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Role of Phosphatidylinositol Phosphate Signaling in the Regulation of the Filamentous-Growth Mitogen-Activated Protein Kinase Pathway

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Reversible phosphorylation of the phospholipid phosphatidylinositol (PI) is a key event in the determination of organelle identity and an underlying regulatory feature in many biological processes. Here, we investigated the role of PI signaling in the regulation of the mitogen-activated protein kinase (MAPK) pathway that controls filamentous growth in yeast. Lipid kinases that generate phosphatidylinositol 4-phosphate [PI(4)P] at the Golgi (Pik1p) or PI(4,5)P2 at the plasma membrane (PM) (Mss4p and Stt4p) were required for filamentous-growth MAPK pathway signaling. Introduction of a conditional allele of *PIK1* (*pik1-83*) into the filamentous (Σ1278b) background reduced MAPK activity and caused defects in invasive growth and biofilm/mat formation. MAPK regulatory proteins that function at the PM, including Msb2p, Sho1p, and Cdc42p, were mislocalized in the *pik1-83* mutant, which may account for the signaling defects of the PI(4)P kinase mutants. Other PI kinases (Fab1p and Vps34p), and combinations of PIP (synaptojanin-type) phosphatases, also influenced the filamentous-growth MAPK pathway. Loss of these proteins caused defects in cell polarity, which may underlie the MAPK signaling defect seen in these mutants. In line with this possibility, disruption of the actin cytoskeleton by latrunculin A (LatA) dampened the filamentous-growth pathway. Various PIP signaling mutants were also defective for axial budding in haploid cells, cell wall construction, or proper regulation of the high-osmolarity glycerol response (HOG) pathway. Altogether, the study extends the roles of PI signaling to a differentiation MAPK pathway and other cellular processes.

MAPK (mitogen-activated protein kinase) pathways are evolutionarily conserved signal transduction modules (1, 2). MAPK cascades regulate the response to environmental challenges, such as changes in osmolarity, nutrient starvation, DNA damage, and damage to cell integrity. In the budding yeast *Saccharomyces cerevisiae*, MAPK pathways regulate cell wall integrity (3), pheromone response or mating (4), filamentous growth (5), and the response to high osmolarity (high-glycerol response [HOG] pathway [6]). Each MAPK pathway in yeast responds to a different stimulus. Under some circumstances, several MAPK pathways are required to mount an appropriate response (7–11).

The filamentous-growth MAPK pathway regulates differentiation to the filamentous cell type (12–14) and the development of biofilms or mats (15). During filamentous growth, the MAPK pathway, together with other pathways (16–18), induces a delay in the cell cycle (19), a reorganization of cell polarity, which leads to a distal-unipolar budding pattern (12, 13, 20, 21), and elevated expression of the cell adhesion molecule Flo11p (22). The developmental foraging responses that occur in *S. cerevisiae* are evolutionarily conserved across many fungal species. In pathogenic fungi, like *Candida albicans*, an orthologous differentiation MAPK pathway (called the Cek1p pathway) regulates filamentous/hyphal growth and biofilm formation (23–25). These behaviors are critical for virulence (24, 26–30). Studies of filamentous growth in a genetically tractable fungal system like *S. cerevisiae* provides information about the genetic basis of fungal behaviors that can be applied to other species, including pathogens.

In *S. cerevisiae*, the filamentous-growth MAPK pathway is regulated by Msb2p (31, 32), a member of the signaling mucin family of glycoprotein receptors (33). Msb2p, the tetraspan adaptor Sho1p (31, 34–36), and the cytosolic adaptor protein Bem4p (37) together regulate the Rho-type GTPase Cdc42p and effector p21-activated kinase Ste20p (38, 39) in the filamentous-growth MAPK pathway. Ste20p activates the MAPK cascade by phosphorylating the MAPKKK Ste11p. Ste11p phosphorylates the MAPKK Ste7p, which in turn phosphorylates the MAPK Kss1p (13, 40). Opy2p is another transmembrane protein that recruits Ste11p to the PM through the adaptor protein Ste50p (35, 36, 41–43). The culmination of these events is the activation of transcription factors, Ste12p and Tec1p, which regulate target genes that control differentiation to the filamentous cell type (14, 44–47).

Phosphorylation of the key lipid phosphatidylinositol (PI) is a critical modification of membrane phospholipids in eukaryotes that is important for normal cellular function (48–50). PIPs are utilized as a mark for organelle identity and impact diverse cellular processes, including the reorganization of the actin cytoskeleton, protein trafficking through the endomembrane system, and protein secretion (51–56). A family of lipid kinases phosphorylates the inositol ring at different positions to designate organelles with specific PIP combinations (54). Two kinases generate PI(4)P in yeast: Pik1p regulates the level of PI(4)P at the Golgi (57), and Stt4p regulates PI(4)P at the PM (58). Mss4p regulates the distribution of $PI(4,5)P_2$ at the PM (59). In addition, Vps34p regulates the level of PI(3)P at the endosome (60–62), and Fab1p regulates $PI(3,5)P_2$ at the vacuole/lysosome (63). PIPs are recognized by

Received 29 January 2015 **Accepted** 23 February 2015

Accepted manuscript posted online 27 February 2015

Citation Adhikari H, Cullen PJ. 2015. Role of phosphatidylinositol phosphate signaling in the regulation of the filamentous-growth mitogen-activated protein kinase pathway. Eukaryot Cell 14:427–440. doi:10.1128/EC.00013-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00013-15.

doi:10.1128/EC.00013-15

TABLE 1 Yeast strains

Strain	Genotype[a]	Reference
PC313	*MATa ura3-52*	14
PC538	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52*	31
PC948	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 msb2::KanMX6*	31
PC1029	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KanMX6*	156
PC1531	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sho1::HYG*	78
PC2053	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pbs2::KanMX6*	106
PC2382	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KanMX6*	78
PC776	*MAT***a** *his3 ura3-52 rsr1::HIS3*	20
PC677	*MAT***a** *his3 ura3-52 bud3::KlURA3*	20
PC2613	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 trp1::NAT*	This study
PC4305	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl1::KlURA3*	This study
PC4306	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl2::KlURA3*	This study
PC4307	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl3::KlURA3*	This study
PC4308	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl1::KlURA3 sjl2::NAT*	This study
PC4309	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl3::KlURA3 sjl2::NAT*	This study
PC4312	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl1::KlURA3 sjl3::NAT*	This study
PC4990	*SEY6210 MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*[b]	94
PC4991	*SEY6210 MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 sjl3::TRP1 sjl2::HIS3 sjl1::HISG pRS314sjl2*$t^{,s}$*-8 (LEU2 CEN6 sjl2*^{ts}*-8)*[b]	68
PC4992	*SEY6210; stt4::HIS3 pRS415stt4-4 (LEU2 CEN6 stt4-4)*[b]	57
PC4993	*SEY6210; mss4::HIS3MX6 Ycplacmss4-102 (LEU2 CEN6 mss4-102)*[b]	68
PC4994	*SEY6210; pik1::HIS3 pRS314pik1-83 (TRP1 CEN6 pik1-83)*[b]	57
PC5260	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl2::NAT sjl3::HYG*	This study
PC5276	*MATa ura3-52 sjl2::NAT sjl3::HYG*	This study
PC5294	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sjl2::NAT sjl3::HYG rga1::KlURA3*	This study
PC5303	*MATa ura3-52 sjl2::NAT sjl3::HYG ssk1::KlURA3*	This study
PC5319	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 trp1::NAT pik1::KLURA3 pRS314pik1-83 (TRP1 CEN6 pik1-83)*	This study
PC5433	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 fab1::HYG*	This study
PC5436	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sac1::KlURA3*	This study
PC5438	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 trp1::NAT pik1::KLURA3 pRS314pik1-83 (TRP1 CEN6 pik1-83) URA3*	This study
PC5473	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 vps34::NAT*	This study
PC5659	*RSY255 MATa leu2-3,112 ura3-52*	157
PC5661	*MATα leu2-3,112 his4-519 ura3-52 suc2-Δ9 sec7-1*	116
PC5562	*RSY263 MATa leu2-3,112 ura3-52 sec12-4*	157
PC5563	*RSY271 MATa his4-619 ura3-52 sec18-1*	157
PC5564	*RSY529 MATa sec62-1 ura3-52, leu2-3,112, his4-619*	157
PC5712	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 msb2::KanMX6 sho1::NAT*	This study
PC6324	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 inp54::KlURA3*	This study

[a] All strains were made in the Σ1278b background unless otherwise indicated.
[b] Strain was made in the SEY6210 background.

specific domains on effector proteins (64–67). PIP modification is reversible by the action of phosphatases that dephosphorylate PIPs (54). Synaptojanin-type proteins Sjl1p, Sjl2p, and Sjl3p are the major PIP phosphatases in yeast. These proteins exhibit a high degree of functional redundancy (68).

Lipid signaling has established connections to the regulation of morphogenetic pathways (69). For example, activation of the yeast mating pathway requires recruitment of the mating-pathway specific scaffold Ste5p to the PM by recognition of PI(4,5)P2 (70). The pheromone response and HOG pathways are regulated by Pik1p at the level of Ste11p (71). The guanine nucleotide exchange factor for the cell wall integrity pathway, Rom2p, interacts with PI(4,5)P2 at the PM by its PH domain to regulate the PKC pathway activity (58). A clear link between PIP signaling and the regulation of the filamentous-growth MAPK pathway has yet to be defined. Intriguingly, in *C. albicans*, steep PI(4,5)P2 gradients occur at hyphal tips and promote filamentous growth and invasion (72–74).

Here, we examined the impact of PI signaling on the regulation of the filamentous-growth MAPK pathway in *S. cerevisiae*. We show that conditional PI kinase mutants exhibit defects in the filamentous-growth pathway. Membrane-associated regulators of the filamentous-growth MAPK pathway (including Msb2p, Sho1p, and Cdc42p) were mislocalized in PI(4)P kinase mutants, which may account for the reduction in MAPK activity in this context. Perhaps unexpectedly, other PI kinases, including Vps34p and to some degree Fab1p, were also involved. Polarity defects in these mutants might explain the MAPK signaling defects, as disruption of the actin cytoskeleton led to ablation of MAPK activity. Roles for PI kinases and PIP phosphatases in axial bud site selection in haploid cells, the maintenance of the yeast cell wall, and the HOG pathway were also uncovered. Therefore, PIP signaling plays a critical role in the regulation of a differentiation MAPK pathway and other aspects of cellular biogenesis and decision-making.

MATERIALS AND METHODS

Strains, media, and growth conditions. Yeast strains are listed in Table 1.

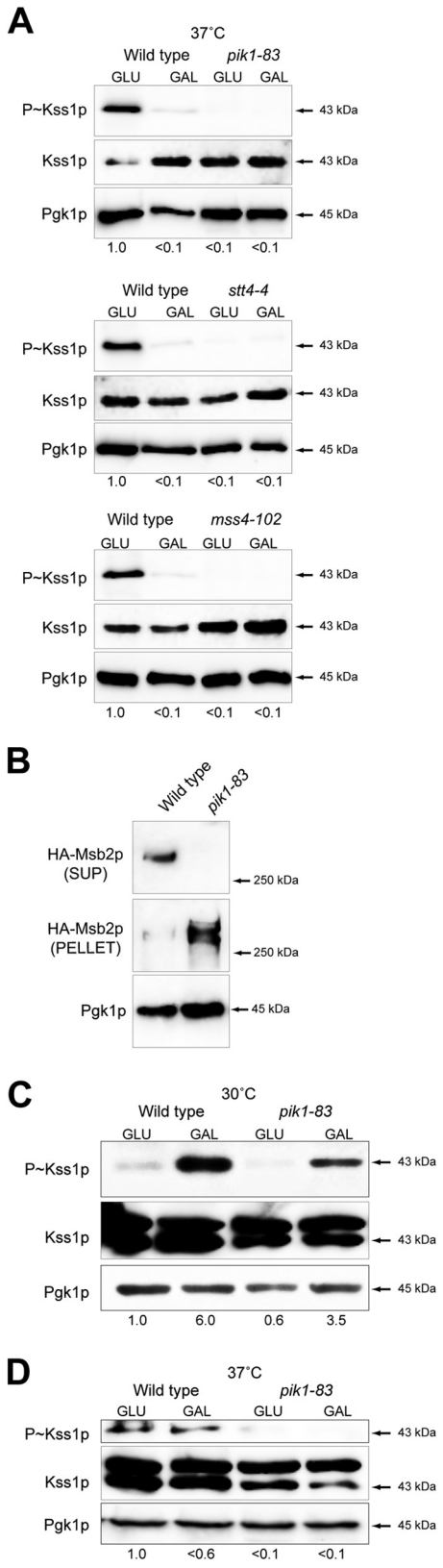


FIG 1 Role of PI(4)P kinases in regulating the filamentous-growth MAPK pathway. (A) Phosphorylation of Kss1p in wild-type cells and the *pik1-83*, *stt4-4*, and *mss4-102* mutants in the SEY6210 background. Cells were incubated at 37°C for 4 h and harvested for immunoblot analysis. Antibodies against Pgk1p were used to compare total protein levels between samples. (B) Evaluation of Msb2p-HA secretion in the *pik1-83* mutant. The wild type and the *pik1-83* mutant harboring pMsb2p-HA were grown to saturation at 37°C for 4 h and separated into supernatant (SUP) and pellet fractions by centrifugation. Cell extracts were prepared and examined by immunoblot analysis for HA-Msb2p levels and Pgk1p levels as a control for protein loading. (C) Phosphorylation of Kss1p in wild-type cells and the *pik1-83* mutant in the Σ1287b background. Cells were incubated in YEPD and YEP-Gal medium at 30°C for 4 h. (D) Cells used for panel C were incubated at 37°C for 4 h.

Strains were grown under standard laboratory conditions (75). Strains were maintained at 30°C unless otherwise indicated. The medium used was YEPD (yeast extract-peptone-dextrose, 2% Glu) or YEP-Gal (2% Gal) for most experiments. For temperature shift experiments, cells were incubated at 37°C in prewarmed medium for 4 to 5 h. Plasmids were maintained on synthetic medium (generally on SD-Ura). Bacterial cultures of *Escherichia coli* were propagated in LB+CARB (carbenicillin) by standard methods (76).

Plasmid pAxl1p-HA was provided by the Boone lab (77). pMsb2p-HA (78), pMsb2p-GFP (78), pSho1p-GFP (79), p*pik1-83* (80), pGFP-Cdc42p (81), and Sec7p-DsRed (82) have been described previously.

Standard gene disruption techniques were used (83). Antibiotic resistance (84) and heterologous auxotrophic markers were used for gene disruption (85) and to create integrated fusion proteins (86). The *pik1-83* allele was introduced into the Σ1278b strain background by allele replacement. Wild-type Σ1278b cells (PC538) were transformed with a plasmid containing the *pik1-83* allele. The *PIK1* gene was subsequently disrupted in cells harboring the *pik1-83* plasmid. Gene disruptions were confirmed by PCR analysis.

Biological assays for filamentous growth and biofilm/mat formation. The plate-washing assay was performed as described previously (13). Biofilm/mat assays were performed as described previously (15). Cells were spotted onto low-agar medium (0.3% YEPD) for 3 days and photographed. Calcofluor white (CFW) staining was performed as described in reference 87. Cells were grown to saturation in YEPD medium at 30°C. Cells were fixed in 3.9% formaldehyde for 30 min at 30°C. Cells were harvested by centrifugation, washed with 1× PBS (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.4]), and resuspended in 0.01% CFW for 5 min. Cells were washed 3 times in 1× PBS and observed by fluorescence microscopy using the DAPI (4′,6-diamidino-2-phenylindole) channel (350 to 400 nm). Cells stained with CFW were also analyzed for budding pattern. Budding pattern determination was based on previous methods (88), except that cell position was also taken into consideration among adherent cells (20, 89).

Protein immunoblot analysis. Cells were harvested, and pellets were frozen at −80°C. Proteins were extracted from cell lysates using the trichloroacetic acid (TCA) precipitation method as described previously (90). Protein extracts were separated by 10% SDS-PAGE analysis and transferred onto nitrocellulose membrane. Phosphorylated Kss1p was detected using p42/p44 antibodies (1:4,000 dilution; no. 4370; Cell Signaling Technology). Antibodies to total Kss1p (1:5,000; no. sc6775; Santa Cruz), Hog1p (1:5,000; yc-20; no. sc6815; Santa Cruz), and Pgk1p (1:5,000 dilution; catalog no. 459250; Life Technologies) were purchased and used according to the manufacturers' specifications. Antibodies to green fluorescent protein (GFP; clones 7.1 and 13.1; no. 11814460001; Roche Diagnostics) and the hemagglutinin (HA) epitope (no. 11583816001; Roche) were used to detect epitope-tagged proteins. Secondary antibodies were used, including goat anti-mouse IgG conjugated to horseradish peroxidase (HRP; 170-6516; Bio-Rad) and goat anti-rabbit IgG–HRP (111-035-144; Jackson ImmunoResearch Laboratories, Inc.). Msb2p-HA secretion was evaluated as described previously (78). Protein bands were quantified by ImageJ by densitometry scanning analysis. The numbers indicate the ratio of phosphorylated protein to total protein, or the ratio of total protein to loading control where appropriate. To determine the change in phosphorylated proteins, total protein levels were normalized to the loading control (Pgk1p).

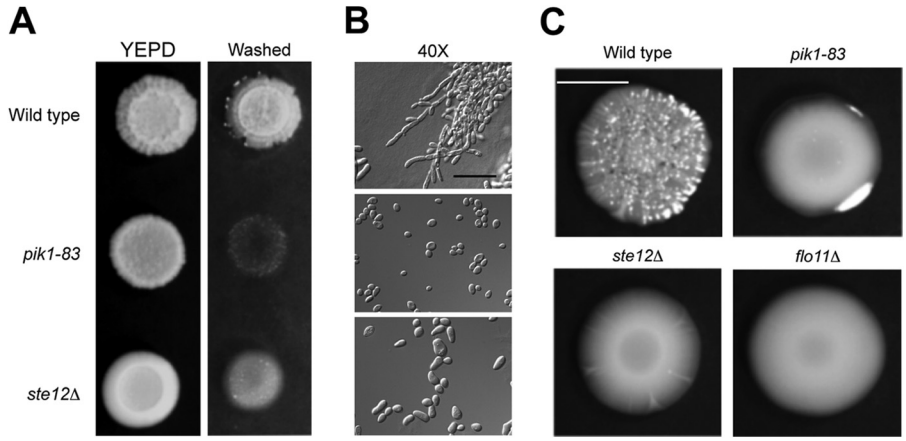


FIG 2 The role of Pik1p in regulating haploid invasive growth and biofilm/mat formation. (A) Plate-washing assay. Wild-type cells and the *pik1-83* and *ste12*Δ mutants were spotted onto YEPD medium and incubated at 30°C for 2 days. Plates were photographed (left), washed in a stream of water, and photographed again (right). (B) Cells scraped from the invasive scars on the washed YEPD plate were examined by differential interference contrast (DIC) microscopy at a magnification of ×40. Bar, 20 μm. (C) Biofilm/mat formation by the indicated strains on YEPD+0.3% agar medium. Wild-type cells and the *pik1-83*, *ste12*Δ, and *flo11*Δ mutants were spotted onto plates and incubated for 5 days at 30°C. Representative colonies are shown. Bar, 1 cm.

Microscopy and protein localization experiments. For protein localization experiments, cells were grown in SD-Ura for 16 h at 30°C, shifted to 37°C for 4 h, and examined at 37°C. Differential interference contrast (DIC) and fluorescence microscopy using fluorescein isothiocyanate (FITC) filter sets were performed using an Axioplan 2 fluorescence microscope (Zeiss) with a Plan-Apochromat 100×/1.4 (oil) objective (numerical aperture [NA], 0.17). Digital images were obtained with the Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition and analysis. Cells were examined by oil immersion on glass slides (no. 2947-75; Corning, Inc., Corning, NY) with a glass coverslip (VWR 48366-227) using the 100× objective. Cells were photographed at 37°C using a slide warmer (no. 0115.000; PeCon GmbH, Germany).

RESULTS

PI(4)P kinases regulate the filamentous-growth MAPK pathway. The role of PI kinases in regulating the filamentous-growth pathway was examined. The filamentous-growth pathway was evaluated by measuring phosphorylation of the filamentous-growth MAPK Kss1p (91–93). Conditional (temperature-sensitive) alleles of PI kinases *pik1-83*, *stt4-4*, and *mss4-102* were tested, which showed reduced phosphorylation of Kss1p at 37°C (Fig. 1A). Thus, the generation of PI(4)P is necessary for activation of the filamentous-growth MAPK pathway. The above-described test was performed in a laboratory strain (SEY6210 background [94]). Many lab strains have lost the ability to undergo filamentous growth (95). Indeed, growth in the nonpreferred carbon source galactose (Gal) induces the filamentous-growth MAPK pathway (36, 78, 96) but led to a decrease in levels of phosphorylated Kss1p (P∼Kss1p) in the SEY6210 background (Fig. 1A, compare GLU lanes to GAL lanes). To better evaluate the role of PI(4)P signaling in regulating the filamentous-growth pathway, the *pik1-83* allele was introduced into the filamentous (Σ1287b) background (12, 95). The *pik1-83* strain behaved as expected, based on temperature sensitivity at 37°C (97), aberrant Golgi morphology (see Fig. S1 in the supplemental material) (57), and a defect in protein secretion (98).

The extracellular domain of Msb2p is highly glycosylated and migrates as a smear at ∼250 kDa (78). The extracellular inhibitory domain is proteolytically processed by the aspartyl protease Yps1p and is shed from cells (78). Consistent with the secretion defect of the *pik1-83* mutant, the large extracellular domain of HA-Msb2p was not shed (Fig. 1B, SUP) and accumulated in cell pellets (Fig. 1B, PELLET). The *pik1-83* mutant is also partly compromised for function at semipermissive temperatures (30°C) (70). The *pik1-83* mutant showed reduced levels of P∼Kss1p at 30°C (Fig. 1C, 30°C) and complete loss of P∼Kss1p at 37°C (Fig. 1D). At 37°C, P∼Kss1p levels were equivalent in glucose and galactose in wild-type cells. These results establish a requirement for Pik1p and other PI(4)P kinases in regulating the filamentous-growth MAPK pathway.

Generation of PI(4)P is required for filamentous growth and biofilm/mat formation. The fact that Pik1p regulates the filamentous-growth MAPK pathway suggested a role for PI(4)P in the regulation of filamentous growth (12) and biofilm/mat formation (15). At 30°C, the *pik1-83* mutant showed a defect in invasive growth by the plate-washing assay (Fig. 2A). The *pik1-83* mutant was more defective for invasive growth than a mutant that completely lacks pathway activity (Fig. 2A, *ste12*Δ). This may indicate that Pik1p has roles in regulating filamentous growth that extend beyond the regulation of the MAPK pathway. Microscopic analysis of the *pik1-83* mutant showed that cells were defective for cell elongation and filament formation compared to wild-type cells (Fig. 2B). These phenotypes are controlled by the filamentous-growth MAPK pathway (Fig. 2B, *ste12*Δ).

Like many microbial species, including fungal pathogens (99, 100), budding yeast forms biofilms or mats (15). On low-agar medium, the *pik1-83* mutant was defective for biofilm/mat formation (Fig. 2C). Specifically, *pik1-83* mats were small and smooth in appearance. In contrast, wild-type mats showed a wrinkled pattern, which was dependent on the filamentous-growth MAPK pathway (Fig. 2C, *ste12*Δ) and the cell adhesion molecule Flo11p (Fig. 2C, *flo11*Δ). Thus, generation of PI(4)P is required for filamentous growth and biofilm/mat formation in yeast.

PI(4)P is required for localization of membrane-associated regulators of the filamentous-growth MAPK pathway. PI kinases are critical regulators of membrane trafficking (51). Modification of PI(4)P at the Golgi (98, 101) and modification of

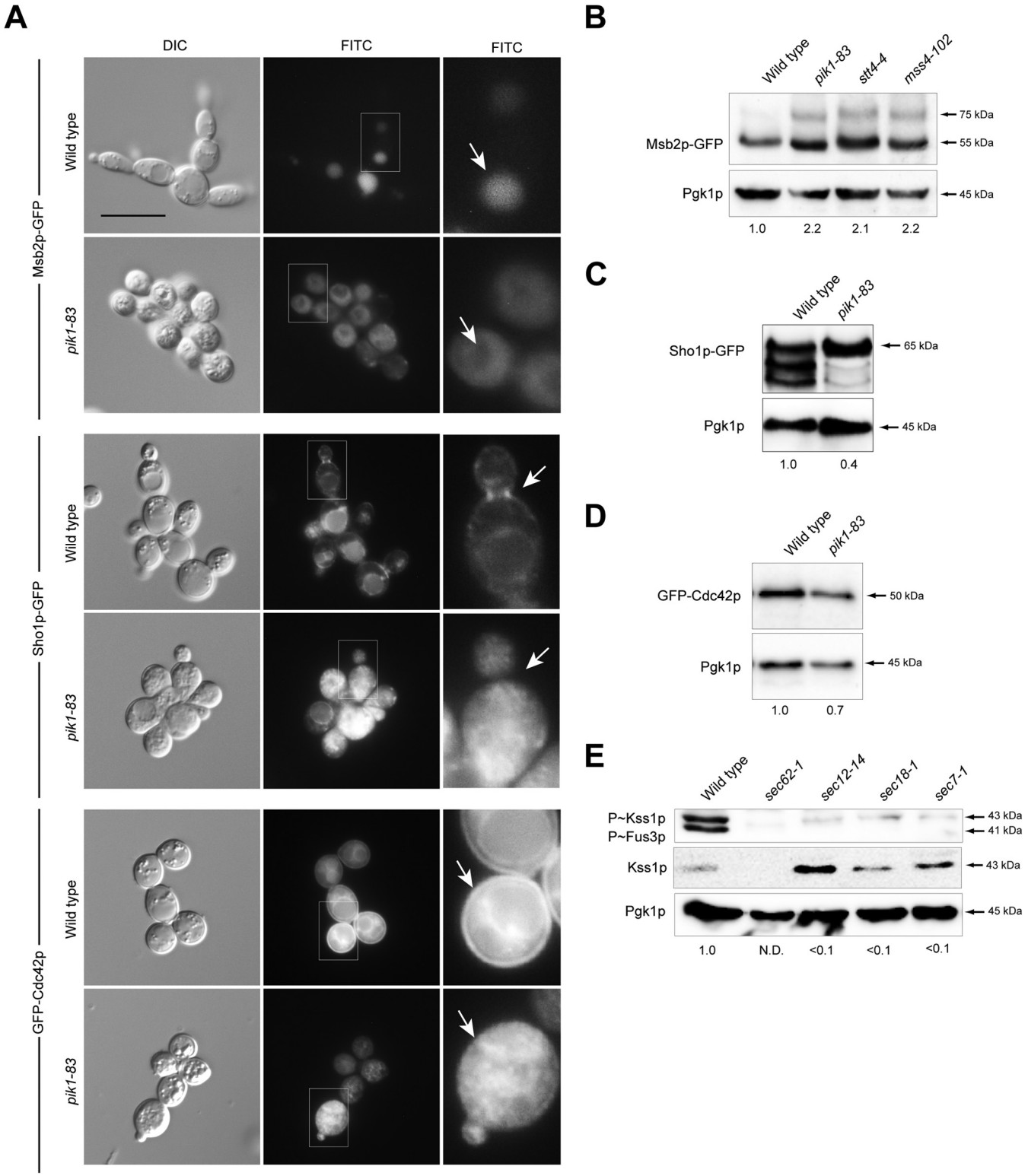


FIG 3 The localization and relative levels of PM regulators of the filamentous-growth MAPK pathway evaluated in the *pik1-83* mutant. (A) Localization of Msb2p-GFP, Sho1p-GFP, and GFP-Cdc42p in wild-type cells and the *pik1-83* mutant. Cells were incubated to mid-log phase at 30°C and shifted to 37°C for 4 h. Bar, 5 μm. The localization patterns shown are representative of the patterns seen in most cells over multiple independent trials. More than 200 cells were examined. The panels on the right show higher magnifications of the areas marked by rectangles in the middle column. Arrows indicate differences between wild type and the *pik1-83* allele. (B) Relative levels of Msb2p-GFP in wild-type cells and the *pik1-83* mutant compared to a loading control, Pgk1p. Cells were grown in YEPD medium to mid-log phase at 30°C and shifted to 37°C for 4 h. (C and D) Relative levels of Sho1p-GFP (C) and GFP-Cdc42p (D). (E) Phosphorylation of Kss1p in wild-type cells and the indicated trafficking mutants at 37°C for 4 h.

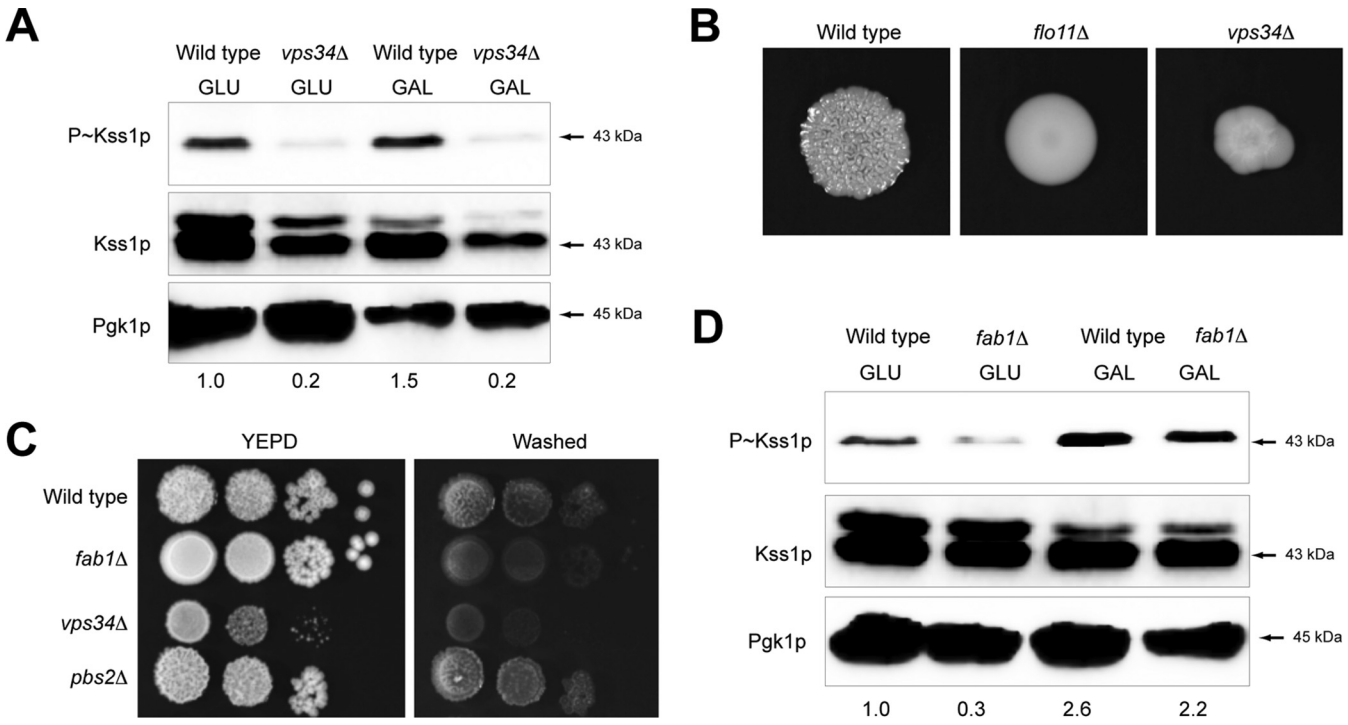


FIG 4 The role of PI kinases Vps34p and Fab1p in regulating the filamentous-growth MAPK pathway. (A) Kss1p phosphorylation in the *vps34*Δ mutant incubated at 30°C for 6 h in YEPD (GLU) and YEP-Gal (GAL) medium. (B) Biofilm/mat formation in the *vps34*Δ mutant and control strains incubated in YEPD+0.3% agar at 30°C for 3 days. (C) Plate-washing assay of the indicated strains incubated at 30°C after 3 days. The *pbs2*Δ mutant was used as a hyperinvasive growth mutant as a control. (D) Kss1p phosphorylation in the *fab1*Δ mutant incubated at 30°C for 6 h in YEPD (GLU) and YEP-Gal (GAL) medium.

PI(4,5)P2 at the PM (102, 103) are required for delivery of vesicles and cargoes to the PM. The signaling defect of the *pik1-83* and other PI(4)P mutants might result from mislocalization of PM proteins that regulate the filamentous-growth MAPK pathway. A functional Msb2p-GFP fusion shows primarily vacuolar localization (78), which results from turnover of the protein from the PM (104). Msb2p-GFP failed to show precise vacuolar localization in the *pik1-83* mutant, which indicates that the protein is mislocalized (Fig. 3A). Msb2p may fail to be delivered to the vacuole in the *pik1-83* mutant because it does not reach the PM, although we cannot rule out the possibility that Msb2p is trafficked from the Golgi to the vacuole directly.

Sho1p is a transmembrane protein and adaptor for the filamentous-growth MAPK pathway (31, 32, 34) and the HOG MAPK pathway (105). Sho1p-GFP localizes to the PM and the mother-bud neck (Fig. 3A) (106–108). Sho1p-GFP was also mislocalized in the *pik1-83* mutant (Fig. 3A). Sho1-GFP was specifically identified in internal sites in the *pik1-83* mutant. Sho1p-GFP was also mislocalized in the *stt4-4* and *mss4-102* mutants (see Fig. S2 in the supplemental material).

Cdc42p is a Rho-type GTPase that regulates (among other things) the filamentous-growth MAPK pathway (38, 39, 109). Cdc42p is localized to the PM membrane by a lipid modification, geranylgeranylation (110–114). GFP-Cdc42p was also mislocal-

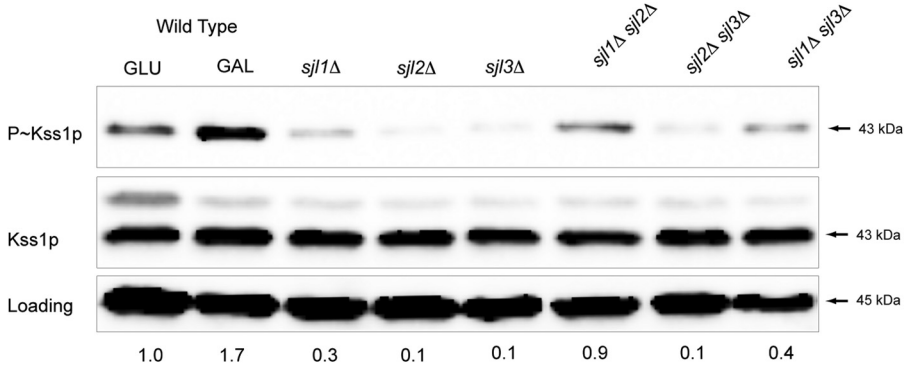
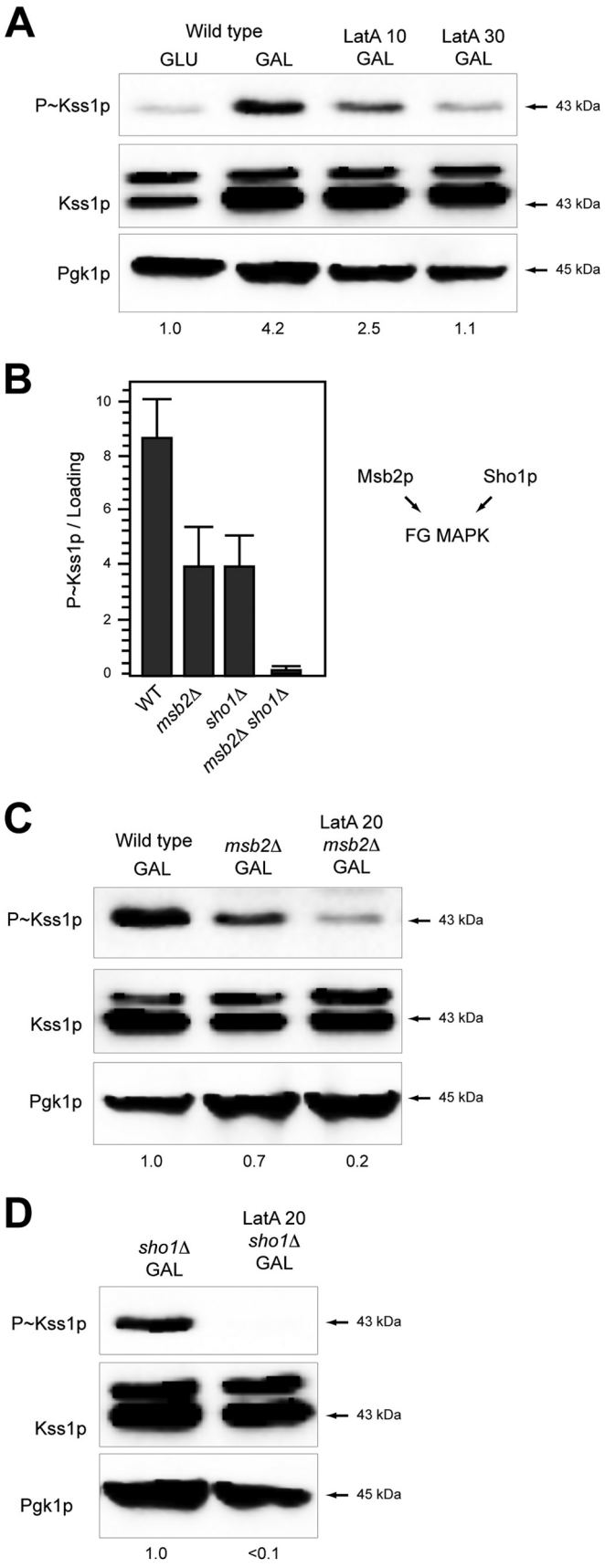


FIG 5 Regulation of the filamentous-growth MAPK pathway by synaptojanin-type PIP phosphatases. Phosphorylation of Kss1p in combinations of PI phosphatase mutants was assessed. Wild-type cells and the *sjl1*Δ, *sjl2*Δ, *sjl3*Δ, *sjl2*Δ *sjl3*Δ, *sjl1*Δ *sjl2*Δ, and *sjl1*Δ *sjl3*Δ mutants were incubated in YEPD (for wild-type [GLU]) or YEP-Gal (GAL) medium for 6 h at 30°C.



ized in the *pik1-83* mutant. Compared to wild-type cells, which show Cdc42p localization at the PM and vacuolar membrane (Fig. 3A), GFP-Cdc42p was seen primarily in internal sites in the *pik1-83* mutant (Fig. 3A). GFP-Cdc42p was also mislocalized in the *stt4-4* and *mss4-102* mutants (see Fig. S3 in the supplemental material).

It was previously reported that Cdc42p levels are reduced in the *mss4-102* mutant (103). The localization defects of Cdc42p and other filamentous-growth MAPK regulatory proteins might result from a decrease in protein stability. The processed form of Msb2p-GFP, Msb2^{P}p, migrates at 55 kDa, and a minor product migrates at 75 kDa (104). The level of Msb2^{P}p-GFP was not reduced in the *pik1-83*, *stt4-4*, or *mss4-102* mutants (Fig. 3B). The higher levels of Msb2p seen in these mutants might result from a delay in turnover of the protein. The level of Sho1p-GFP was reduced in the *pik1-83* mutant (Fig. 3C). The levels of GFP-Cdc42p were not reduced in the *pik1-83* mutant (Fig. 3D). Thus, defects in trafficking of pathway components to the PM in a PI(4)P-dependent manner, not loss of protein abundance, may account for the defect in activation of the filamentous-growth MAPK pathway in PI kinase mutants.

To further test this possibility, protein trafficking mutants that trap PM cargoes in the secretory pathway were tested for filamentous-growth MAPK pathway activity. Specifically, the *sec62-1*, *sec12-14*, *sec18-1*, and *sec7-1* mutants, which are defective for protein trafficking at various steps in the secretory pathway (115–120), were tested. Like PI kinase mutants, these mutants showed a decrease in MAPK activity (Fig. 3E) and mislocalization of PM regulators of the filamentation MAPK pathway (data not shown). These results are consistent with the idea that delivery of membrane-associated regulators of the filamentous-growth MAPK pathway to the PM is required for activation of the filamentous-growth MAPK pathway.

Other PI kinases regulate the filamentous-growth MAPK pathway. Other PI kinases may also regulate the filamentous-growth MAPK pathway. Vps34p regulates PI(3)P levels at the endosome/multivesicular body and is required for protein trafficking to the vacuole/lysosome (62, 121–123). The *vps34*Δ mutant showed reduced P~Kss1p levels (Fig. 4A). The levels of total Kss1p were also reduced, which may result from a positive-feedback loop, given that the *KSS1* gene is a transcriptional target of the filamentous-growth pathway (124).

The *vps34*Δ mutant was also defective for biofilm/mat formation (Fig. 4B). The *flo11*Δ mutant was used as a control in evaluating biofilm/mat formation. The *vps34*Δ mutant also had a growth defect; however, *vps34*Δ mats were smaller than wild-type mats and smooth in appearance. The unstructured appearance of these mats suggests that Vps34p plays a role in their development. Vps34p was also required for invasive growth (Fig. 4C), although its growth defect on YEPD (Glu) medium may contribute to its invasive growth defect. The PI(3,5)P2 kinase Fab1p (63, 125) was also tested. The *fab1*Δ mutant showed a modest reduction in

FIG 6 The role of LatA on the activity of the filamentous-growth MAPK pathway. (A) Wild-type cells were grown in YEPD (GLU) or YEP-Gal (GAL) for 6 h at 30°C and treated with LatA at the indicated concentrations (μM) for 3 h at 30°C. (B) Phosphorylation of Kss1p in wild-type cells and *msb2*Δ, *sho1*Δ, and *msb2*Δ *sho1*Δ mutants. Data are the ratios of the phosphorylated Kss1p levels to total Kss1p quantified by ImageJ. (C and D) Same experiment performed in the *msb2*Δ (C) and *sho1*Δ (D) mutants with 20 μM LatA for 3 h at 30°C.

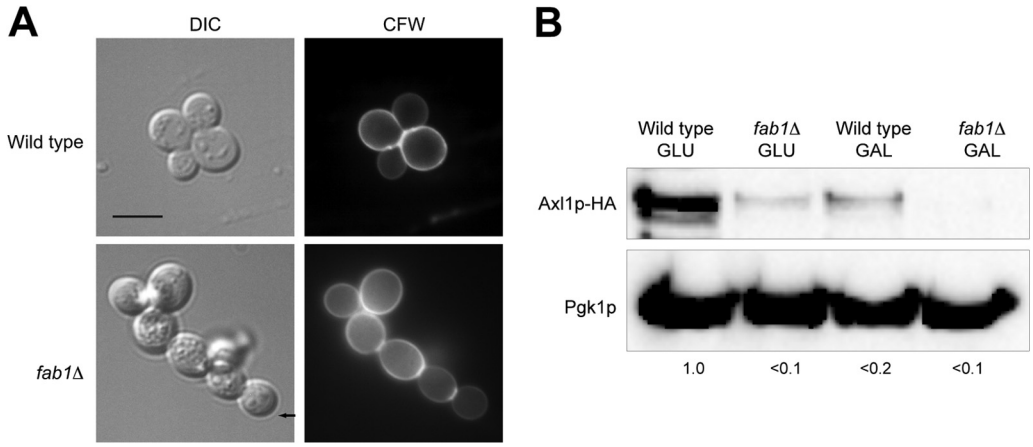


FIG 7 Role of the PI kinase Fab1p in regulating axial budding pattern and Axl1p protein levels. (A) CFW staining of the *fab1*Δ mutant and control strain grown to saturation in YEPD medium at 30°C. (B) Levels of Axl1p-HA in the wild-type cells and *fab1*Δ mutant at 30°C. Cells were incubated for 6 h in YEPD (GLU) and YEP-Gal (GAL) medium.

P∼Kss1p levels in high-glucose (basal) conditions (Fig. 4D) and exhibited a defect in invasive growth (Fig. 4C). Thus, Fab1p may play a minor role in regulating the filamentous-growth MAPK pathway and may have several roles in regulating filamentous growth.

Synaptojanin-type PIP phosphatases regulate the filamentous-growth pathway. PIP phosphatases dephosphorylate PI(4,5)P2 at different cellular locations to maintain PIP balance (54, 68, 126). The role of PIP phosphatases in regulating the filamentous-growth MAPK pathway was evaluated. The level of Kss1p∼P was reduced in PIP phosphatase *sjl1*Δ, *sjl2*Δ, and *sjl3*Δ single mutants (Fig. 5). *sjl1*Δ *sjl2*Δ, *sjl2*Δ *sjl3*Δ, and *sjl1*Δ *sjl3*Δ double mutants also showed reduced MAPK activity (Fig. 5). The *sjl1*Δ *sjl2*Δ mutant showed higher P∼Kss1p levels than the single mutants. The Sjl proteins are functionally redundant, and it is possible that Sjl3p alone carries out a new function in this genetic context. Therefore, PIP phosphatases are involved in regulating the filamentous-growth MAPK pathway. Two other phosphatases, Sac1p and Inp54p, were also tested but did not regulate the filamentous-growth MAPK pathway (data not shown). Thus, altering the balance of PIP signaling through perturbation of PI kinases or PIP phosphatases impacts the filamentous-growth MAPK pathway.

An intact actin cytoskeleton is required for filamentous-growth pathway activity. The trafficking defect of the PM regulators Msb2p, Sho1p, and Cdc42p in PI(4)P kinase mutants may explain the defect in filamentous-growth MAPK pathway activity. Other PI kinase mutants and PIP phosphatase mutants did not show dramatic localization defects of these proteins (data not shown). PI kinases and PIP phosphatases also regulate cell polarity and the actin cytoskeleton (58, 59, 68, 82, 127–129). Defects in the actin cytoskeleton may underlie the MAPK signaling defects of these mutants. To test this possibility, a pharmacological inhibitor of filamentous actin, latrunculin A (LatA) (130), was tested. Addition of a minimal concentration of LatA (10 μM) led to reduced P∼Kss1p levels (Fig. 6A), despite the fact that total Kss1p levels are higher under this condition. Addition of higher concentration of LatA (30 μM) caused a more severe reduction in P∼Kss1p levels (Fig. 6A). Thus, an intact cytoskeleton is required for filamentous-growth MAPK pathway signaling.

The actin cytoskeleton is required for many different cellular processes, such as delivery of vesicles and cargoes to the PM and turnover of proteins from the PM by endocytosis (131, 132). The individual contributions of Msb2p and Sho1p in regulating MAPK signaling were examined in response to treatment with LatA. Under nutrient-limiting conditions, Msb2p and Sho1p were partly redundant for activation of the filamentous-growth MAPK pathway (Fig. 6B), which is consistent with the invasive growth phenotypes of the *msb2*Δ and *sho1*Δ mutants (31). The *msb2*Δ mutant, which is somewhat defective for MAPK activity, showed a further reduction in P∼Kss1p levels upon treatment with LatA (Fig. 6C). Thus, in the *msb2*Δ mutant, the Sho1p-dependent signal requires an intact actin cytoskeleton. The *sho1*Δ mutant showed a similar response (Fig. 6D), indicating that the Msb2p-dependent signal also requires an intact actin cytoskeleton. Therefore, the actin cytoskeleton is required to facilitate filamentous-growth MAPK pathway signaling by a mechanism that is dependent on Msb2p and Sho1p.

PIP signaling contributes to axial budding in haploid yeast. Cell polarity in yeast is also controlled by the actin cytoskeleton

TABLE 2 Budding patterns of mutants defective for PI signaling and control strains in haploid yeast cells

Mutation(s)[a]	Percentage of cells with pattern		
	Distal unipolar	Random	Axial
None (wild type)	8	<1	92
*rsr1*Δ[b]	10	32	58
*bud3*Δ[c]	36	1	63
*fab1*Δ	20	<1	80
*sac1*Δ	19	1	80
*sjl1*Δ	11	1	88
*sjl2*Δ	7	3	90
*sjl3*Δ	6	<1	81
*sjl1*Δ *sjl2*Δ	21	<1	79
*sjl1*Δ *sjl3*Δ	18	1	81
*sjl2*Δ *sjl3*Δ	30	6	64

[a] Cells were grown to saturation in YEPD medium, fixed, and stained with CFW. More than 200 cells were counted for each mutant.
[b] The *rsr1*Δ mutant exhibits a random budding pattern (135) and was used as a control.
[c] The *bud3*Δ mutant exhibits a distal-pole budding pattern (135) and was used as a control.

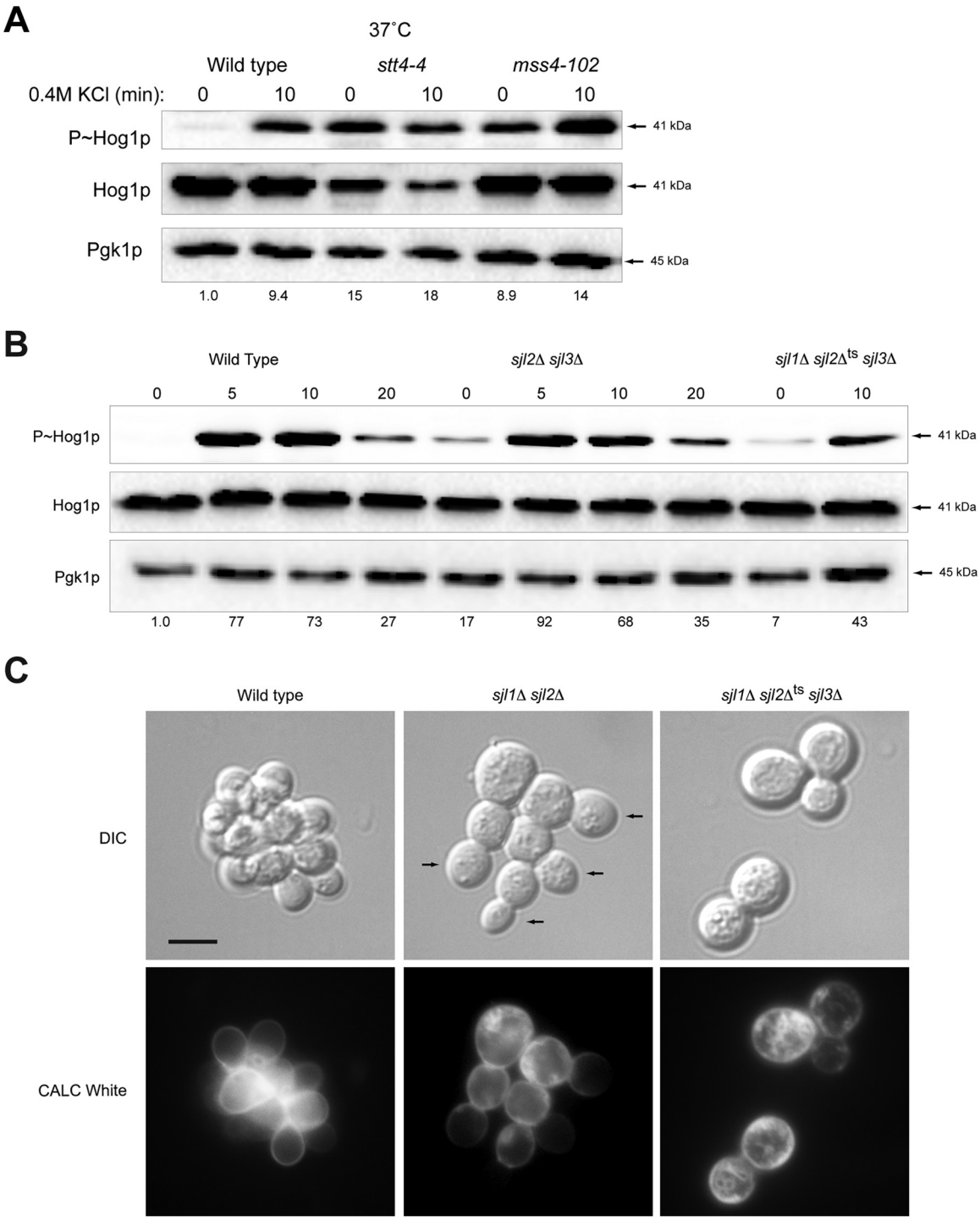


FIG 8 Role of the PI kinase and PIP phosphatase mutants in regulating the HOG pathway. (A) Hog1p phosphorylation in essential PI kinase mutants. Cells were grown for 37°C for 4 h. Cells were treated with 0.4 M KCl for the indicated times. (B) Hog1p phosphorylation in the indicated combinations of PIP phosphatase mutants. (C) CFW staining of control and PIP phosphatase mutants grown to saturation in YEPD medium at 30°C.

(133, 134). Depending on cell type and growth condition, yeast cells bud at the proximal or distal poles (88, 135–138). Many genes regulate bud site selection in haploid and diploid cells (135, 139, 140). PI kinase mutants showed a bud site selection defect. Specifically, at 30°C, haploid *pik1-83* and *fab1*Δ mutants failed to bud axially and showed an increase in distal-unipolar budding (Fig. 7A shows data for *fab1*Δ; results for other mutants are shown in Fig. S4 in the supplemental material [arrows]). The increased distal-pole budding in the *pik1-83* mutant, which would be expected to promote filamentous growth, was not sufficient to restore agar invasion to that mutant (Fig. 2A). This phenotype was quantitated for the *fab1*Δ mutant (Table 2) but not for the essential kinase

mutants, because at 37°C the mutants fail to produce buds due to their growth defect. Combinations of PIP phosphatase mutants also showed axial budding defects, including the *sjl1*Δ *sjl2*Δ, *sjl2*Δ *sjl3*Δ, and *sjl1*Δ *sjl3*Δ double mutants (Table 2; also, see Fig. S4 [arrows]). The Golgi PI(4)P phosphatase mutant *sac1*Δ also showed an axial budding defect (Table 2; also, see Fig. S4). Thus, PIP signaling contributes to axial budding in haploid yeast.

The defect in axial budding might be related to the levels of Axl1p, an axial-specific protein expressed in haploid cells (141). Axl1p is not produced in diploid cells (77, 141–143) or haploid cells grown under nutrient-limiting conditions (20). One of the PI kinase mutants that showed an axial budding defect was tested for the levels of Axl1p. The *fab1*Δ mutant showed reduced Axl1p-HA levels by immunoblot analysis, as seen under glucose-rich and glucose-limited conditions (Fig. 7B). Therefore, changes in Axl1p levels may provide a connection between axial budding and PI signaling.

Role of PI signaling in regulating the HOG pathway and yeast cell wall. The filamentous-growth and HOG pathways share a subset of components (1, 144–147). PIP regulators may impact the activity of the HOG pathway, which is measured by phosphorylation of the MAPK Hog1p (148, 149). It was previously shown that Pik1p plays a positive role in regulating the HOG pathway (71). We found that the *stt4-4* and *mss4-102* mutants showed constitutive HOG pathway activity at the restrictive temperature (Fig. 8A), although not to the levels seen under activating conditions. Therefore, PI(4)P kinases that function at the PM play an inhibitory role in regulating the HOG pathway.

Several PI kinase and PIP phosphatase mutant combinations showed growth defects in high-osmolarity medium. The *vps34*Δ mutant had a growth defect on YEPD supplemented with 1 M KCl that was similar to the *pbs2*Δ mutant (data not shown). No significant reduction in P~Hog1p was observed in the *vps34*Δ mutant (data not shown). Some combinations of PIP phosphatase mutants also showed osmotic sensitivity, such as the *sjl2*Δ *sjl3*Δ mutant (128). The *sjl2*Δ *sjl3*Δ mutant showed normal HOG pathway activity compared to wild-type cells (Fig. 8B). This may indicate that PIP phosphatases play roles in regulating osmotic tolerance through a mechanism that is independent of the HOG pathway.

A connection between the cell wall integrity pathway and PIP signaling has been described (58). Calcofluor white (CFW), which stains chitin in the yeast cell wall (150, 151), showed an irregular pattern in the *sjl1*Δ *sjl2*Δ mutant (Fig. 8C). A triple PIP phosphatase mutant, the *sjl1*Δ *sjl2*Δ^{ts} *sjl3*Δ mutant, also showed a defect in chitin deposition at 37°C (Fig. 8C). Single *sjl* mutants showed uniform distribution of CFW on the cell surface (see Fig. S4 in the supplemental material). Thus, combinations of Sjl-type phosphatases have a function in maintenance of the yeast cell wall.

DISCUSSION

PIP signaling is an essential cellular process that is critical for the regulation of protein secretion, actin cytoskeleton reorganization, and organelle identity, biogenesis, transport, and inheritance. Here, we describe a role for PIP signaling in the regulation of an ERK-type MAPK pathway that controls filamentous growth in yeast. We specifically show that generation of PI(4)P is required for filamentous-growth MAPK pathway signaling. This may result from mislocalization of PM regulators of the MAPK pathway in PI(4)P kinase mutants. Failure of PM regulators to reach the PM may be expected to result in MAPK signaling defects. Previous studies have implicated PI signaling in the regulation of filamentous growth, particularly in *C. albicans* (72–74). Here, we posit that this connection can be explained, at least in part, at the level of the MAPK pathway.

We also demonstrate that the filamentous-growth MAPK pathway requires other PI kinases that generate PI(3)P and PI(3,5)P2 and Sjl-type PIP phosphatases. PM MAPK regulatory proteins are not mislocalized in these mutants (data not shown); thus, how the MAPK is functionally connected to these PIP species is not clear. We show that perturbation of PIP signaling influences cell polarity. Thus, the MAPK signaling defect in these mutants may result from problems in cell polarity. In support of this possibility, pharmacological disruption of filamentous actin results in a defect in filamentous-growth MAPK pathway activity. Previous reports have suggested a link between actin cytoskeleton and filamentous growth (152). Thus, the observations reported here extend this connection by linking the actin cytoskeleton to the activity of the filamentous-growth MAPK pathway.

We also show that PIP signaling is required for a specific aspect of cell polarity regulation, that of axial bud site selection in haploid cells. This may result from a general defect in the actin cytoskeleton. However, the levels of the axial-specific factor Axl1p are reduced in at least one PI kinase mutant and may reflect a specific connection between the two pathways. PIP signaling in yeast is required for other cellular processes, including proper regulation of the cell wall (58), and we identify cell wall defects in some *sjl* mutant combinations. Intriguingly, different PIP combinations influence each of these processes, which indicates a high degree of functional specialization of PIP regulators.

Different PIP species differentially regulate the HOG pathway. A Golgi PI(4)P kinase, Pik1p, positively regulates the HOG pathway (71), whereas PM PI(4)P kinases negatively regulate the HOG pathway. The mechanistic basis for the antagonistic roles of these PI(4)P kinases is not clear and underscores the importance of future studies of PI(4)P in regulation of the p38 MAPK pathway. The filamentous-growth (ERK-type) and HOG (p38-type) MAPK pathways have opposing functions in the cell (7, 106, 153–155). The fact that PI(4)P has different effects on ERK and p38 MAPK pathways could, in principle, influence the specificity of MAPK outputs. Future studies on how PIP signaling differentially activates MAPK pathways will shed light on the overall regulation of signaling pathways in this system.

ACKNOWLEDGMENTS

We thank Scott Emr (Cornell University), Chris Stefan (University Medical College London), Alan Tartakoff (Case Western Reserve University), Doug Johnson (University of Vermont), and C. Boone (University of Toronto) for reagents. We thank Hay-Oak Park for discussions and assistance.

P.J.C. is supported by the U.S. Public Health Service (GM098629).

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