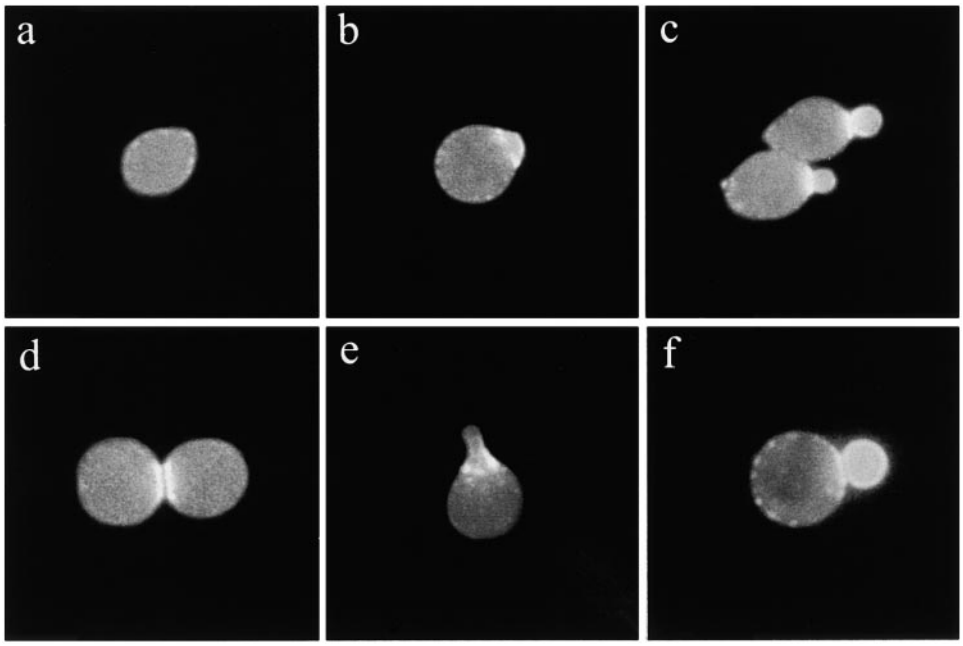

FIGURE 5.—Distribution of Syp1p changes during the cell cycle. The Syp1p-GFP fusion protein was expressed from a centromeric plasmid in WT cells (a–e) and from a multicopy plasmid in the *pfy1*Δ strain DJP102 (f). The Syp1p-GFP was viewed by fluorescent microscopy in exponentially growing cells at different stages of the cell cycle (a–d and f) and during shmoo formation (e). To form shmoos, *MAT***a** wild-type cells (DJP100) expressing Syp1p-GFP were incubated with 6 μM α-factor for 3 hr prior to observation by fluorescence microscopy.

mid in *pfy1*Δ cells gives an intense fluorescence. The location of the fusion protein is similar in these cells and in wild-type cells expressing the protein from a centromeric plasmid, although the fluorescence is much more intense in the *pfy1*Δ cells with the multicopy plasmid. A cell with a medium size bud is shown (Figure 5f). The mother cell has a peripheral fluorescence, usually in the form of small dots. The mother-bud neck junction and the contour of the bud are intensely stained, while a diffuse fluorescence is seen within the bud.

The expression of the Syp1-GFP fusion protein from a multicopy plasmid in wild-type cells gives an intense staining mainly in the 10% of the population with long projections. In G1 cells with projections, the fusion protein is found throughout the mother cell and the projection, except at the junction between the two where a pale region is seen. The most intense fluorescence is seen in the contour of the mother cell and the projection (results not shown).

Genetic interactions: On the basis of previously published work, we hypothesized that Mid2p, Rom1p, Rom2p, and Rho2 act through the same signaling pathway to correct the profilin-deficient phenotype. To test this hypothesis and to obtain more information about all the suppressors, we constructed various double deletion strains. We first checked for possible synthetic lethal interactions among the suppressor genes. Haploid strains *mid2*Δ/*rho2*Δ, *mid2*Δ/*smy1*Δ, *mid2*Δ/*syp1*Δ, *rho2*Δ/*smy1*Δ, and *rom2*Δ/*syp1*Δ are all viable. Also, as with cells carrying single deletions for any of these genes, no obvious morphological or growth defects were observed (Table 4). Thus, these suppressor genes do not show a synthetic lethality. We attempted to obtain double mutants with the *pfy1*Δ allele and the deleted suppressor genes, but our repeated attempts were unsuccessful. We assumed that this was due to the severely compromised growth of the *pfy1*Δ cells. We therefore constructed double mutants with the *pfy1-111* allele (HAARER *et al.* 1993). This allele renders cells partly temperature sensitive; they grow slowly at an elevated temperature and a fraction of them undergo cell lysis. At the restrictive temperature, cells adopt a phenotype similar to but not as severe as the *pfy1*Δ cells. Phalloidin staining of *pfy1-111* cells incubated at 37° shows a partial loss of actin patch polarization and also the absence of visible actin cables (Figure 6). Using this allele, we studied the double mutants *pfy1-111*/*mid2*Δ, *pfy1-111*/*rho2*Δ, *pfy1-111*/*smy1*Δ, and *pfy1-111*/*syp1*Δ.

TABLE 4

Genetic interactions between the suppressor genes and between these genes and the *pfy1-111* allele

Genotype	Genetic interaction
*mid2*Δ/*rho2*Δ	−
*mid2*Δ/*smy1*Δ	−
*mid2*Δ/*syp1*Δ	−
*rho2*Δ/*smy1*Δ	−
*rom2*Δ/*syp1*Δ	−
pfy1-111/*mid2*Δ	+
pfy1-111/*rho2*Δ	+
pfy1-111/*smy1*Δ	Lethal
pfy1-111/*syp1*Δ	−

The morphology in the light microscope and distribution of actin cortical patches was determined for strains growing at 37°. A plus (+) indicates a genetic interaction as defined by larger cells and more delocalized actin patches than is seen in cells carrying either of the single mutations. The minus (−) indicates no genetic interaction.

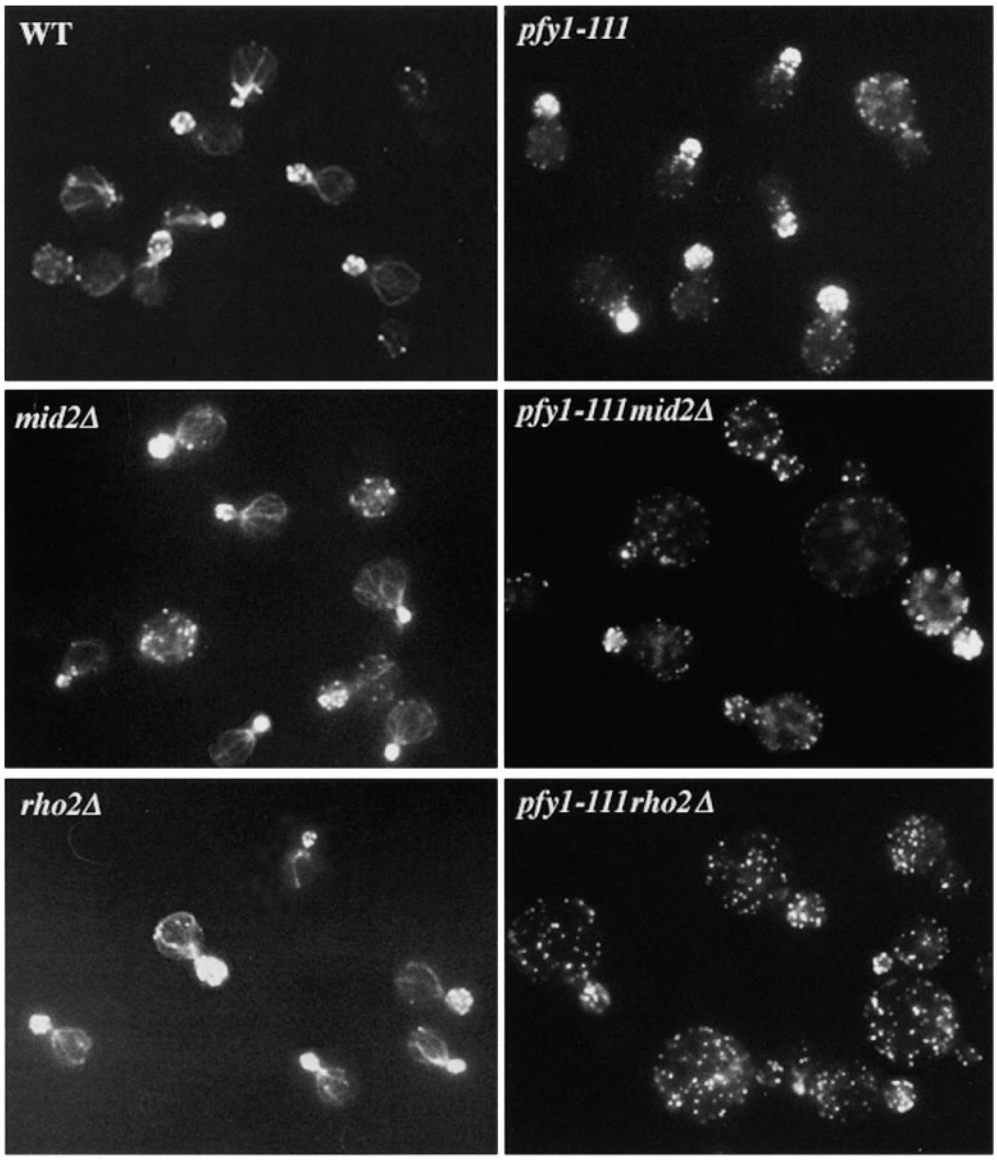

FIGURE 6.—Actin filament distribution in different mutant strains. Strains were grown to exponential phase at 30° then shifted to 37° for 2 hr 30 min prior to staining with FITC-phalloidin. Cortical patches are seen in all the strains but are delocalized in the *pfy1-111*, *pfy1-111/mid2*Δ, and *pfy1-111/rho2*Δ strains. Actin cables are seen in the WT, *mid2*Δ, and *rho2*Δ strains only.

Deletion of *SYP1* in the *pfy1-111* background does not increase the morphological defects or the aberrant actin distribution of the *pfy1-111* cells. In contrast, the *pfy1-111/mid2*Δ and *pfy1-111/rho2*Δ double mutants have a nearly completely depolarized actin distribution at the restrictive temperature. The *pfy1-111/mid2*Δ and *pfy1-111/rho2*Δ strains grow very slowly at 37° and few cells at this temperature have buds. Therefore, exponential phase cultures grown at 30° were shifted to 37° for 2 hr 30 min prior to staining with FITC-conjugated phalloidin. Under these conditions, both *mid2*Δ and *rho2*Δ cells contain abundant actin cables and show the normal polarized distribution of cortical patches (Figure 6). In contrast, the *pfy1-111/mid2*Δ and the *pfy1-111/rho2*Δ cells are larger than either single mutant, and actin patches are now completely depolarized compared to the *pfy1-111* strain (Table 5, Figure 6). We attempted to obtain a *pfy1-111/smy1*Δ strain by sporulation and dissection of a *pfy1-111*, *smy1*Δ heterozygous diploid strain. A total of 80 tetrads originating from two different sporulations were dissected and incubated at 26° or 30°. No *pfy1-111/smy1*Δ double mutant was obtained, suggesting a synthetic lethal interaction between these two genes.

We overexpressed the suppressors in the *pfy1-111/mid2*Δ and *pfy1-111/rho2*Δ strains as a means for determining whether suppression by a given protein requires the presence of either Mid2p or Rho2p. In the *pfy1-111/mid2*Δ strain, overexpression of Pfy1p, Mid2p, Rho2p, or Rom2p all repolarize cortical patches to small buds (Table 5). However, overexpression of Smy1p does not correct the actin distribution or the growth defects of the *pfy1-111/mid2*Δ strain, indicating that Smy1p requires the presence of Mid2p for suppression. These results are compatible with a model placing Mid2p, Rom2, and Rho2 on the same signaling pathway. The results with Smy1p are more complex and do not allow us to include it in a model (see DISCUSSION).

TABLE 5

Redistribution of cortical patches in the mutant strains *pfy1-111/mid2*Δ and *pfy1-111/rho2*Δ by Pfy1p and five of the suppressors

	Strain	
Suppressor	*pfy1-111/mid2*Δ	*pfy1-111/rho2*Δ
Pfy1p	+	+
Mid2p	+	−
Syp1p	+/−	−
Smy1p	−	−
Rho2p	+	+
Rom2p	+	+/−

The plus (+) indicates a polarized distribution, the +/− a partially polarized distribution, and the minus (−) a delocalized distribution of cortical patches in the bud.

In contrast, the results with the overexpression of Syp1p in the *pfy1-111/mid2*Δ cells are ambiguous. Approximately half the cells are large and round with nonpolarized cortical patches while the other half are small cells with polarized cortical patches. These two populations may be the result of varying numbers of plasmids and consequently the amount of Syp1p in the cells. These results suggest that Syp1p can function independently of Mid2p but that it is a more efficient suppressor in the presence of Mid2p.

In the *pfy1-111/rho2*Δ strain, the overexpression of Mid2p, Syp1p, or Smy1p does not repolarize cortical patches and only the overexpression of Pfy1p or Rho2p restores cortical patch polarity (Table 5). Overexpression of Rom2p partly corrects the distribution of cortical patches, but not as well as it does in cells with a functional Rho2p. Collectively these results again suggest a signaling pathway in which Mid2p signals through Rho2p to modify the actin cytoskeleton. They also indicate that Smy1p and Syp1p require Rho2p to repolarize cortical patches in *pfy1*Δ cells.

DISCUSSION

We have identified a novel gene as a suppressor of *pfy1*Δ cells that we named *SYP1*. Overexpression of Syp1p in haploid wild-type cells causes the formation of a long projection in a fraction of the cells. In diploid cells, a small protuberance forms at the opposite end from the growing bud. However, no morphological or growth phenotype was observed following deletion of *SYP1*. In particular, there is no effect on the budding pattern of either haploid or diploid cells. Localization of Syp1p resembles that of the actin-binding protein Aip3p/Bud6p, which is also found in the bud tip and in the mother-bud neck (Amberg *et al.* 1997). Both proteins have a putative coiled-coil domain and Aip3p has been shown to form homodimers. Apart from the coiled-coil domain, there is no obvious sequence homology between the two proteins. Unlike the deletion of *SYP1*, the deletion of *AIP3* leads to a random budding pattern in diploid cells (Amberg *et al.* 1997). Mutants of several other proteins of the actin cytoskeleton, including *act1* mutants, specifically affect the budding pattern of diploid cells (Zahner *et al.* 1996; Yang *et al.* 1997). This implicates the actin cytoskeleton and proteins like Aip3p in localizing the positional signals for bipolar budding. Syp1p does not appear to be involved in this process.

All of the suppressors restore the polarized distribution of cortical patches in profilin-deficient cells, but none restore visible actin cables. This indicates that profilin is essential only for the polymerization of actin filaments that form actin cables. There is evidence that actin cables are responsible for polarization of cortical patches. Tropomyosin binds to and stabilizes actin cables. There are two tropomyosins in yeast, the major form, Tmp1p (Liu and Bretscher 1989), and the minor form, Tpm2p (Drees *et al.* 1995). Deletion of *TPM1* causes the apparent loss of actin cables (Liu and Bretscher 1989) and deletion of both tropomyosins is lethal (Drees *et al.* 1995). A detailed study of the temperature-sensitive strain *tpm1-2/tpm2*Δ shows that at the permissive temperature these cells contain abundant actin cables (Pruyne *et al.* 1998). Shifting cells to the restrictive temperature leads to a rapid disappearance of cables and subsequent depolarization of cortical patches. Reincubation at the permissive temperature causes the reappearance of cables oriented toward growing buds and a gradual repolarization of cortical patches. This suggests a role for actin cables in the establishment of polarity in *S. cerevisiae* cells.

Studies with the actin depolymerizing drug latrunculin-A (Lat-A) support this conclusion. Lat-A binds to actin monomers and inhibits their polymerization without directly causing depolymerization of actin filaments. Incubating yeast cells in the presence of Lat-A results in the disappearance of both actin cables and cortical patches (Ayscough *et al.* 1997). Actin cables are the first to depolymerize, and, as a result, cortical patches become depolarized before they are also depolymerized. Therefore, delocalization of cortical patches is in part due to the absence of actin cables.

Most mutants of actin-binding proteins, such as *cap2*Δ and *srv2*Δ, have depolarized cortical patches but still retain actin cables (Karpova *et al.* 1998). There must therefore exist another mechanism for establishing cell polarity and polarization of cortical patches distinct from actin cables. The results presented here suggest that the Rho2p pathway is implicated in this process. In the absence of profilin, actin cables disappear, leading to the depolarization of the cortical patches. The suppressors that we isolated can bypass the need for actin cables and somehow reestablish the positional signals needed to establish cell polarity.

The membrane protein Mid2p, which is a sensor for

cell wall integrity, is a suppressor of the profilin-deficient phenotype. Overexpression of this protein increases chitin synthesis and probably other cell wall components, at least in part through the activation of Rho1p and the PKC1-MPK1 pathway (Ketela *et al.* 1999; Rajavel *et al.* 1999). Our electron micrographic studies on the formation of a thick cell wall following overexpression of Mid2p and Rho1p are in agreement with a role for these proteins in cell wall biosynthesis.

Although they were not selected as suppressors in our screen, we also tested directly two additional plasma membrane proteins that are involved in cell wall integrity. One of the proteins is Mtl1p for *MID2*-like, which has 50% sequence identity to Mid2p. The high-copy-number expression of *MTL1* from its own promoter did not allow *pfy1*Δ cells to grow in the presence of 1.25 mg/ml caffeine. These cells grow slowly at 30° and at 37°, with a doubling time of ~6 hr, and they are much larger than *pfy1*Δ cells overexpressing Mid2p (results not shown). We conclude from these results that Mtl1p only partially suppresses the *pfy1*Δ phenotype.

Wsc1p, also called Slg1p or Hcs77p, is another plasma membrane protein that shows partial overlapping functions with Mid2p. The *wsc1*Δ cells undergo cell lysis at high temperatures, a phenotype that is corrected by the overexpression of Mid2p. The mating-induced death phenotype seen after the treatment of a *MAT***a** *mid2*Δ mutant with α-factor is partially corrected by the overexpression of Wsc1p. Finally, cells carrying either the *mid2*Δ or the *wsc1*Δ single mutations are viable at room temperature while the double mutant is inviable (Ketela *et al.* 1999; Rajavel *et al.* 1999). The *pfy1*Δ strain DJP102 overexpressing Wsc1p from its own promoter grows at 37° and in the presence of 1.25 mg/ml caffeine at 30°. They are smaller than the *pfy1*Δ cells and are morphologically similar in the light microscope to *pfy1*Δ cells overexpressing Mid2p (results not shown). On the basis of these findings, we conclude that Wsc1p is a multicopy suppressor of the profilin-deficient phenotype (Table 2).

Although Mid2p and Wsc1p signal through Rho1p, this protein is not a suppressor of the *pfy1*Δ phenotype. Rho1p mediates bud growth by controlling polarization of the actin cytoskeleton and cell wall synthesis (Drgonova *et al.* 1999). The downstream effectors of Rho1p are Skn7p (Alberts *et al.* 1998), a two-component signaling protein, Pkc1p (Nonaka *et al.* 1995; Kamada *et al.* 1996) and Fks1p (β-1,3-glucan synthase; Drgonova *et al.* 1996; Qadota *et al.* 1996), and Bni1p, a member of the formin family (Kohno *et al.* 1996). It is postulated that Rho1p controls the actin cytoskeleton via its interaction with Bni1p, which binds to profilin through its formin-homology (FH1) domain (Evangelista *et al.* 1997; Imamura *et al.* 1997). Presumably, this locates the profilactin complex to the growing bud, where it can be used for polymerization of F-actin. In the *pfy1*Δ strain used in this study, the profilactin complex does not exist, and it cannot be recruited to regions of active growth by Bni1p. It is therefore reasonable the *RHO1* is not a multicopy suppressor of the profilin-deficient phenotype.

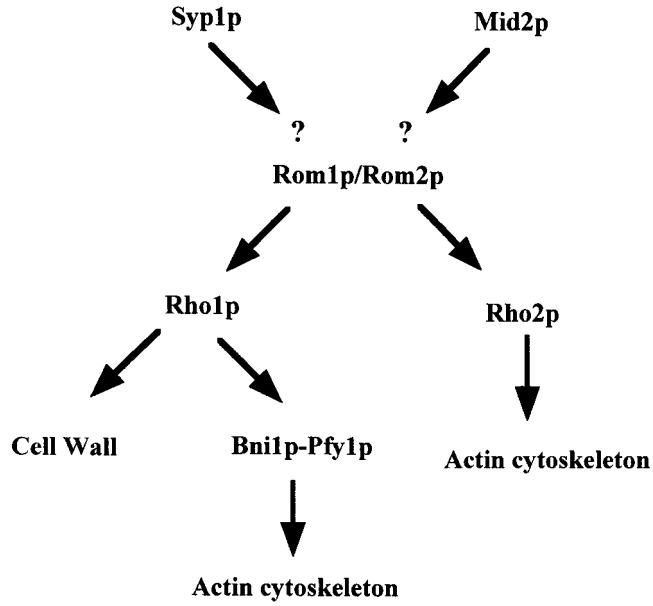

Figure 7.—Proposed model for the organization of the actin cytoskeleton by the Rho1p and Rho2p signaling pathways. Mid2p and Syp1p are postulated to activate Rho1p and Rho2p through their exchange factors Rom1p and Rom2p, either directly or through an intermediate.

Rho2p is the only yeast Rho family member that can repolarize the actin cortical patches in the *pfy1*Δ strain. The two exchange factors for Rho2p, Rom1p and Rom2p, are also suppressors of the profilin-deficient phenotype, further supporting the role for the Rho2p signaling pathway in actin cortical patch polarization. We therefore propose a model in which Mid2p, and possibly Wsc1p, act through the exchange factors Rom1p and Rom2p. These proteins would then activate the Rho1p signaling pathway to stimulate cell wall synthesis and would activate Rho2p to repolarize the actin cytoskeleton (Figure 7).

The overexpression of Syp1p leads to a thick cell wall, suggesting that this protein is upstream of Rom1/Rom2p (Figure 7). This is further strengthened by the finding that Syp1p does not correct the phenotype of the *pfy1-111/rho2*Δ mutant phenotype. A thick cell wall is produced by the overexpression of Mid2p in *syp1*Δ cells suggesting that Mid2p does not signal through Syp1p and that Mid2p and Syp1p may be on parallel pathways. In support of this hypothesis, deletion of *SYP1* in the *pfy1-111* strain does not exaggerate the profilin mutant phenotype. In contrast, cortical patches of *pfy1-111/mid2*Δ cells are completely depolarized, and the cells are larger than the *pfy1-111* cells.

The overexpression of Rho2p corrects the depolarized actin cortical patch phenotype, but it does not

affect the diameter of the yeast cell wall. This is in agreement with Rho2p acting downstream of Rom1p and Rom2p. None of the suppressors, except for Rho2p, is able to fully correct the distribution of the actin cortical patches in a *pfy1-111/rho2*Δ strain, suggesting that all the suppressors tested act through the Rho2p signaling pathway or that they are dependent on the presence of Rho2p.

It is difficult, however, to position Smy1p precisely in the signaling pathway. Smy1p is localized in the cortical patch region of budding cells and is necessary for the proper localization of Myo2p, which is located in the same region (Lillie and Brown 1994). The amino acid sequence of Smy1p suggests that it is a kinesin-like protein, but microtubular organization in *smy1*Δ cells is normal and disruption of microtubules does not affect the localization of Smy1p. The *smy1*Δ strain shows synthetic lethality with the *myo2-66* mutation but not with the yeast kinesin genes, suggesting a role for Smy1p in the organization of the actin cytoskeleton (Lillie and Brown 1992, 1998).

The presence of a normal cell wall in strains overexpressing Smy1p argues that it is downstream of Rom1p/Rom2p or on a parallel pathway. The overexpression of Smy1p, however, does not correct the *pfy1-111/rho2*Δ mutant phenotype, suggesting that it is not downstream of Rho2p. Finally, the *pfy1-111/rho2*Δ double mutant is viable and the *pfy1-111/smy1*Δ is inviable. These results suggest a complex interaction between Smy1p and Rho2p that will require further study to resolve.

Evidence for a pathway leading from Mid2p to Rho2p via the exchange factor Rom2p was also obtained in a study of the microtubule cytoskeleton. The *rho2*Δ mutant shows no morphological or growth defects except for an increased sensitivity to benomyl, a microtubule depolymerizing drug (Manning *et al.* 1997). Cik1p, for chromosome instability and karyogamy, is located in the spindle pole body and is essential for proper spindle organization. The *cik1*Δ mutant show defects in karyogamy at 30° and is inviable at 37° (Page and Snyder 1992; Page *et al.* 1994). Cik1p interacts physically with Kar3p, which is a kinesin-like motor protein involved in nuclear migration during karyogamy (Rose 1996). The *kar3*Δ mutant has a temperature-sensitive growth defect. Overexpression of Mid2p, Rho2p, or Rom2p allows both the *cik1*Δ and *kar3*Δ mutants to grow at the restrictive temperature (Manning *et al.* 1997).

Genetic interactions have also implicated Rho2p in the organization of the actin cytoskeleton. Tor2p, a phosphatidylinositol kinase homologue, plays a role in cell cycle control and the organization of the actin cytoskeleton by activating Rom2p (Schmidt *et al.* 1997). Class A *tor2-ts* mutants, which have actin cytoskeleton defects, can be suppressed by the overexpression of several proteins, including Mss4p, a phosphatidylinositol-4-phosphate 5-kinase, Rom2p and Rho2p (Helliwell *et al.* 1998). The overexpression of Rho2p can also rescue the lethality and the actin cytoskeleton defects of a *mss4-ts* mutations (Desrivieres *et al.* 1998). In addition, the *mss4-1* mutant is synthetically lethal with the profilin mutant *cls5-1*. It is noteworthy that the *mss4-1* mutant, with its large cell size and the random distribution of cortical actin patches and random chitin localization, has a phenotype resembling that of the *pfy1*Δ cells (Homma *et al.* 1998).

Additional evidence implicating Rho2p in the organization of the actin cytoskeleton is seen in the work on *GLC7*, which encodes the catalytic subunit of a type 1 serine/threonine phosphatase. It controls glycogen accumulation by the dephosphorylation of glycogen synthase (Cannon *et al.* 1994; Ramaswamy *et al.* 1998). A temperature-sensitive *glc7* mutant has aberrant bud morphology, delocalized cortical actin patches, and few actin cables. These defects are suppressed by the overexpression of the protein kinase C homologue Pkc1p, but not by the components of the downstream mitogen-activated protein kinase cascade. Interestingly, Mid2p, Rho2p, Rom2p, and Wsc1p are also suppressors (Andrews and Stark 2000). We point out that these proteins are also suppressors of the *pfy1*Δ phenotype. Thus, Rho2p and other proteins affecting Rho2p signaling are involved in the control of the actin cytoskeleton. The eventual identification of downstream effectors of the Rho2p pathway should help determine how this is accomplished.

We thank Howard Bussey, Brian Haarer, Michael Hall, David Levin, and Michael Snyder for strains and plasmids. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la formation des Chercheurs et l'Aide à la Recherche of Québec.

LITERATURE CITED

Alberts, A. S., N. Bouquin, L. H. Johnston and R. Treisman, 1998 Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein beta subunits and the yeast response regulator protein Skn7. J. Biol. Chem. **273:** 8616–8622.

Amberg, D. C., J. E. Zahner, J. W. Mulholland, J. R. Pringle and D. Botstein, 1997 Aip3p/Bud6p, a yeast actin-interacting protein that is involved in morphogenesis and the selection of bipolar budding sites. Mol. Biol. Cell **8:** 729–753.

Andrews, P. D., and M. J. Stark, 2000 Type 1 protein phosphatase is required for maintenance of cell wall integrity, morphogenesis and cell cycle progression in *Saccharomyces cerevisiae*. J. Cell Sci. **113:** 507–520.

Ayscough, K. R., J. Stryker, N. Pokala, M. Sanders, P. Crews *et al.*, 1997 High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. J. Cell Biol. **137:** 399–416.

Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute and C. Cullin, 1993 A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res. **21:** 3329–3330.

Cannon, J. F., J. R. Pringle, A. Fiechter and M. Khalil, 1994 Characterization of glycogen-deficient glc mutants of *Saccharomyces cerevisiae*. Genetics **136:** 485–503.

Carlson, M., and D. Botstein, 1982 Two differentially regulated mRNAs with different 5′ ends encode secreted with intracellular forms of yeast invertase. Cell **28:** 145–154.

Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero and P.

Hieter, 1992 Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.

Cormack, B. P., G. Bertram, M. Egerton, N. A. Gow, S. Falkow *et al.*, 1997 Yeast-enhanced green fluorescent protein (yEGFP) a reporter of gene expression in Candida albicans. Microbiology **143:** 303–311.

Daniel, J., 1993 Potentially rapid walking in cellular regulatory networks using the gene-gene interference method in yeast. Mol. Gen. Genet. **240:** 245–257.

Desrivieres, S., F. T. Cooke, P. J. Parker and M. N. Hall, 1998 MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. J. Biol. Chem. **273:** 15787–15793.

Drees, B., C. Brown, B. G. Barrell and A. Bretscher, 1995 Tropomyosin is essential in yeast, yet the TPM1 and TPM2 products perform distinct functions. J. Cell Biol. **128:** 383–392.

Drgonova, J., T. Drgon, K. Tanaka, R. Kollar, G. C. Chen *et al.*, 1996 Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science **272:** 277–279.

Drgonova, J., T. Drgon, D. H. Roh and E. Cabib, 1999 The GTP-binding protein Rho1p is required for cell cycle progression and polarization of the yeast cell. J. Cell Biol. **146:** 373–387.

Evangelista, M., K. Blundell, M. Longtine, C. Chow, N. Adames *et al.*, 1997 Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. Science **276:** 118–122.

Goldschmidt-Clermont, P. J., L. M. Machesky, J. J. Baldassare and T. D. Pollard, 1990 The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. Science **247:** 1575–1578.

Gray, J. V., J. P. Ogas, Y. Kamada, M. Stone, D. E. Levin *et al.*, 1997 A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. EMBO J. **16:** 4924–4937.

Haarer, B. K., S. H. Lillie, A. E. Adams, V. Magdolen, W. Bandlow *et al.*, 1990 Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. J. Cell Biol. **110:** 105–114.

Haarer, B. K., A. S. Petzold and S. S. Brown, 1993 Mutational analysis of yeast profilin. Mol. Cell. Biol. **13:** 7864–7873.

Helliwell, S. B., I. Howald, N. Barbet and M. N. Hall, 1998 TOR2 is part of two related signaling pathways coordinating cell growth in *Saccharomyces cerevisiae*. Genetics **148:** 99–112.

Homma, K., S. Terui, M. Minemura, H. Qadota, Y. Anraku *et al.*, 1998 Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. J. Biol. Chem. **273:** 15779–15786.

Iida, H., H. Nakamura, T. Ono, M. S. Okumura and Y. Anraku *et al.*, 1994 MID1, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca2+ influx and mating. Mol. Cell. Biol. **14:** 8259–8271.

Imamura, H., K. Tanaka, T. Hihara, M. Umikawa, T. Kamei *et al.*, 1997 Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. EMBO J. **16:** 2745–2755.

Jacoby, J. J., S. M. Nilius and J. J. Heinisch, 1998 A screen for upstream components of the yeast protein kinase C signal transduction pathway identifies the product of the SLG1 gene. Mol. Gen. Genet. **258:** 148–155.

Kaiser, C., S. Michaelis and A. Mitchell, 1994 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kamada, Y., H. Qadota, C. P. Python, Y. Anraku, Y. Ohya *et al.*, 1996 Activation of yeast protein kinase C by Rho1 GTPase. J. Biol. Chem. **271:** 9193–9196.

Karpova, T. S., J. G. McNally, S. L. Moltz and J. A. Cooper, 1998 Assembly and function of the actin cytoskeleton of yeast: relationships between cables and patches. J. Cell Biol. **142:** 1501–1517.

Ketela, T., R. Green and H. Bussey, 1999 *Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. J. Bacteriol. **181:** 3330–03340.

Kohno, H., K. Tanaka, A. Mino, M. Umikawa, H. Imamura *et al.*, 1996 Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. EMBO J. **15:** 6060–6068.

Lassing, I., and U. Lindberg, 1985 Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature **314:** 472–474.

Lillie, S. H., and S. S. Brown, 1992 Suppression of a myosin defect by a kinesin-related gene. Nature **356:** 358–361.

Lillie, S. H., and S. S. Brown, 1994 Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smy1p, to the same regions of polarized growth in *Saccharomyces cerevisiae*. J. Cell Biol. **125:** 825–842.

Lillie, S. H., and S. S. Brown, 1998 Smy1p, a kinesin-related protein that does not require microtubules. J. Cell Biol. **140:** 873–883.

Liu, H. P., and A. Bretscher, 1989 Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. Cell **57:** 233–242.

Lodder, A. L., T. K. Lee and R. Ballester, 1999 Characterization of the Wsc1 protein, a putative receptor in the stress response of *Saccharomyces cerevisiae*. Genetics **152:** 1487–1499.

Manning, B. D., R. Padmanabha and M. Snyder, 1997 The Rho-GEF Rom2p localizes to sites of polarized cell growth and participates in cytoskeletal functions in *Saccharomyces cerevisiae*. Mol. Biol. Cell **8:** 1829–1844.

Marcoux, N., Y. Bourbonnais, P. M. Charest and D. Pallotta, 1998 Overexpression of MID2 suppresses the profilin-deficient phenotype of yeast cells. Mol. Microbiol. **29:** 515–526.

Marcoux, N., M. Laforest and D. Pallotta, 1999 The two *Physarum* polycephalum profilins are not functionally equivalent in yeast cells. Protoplasma **210:** 45–51.

Mulholland, J., D. Preuss, A. Moon, A. Wong, D. Drubin *et al.*, 1994 Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. J. Cell Biol. **125:** 381–391.

Nonaka, H., K. Tanaka, H. Hirano, T. Fujiwara, H. Kohno *et al.*, 1995 A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. EMBO J. **14:** 5931–5938.

Ozaki, K., K. Tanaka, H. Imamura, T. Hihara, T. Kameyama *et al.*, 1996 Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. EMBO J. **15:** 2196–2207.

Page, B. D., and M. Snyder, 1992 CIK1: a developmentally regulated spindle pole body-associated protein important for microtubule functions in *Saccharomyces cerevisiae*. Genes Dev. **6:** 1414–1429.

Page, B. D., L. L. Satterwhite, M. D. Rose and M. Snyder, 1994 Localization of the Kar3 kinesin heavy chain-related protein requires the Cik1 interacting protein. J. Cell Biol. **124:** 507–519.

Pringle, J. R., 1991 Staining of bud scars and other cell wall chitin with calcofluor, pp. 732–735 in *Guide to Yeast Genetics and Molecular Biology*, Vol. 194, edited by C. Guthrie and G. R. Fink. Academic Press, San Diego.

Pringle, J. R., A. E. Adams, D. G. Drubin and B. K. Haarer, 1991 Immunofluorescence methods for yeast, pp. 565–602 in *Guide to Yeast Genetics and Molecular Biology*, Vol. 194, edited by C. Guthrie and G. R. Fink. Academic Press, San Diego.

Pruyne, D. W., D. H. Schott and A. Bretscher, 1998 Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. J. Cell Biol. **143:** 1931–1945.

Qadota, H., C. P. Python, S. B. Inoue, M. Arisawa, Y. Anraku *et al.*, 1996 Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science **272:** 279–281.

Rajavel, M., B. Philip, B. M. Buehrer, B. Errede and D. E. Levin, 1999 Mid2 is a putative sensor for cell integrity signaling in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:** 3969–3976.

Ramaswamy, N. T., L. Li, M. Khalil and J. F. Cannon, 1998 Regulation of yeast glycogen metabolism and sporulation by Glc7p protein phosphatase. Genetics **149:** 57–72.

Rose, M. D., 1996 Nuclear fusion in the yeast *Saccharomyces cerevisiae*. Annu. Rev. Cell Dev. Biol. **12:** 663–695.

Schmidt, A., M. Bickle, T. Beck and M. N. Hall, 1997 The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. Cell **88:** 531–542.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Verna, J., A. Lodder, K. Lee, A. Vagts and R. Ballester, 1997 A family of genes required for maintenance of cell wall integrity

and for the stress response in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **94:** 13804–13809.

Wach, A., A. Brachat, R. Pohlmann and P. Philippsen, 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:** 1793–1808.

Yang, S., K. R. Ayscough and D. G. Drubin, 1997 A role for the actin cytoskeleton of *Saccharomyces cerevisiae* in bipolar bud-site selection. J. Cell Biol. **136:** 111–123.

Zahner, J. E., H. A. Harkins and J. R. Pringle, 1996 Genetic analysis of the bipolar pattern of bud site selection in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 1857–1870.

Communicating editor: P. G. Young