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MSS4, a Phosphatidylinositol-4-phosphate 5-Kinase Required for Organization of the Actin Cytoskeleton in *Saccharomyces cerevisiae**

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The *Saccharomyces cerevisiae* protein MSS4 is essential and homologous to mammalian phosphatidylinositol-4-phosphate (PI(4)P) 5-kinases. Here, we demonstrate that MSS4 is a lipid kinase. MSS4 has dual substrate specificity *in vitro*, converting PI(4)P to PI(4,5)P_2 and to a lesser extent PI(3)P to PI(3,4)P_2; no activity was detected with PI or PI(5)P as a substrate. Cells overexpressing *MSS4* contain an elevated level specifically of PI(4,5)P_2, whereas *mss4* mutant cells have only approximately 10% of the normal amount of this phosphorylated phosphoinositide. Furthermore, cells lacking MSS4 are unable to form actin cables and to properly localize their actin cytoskeleton during polarized cell growth. Overexpression of *RHO2*, encoding a Rho-type GTPase involved in regulation of the actin cytoskeleton, restores growth and polarized distribution of actin in an *mss4* mutant. These results suggest that MSS4 is the major PI(4)P 5-kinase in yeast and provide a link between phosphoinositide metabolism and organization of the actin cytoskeleton *in vivo*.

Phosphorylated phosphoinositides play a crucial role in the transduction of signals induced by a variety of stimuli, including growth factors, hormones, and neurotransmitters. Signaling by phosphoinositides is conserved from yeast to mammalian cells. Phosphatidylinositol (PI)[1] is sequentially phosphorylated by a PI 4-kinase to produce PI(4)P and by a PI(4)P 5-kinase to generate PI(4,5)P_2. PI(4,5)P_2 is a substrate for phospholipase C, yielding inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (1). IP_3 binds to specific receptors and induces the release of calcium from intracellular stores, whereas diacylglycerol activates protein kinase C. The increase in intracellular calcium and the activation of protein kinase C act in synergy to regulate transcription and to control other cellular events. More recently, it has been shown that PI(4,5)P_2 can, at least in mammalian cells, be further phosphorylated by PI-3 kinase to generate PI(3,4,5)P_3, a mediator of cell growth and survival (Ref. 2 and references therein). In addition, PI(4,5)P_2 can directly regulate the organization of the actin cytoskeleton through specific interactions with actin-associated proteins (3–10).

Several lipid kinases able to generate PI(4,5)P_2 have been identified in mammalian cells. They fall into two subfamilies (type I and type II), differing both biochemically and immunologically, that together form a novel family of homologous PI kinases with no homology to PI 3-kinases or classical PI 4-kinases (11–16). Zhang *et al.* (17) have presented evidence that both the type I and type II enzymes generate PI(4,5)P_2 by phosphorylating PI(4)P on the D-5 position of the inositol ring. More recently, Rameh *et al.* (18) have demonstrated that both types generate PI(4,5)P_2, but the type I enzyme is a PI(4)P 5-kinase whereas the type II enzyme is a PI(5)P 4-kinase. Both types of enzymes also seem to catalyze the phosphorylation of PI(3)P (17, 18). Hereafter, we collectively refer to the type I and type II enzymes as PIP kinases.

In the yeast *Saccharomyces cerevisiae*, two predicted proteins, FAB1 and MSS4, show extensive sequence similarity to the mammalian PIP kinases. The *FAB1* gene was identified based on the *fab1* mutant phenotype of aberrant chromosome segregation. Further characterization of the *fab1* mutant indicated that loss of FAB1 causes defects in vacuole function and morphology and in cell surface integrity (19). The *MSS4* gene was first identified as a multicopy suppressor of the temperature-sensitive lethality caused by a mutation in the PI 4-kinase gene *STT4* (20). *MSS4* was also isolated recently as a multicopy suppressor of the growth defect caused by loss of TOR2, a yeast homologue of PI 3- and 4-kinases (21). Unlike *FAB1*, *MSS4* is essential, suggesting that these two proteins have different cellular functions. Based on their homology with mammalian PIP kinases, FAB1 and MSS4 are assumed to be the yeast counterparts of the mammalian lipid kinases, although kinase activity for FAB1 and MSS4 has yet to be demonstrated.

This study was undertaken to determine whether, as predicted by its primary structure, MSS4 is a PIP kinase and to identify its cellular function(s). We demonstrate that MSS4 accounts for most of the PI(4)P 5-kinase activity in yeast and controls organization of the actin cytoskeleton.

MATERIALS AND METHODS

Strains, Plasmids, and Media— The *S. cerevisiae* strains used in this study are listed in Table I. All strains were isogenic derivatives of JK9–3d. *Escherichia coli* strain MH4 is Δ*(lac)X74 hsr⁻ hsm⁺ strA leuB600 galE galK*. Plasmids used in this study were as follows. Plasmids pSH22, pSH23, and pSD6 are pSEY18 (2 μ *URA3*), YCplac111 (*CEN LEU2*), and YCplac33 *(CEN URA3)*, respectively, carrying *MSS4* on a 4.1-kb *Bam*HI-*Xho*I fragment. pSD2 (pGST-MSS4) is the yeast GST-fusion vector pEG-KT (2 μ *URA3 leu2-d*) (22) containing the full coding region of *MSS4*. pRHO2 is pC-186, *RHO2* (2 μ *URA3*) (23). Rich media (YPD) and synthetic minimal media (SD, SRaff/Gly) complemented with the appropriate nutrients for plasmid maintenance were as described (24).

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[1] The abbreviations used are: PI, phosphatidylinositol; PI(4,5)P_2, phosphatidylinositol 4,5-biphosphate; PI(3,5)P_2, phosphatidylinositol 3,5-biphosphate; IP_3, inositol 1,4,5-triphosphate; kb, kilobase(s); GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; GroPIns(3,5)P_2, deacylated PI(3,5)P_2.

TABLE I
Yeast strains used in this study

Strain	Genotype
JK9–3d **a**/α	*MAT* ***a***/*MAT* α *leu2–3,112*/*leu2–3,112 ura3–52*/*ura3–52 trp1*/*trp1 his4*/*his4 rme1*/*rme1 HML****a***/*HML****a***
MH272–1d**a**/α	JK9–3d**a**/α *his3*Δ/*his3*Δ *HIS4*/*HIS4*
SD1	MH272–1d**a**/α *mss4::HIS3MX6*/*MSS4*
SD2–5a/pSH22	MH272–1d**a** *mss4::HIS3MX6*/*pSEY18*::*MSS4*
SD100	MH272–1d**a** *mss4::HIS3MX6*/YCplac111::*MSS4*
SD102	MH272–1d**a** *mss4::HIS3MX6*/YCplac111::*mss4–2^{ts}*

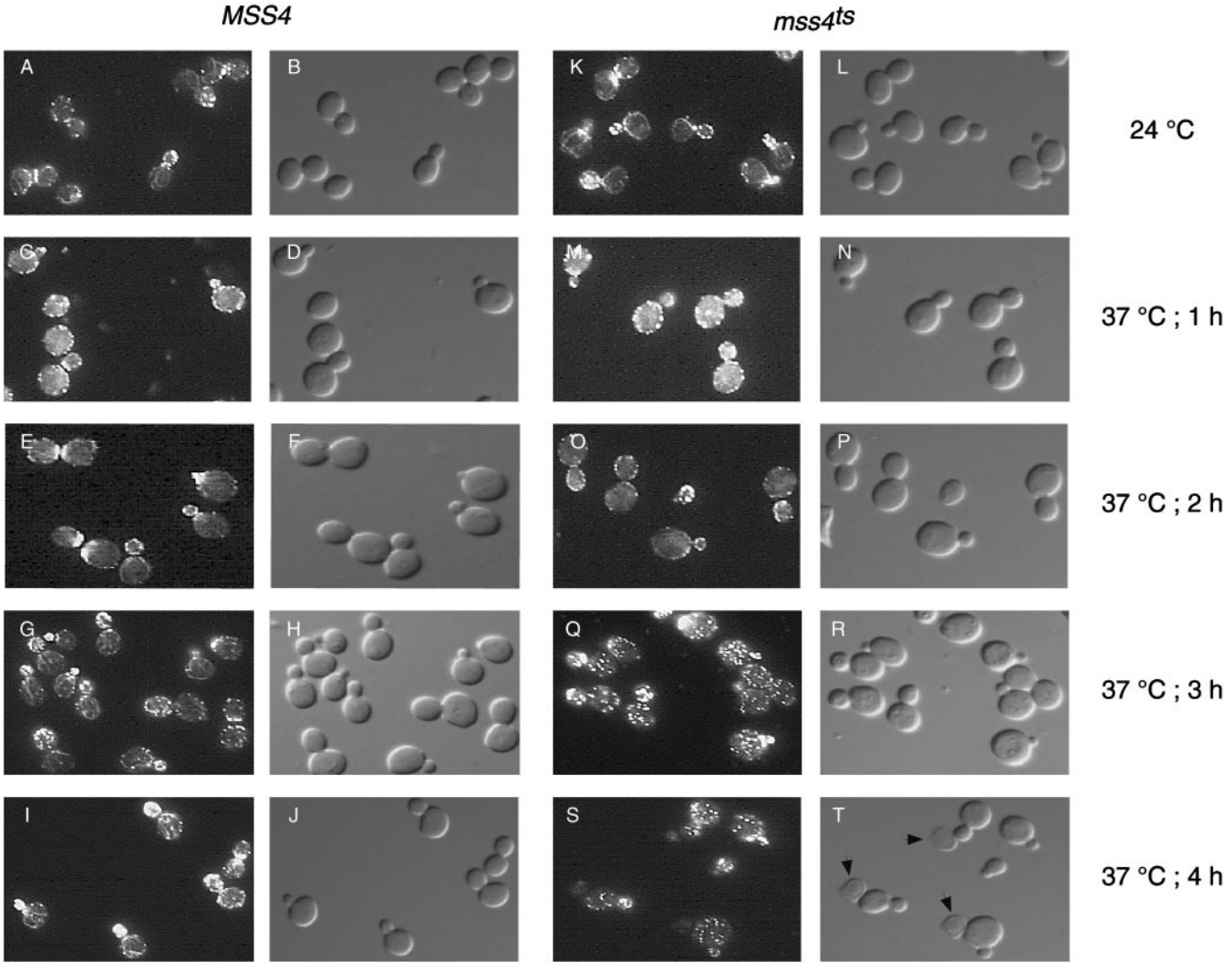

FIG. 1. **Actin distribution in wild-type and *mss4-2^{ts}* cells.** Logarithmic cultures of wild-type *MSS4* (SD100; *A–J*) and *mss4-2^{ts}* (SD102; *K–T*) cells were grown at 24 °C and then shifted to 37 °C. At the indicated times, aliquots were removed, fixed with formaldehyde, and stained with TRITC-phalloidin. The cells were examined with Nomarski optics (*B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*, *R*, *T*) and by fluorescence to visualize the actin (*A*, *C*, *E*, *G*, *I*, *K*, *M*, *O*, *Q*, *S*). *Arrowheads* show "ghost" *mss4-2^{ts}* cells.

Expression and Purification of Recombinant GST-MSS4—GST-MSS4 was expressed in yeast from the *GAL1* promotor. Cells containing pGST-MSS4 (JK9–3d**a**/α/pSD2) were grown at 30 °C in SRaff/Gly-URA to early logarithmic phase. Expression of the GST-MSS4 fusion protein was induced by the addition of 2% (final) galactose for 8 h. The cells were resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride), lysed with glass beads in a bead-beater, and centrifuged at 500 × *g*, 4 °C, for 10 min to remove cell debris. GST-MSS4 was precipitated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. After washing five times with lysis buffer and five times with 50 mM Tris-HCl, pH 7.5, the beads were stored at −70 °C until use.

Phosphatidylinositol Kinase Assay—The lipids used in this assay were PI (Lipid Products), PI(3)P (gift from Dr. G. Painter), PI(4)P (Calbiochem), and PI(5)P (gift from Drs. G. Prestwich and G. Peng). Dried lipids were sonicated into 46 μl of assay buffer (25 mM HEPES, pH 7.4, at 25 °C, 2 mM MgCl, 0.2 mM EDTA, 1 mM EGTA, 5 mM β-glycerophosphate, 1 mM dithiothreitol, and 120 mM NaCl) to give a final concentration in the assay of 100 μM substrate and 300 μM phosphatidylserine (Lipid Products). GST-MSS4 bound to glutathione beads (4 μl) was added to the assay, and the reactions were started by the addition of 15 μl of assay buffer containing 5 μCi [γ-^{32}P]ATP and 43 μM ATP. Kinase activities were tested at 30 °C for 30 min. The reactions were terminated by the addition of 243 μl of methanol/chloroform (2:1), and the lipids products were extracted by the addition of 58 μl of 2.4 M HCl and 245 μl of chloroform. The lower phase was washed with 239 μl of upper phase (47:48:3, 1 M HCl/methanol/chloroform, v/v/v), and the lipids were analyzed by TLC on a Whatman LKD5 TLC plate as described by Munnik *et al.* (25). After autoradiography for 1.5 h at room temperature, the phosphorylated spots were scraped, deacylated, and analyzed by HPLC as described (26), using purified tritiated deacylated PI(3,5)P_2 (GroPIns(3,5)P_2) as an internal standard.

[^{3}H]Inositol Labeling of S. cerevisiae—SD100 and SD102 were grown

at 24 °C to an A_{600} of 0.7 in medium containing 50 μCi/ml [^{3}H]inositol (Amersham). The cells were harvested by centrifugation and resuspended in one-fifth of the original volume. After control samples had been removed, the cells were incubated at 37 °C. Aliquots (200 μl) were collected, and the cells were arrested by the addition of 500 μl of methanol. The samples were dried *in vacuo*, and lipid extraction was performed as described previously (26). Some experiments (Fig. 4*A*) were done without temperature shift. In this case, the cells were labeled at 30 °C, and lipids were immediately extracted, as described above.

Disruption of MSS4—The entire open reading frame of the *MSS4* gene was deleted in MH272–1d**a**/α with a polymerase chain reaction-generated fragment containing the *HIS3*MX6 module (A. Wach, and P. Philippsen, Biozentrum, Basel, Switzerland) flanked by 45 nucleotides homologous to the region either directly upstream of the start codon or downstream of the stop codon of *MSS4*. The oligonucleotides used for generation of the polymerase chain reaction disruption cassette were 5′-GACAGTTTGCCCTATATCGCTTTTCCCTATCAATAGTTTCTAACTCGCGCCAGATCTGTTTAGCTTGCC-3′ and 5′-AGACTGAGTACATAGTCGATAGGTTATTTACCTGTGCCCTACTCACGAGCTCGTTTAAACTGGATGGCG-3′. The disruption was confirmed by Southern blot analysis.

Isolation of an MSS4 Conditional Allele—Chemical mutagenesis and identification of mutants were performed as described previously (27). Briefly, the plasmid pSH23 was mutagenized *in vitro* for various times with 1 M hydroxylamine. The level of mutagenesis was assessed by testing the plasmids for complementation of the leucine auxotrophy of the *E. coli* strain MH4. Plasmid aliquots in which 5% of the *LEU2* genes had been inactivated were used to transform SD2–5a/pSH22 (*URA3*). Leu$^+$ transformants were replica plated at 24 °C on 5-fluoroorotic acid-containing medium to select for loss of the pSH22 plasmid (carrying the wild-type *MSS4*). The colonies were afterward plated on YPD at 24 and 37 °C for detection of a temperature-sensitive phenotype. Plasmids conferring a temperature-sensitive growth were isolated and retested on the original host strain SD2–5a/pSH22, using the same procedure. The recessive *mss4-2^{ts}* allele was isolated from 12,000 transformants. The *mss4-2^{ts}* allele in the temperature-sensitive strain SD102 was complemented by a plasmid-borne copy of *MSS4* (pSD6).

Fluorescence Microscopy—Cells were grown to early logarithmic phase, fixed in formaldehyde, and stained with TRITC-phalloidin (Sigma) to visualize actin, as described previously (28).

RESULTS

Disruption of MSS4 and Isolation of a Conditional Mutant—To determine the cellular function of MSS4, we constructed a strain deleted for *MSS4*. The disruption replaced the entire open reading frame of *MSS4* with *HIS3*. In agreement with a previous report (20), we found that MSS4 is essential for growth in our strain background. When a heterozygous diploid *mss4Δ::HIS3/MSS4* strain (SD1) was sporulated and dissected, only the two His$^-$ spores gave rise to colonies on rich medium containing either glucose or galactose as a carbon source (data not shown). Because deletion of *MSS4* is lethal, we isolated a temperature-sensitive (*ts*) mutant to facilitate the analysis of MSS4 function (see "Materials and Methods"). The temperature-sensitive mutant SD102, carrying the *mss4-2^{ts}* allele, grew like wild type at the permissive temperature (24 or 30 °C) but stopped growing 4 h (corresponding to approximately 3 generations) after shift to the restrictive temperature (37 °C).

Inactivation of MSS4 Causes Loss of Cell Viability—To further assess the consequences of loss of MSS4, the *mss4-2^{ts}* strain was examined under the light microscope after incubation at the restrictive temperature. The only striking observation was the appearance of "ghost" cells (see Fig. 1), suggesting a loss of viability of the mutant cells at 37 °C. Indeed, the mutant cells started to die after 2 h at 37 °C, as evidenced by staining with the vital dye methylene blue. After 4 h at 37 °C, ~60% of the *mss4-2^{ts}* cells stained intensely with methylene blue (data not shown), whereas no appreciable staining was observed for a wild-type control.

MSS4 Is Required for Organization of the Actin Cytoskeleton—MSS4 was identified in our laboratory in a screen for multicopy suppressors of the growth defect of a *tor2^{ts}* strain (21). *TOR2* encodes a putative phosphatidylinositol kinase with

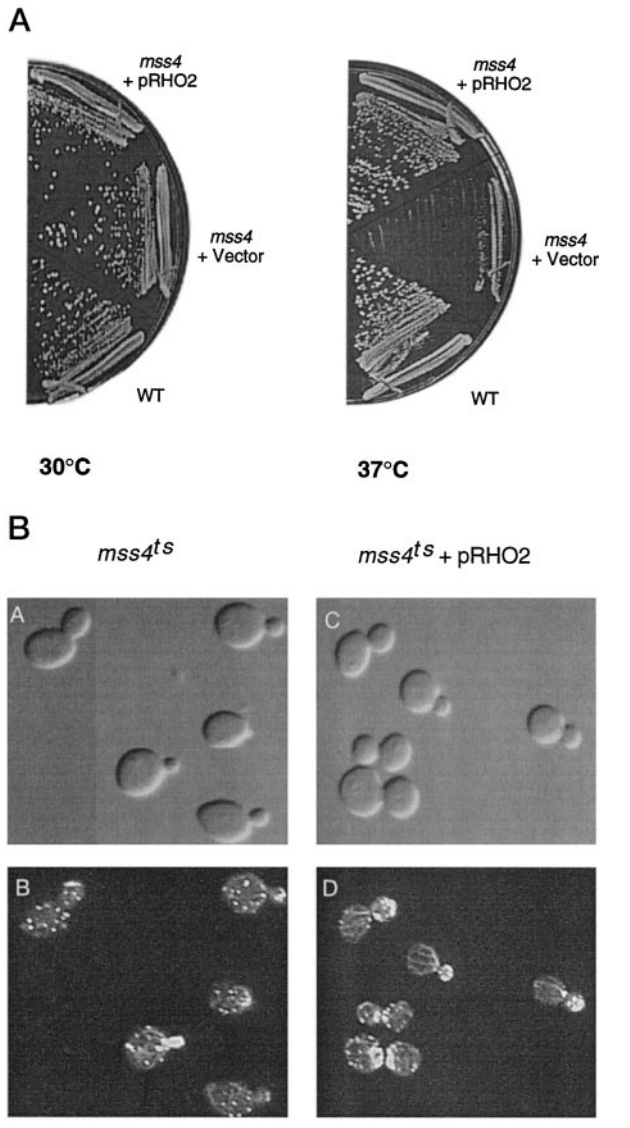

FIG. 2. **Overexpression of RHO2 suppresses an *mss4-2^{ts}* mutation.** *A*, overexpression of *RHO2* suppresses the growth defect of an *mss4-2^{ts}* mutant. Wild-type cells (SD100) and *mss4-2^{ts}* (SD102) cells carrying either an empty vector or pRHO2 were streaked on solid rich medium (YPD) and incubated for 2 days at 30 or 37 °C. *B*, overexpression of *RHO2* suppresses the actin organization defect of *mss4-2^{ts}* cells. *mss4-2^{ts}* (SD102) cells carrying an empty vector (*A* and *B*) or pRHO2 (*C* and *D*) were grown in selective media (SD-URA) at 24 °C to logarithmic phase and then shifted to 37 °C for 4 h. The actin content was visualized as described in Fig. 1.

two essential functions (29–32). One function is to control translation initiation and G1 progression in response to nutrients (33, 34); the second function is to control the organization of the actin cytoskeleton via a Rho-type GTPase switch (35, 36). Overexpression of *MSS4* restores both growth (21) and actin organization (data not shown) of a *tor2^{ts}* mutant defective in the organization of the actin cytoskeleton.

The above results suggest that MSS4, like TOR2, is also involved in organization of the actin cytoskeleton. To test this, we examined if the lack of MSS4 also caused a defect in the polarized distribution of the actin cytoskeleton. Wild-type (SD100) and *mss4-2^{ts}* (SD102) cells were grown at 24 °C and then shifted to 37 °C. At time points, aliquots were removed, and the cells were fixed and stained with TRITC-phalloidin to visualize F-actin (Fig. 1). When grown at 24 °C, both wild-type cells and *mss4-2^{ts}* cells displayed the normal cell cycle-dependent distribution of actin (37, 38), with cortical actin patches

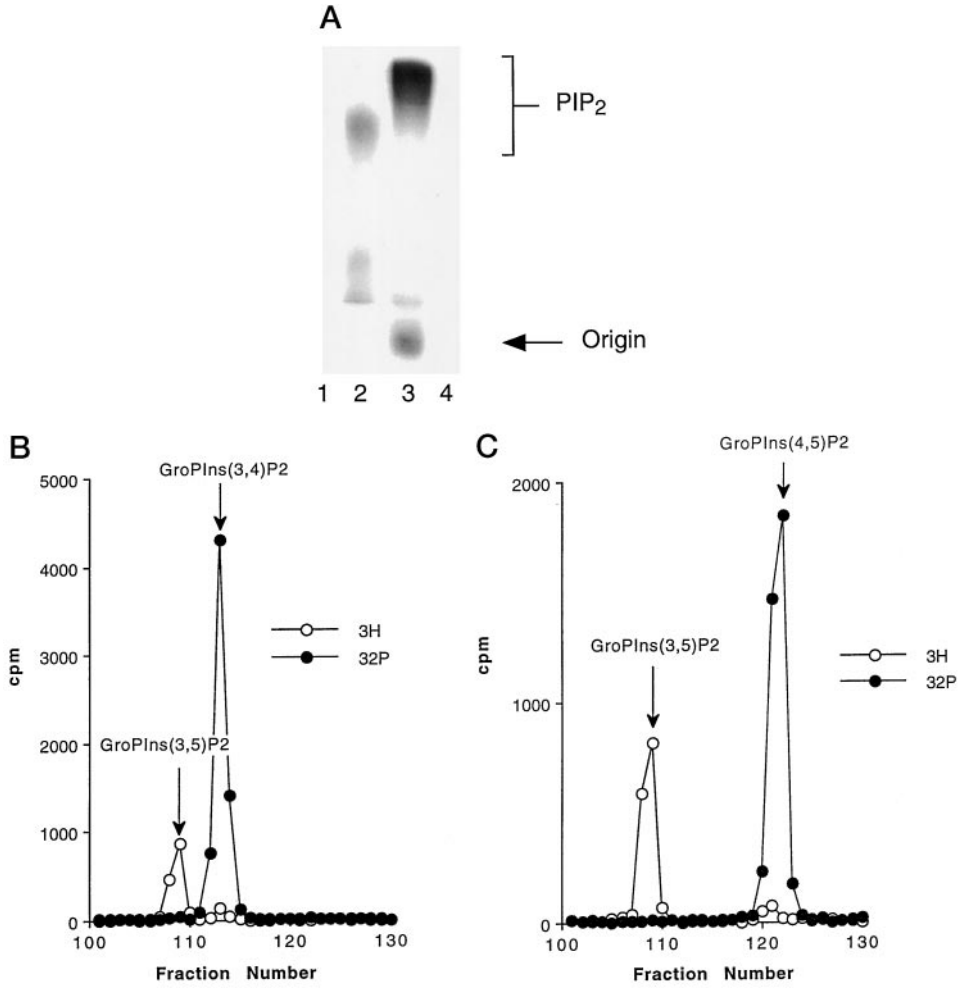

FIG. 3. **Phosphorylation of PI(3)P and PI(4)P by purified MSS4.** *A*, autoradiograph of *in vitro* kinase reaction products separated by TLC. The activity of purified GST-MSS4 was assayed using 100 μM of PI (*lane 1*), PI(3)P (*lane 2*), PI(4)P (*lane 3*), or PI(5)P (*lane 4*) as a substrate. The PIP_2 spots in *lanes 2* and *3* contained 12,411 cpm and 65,338 cpm, respectively. *B* and *C*, HPLC chromatogram of the deacylated products of the kinase reactions (*closed circles*), with deacylated [^{3}H]PI(3,5)P_2 (GroPIns(3,5)P_2) as internal standard. *B*, the deacylated PIP_2 product of MSS4, using PI(3)P as substrate is identified as GroPIns(3,4)P_2. *C*, the deacylated PIP_2 product of MSS4, using PI(4)P as substrate, is identified as GroPIns(4,5)P_2.

concentrated in the bud or septum, and actin cables aligned toward the concentrated patches. A shift from 24 to 37 °C caused, in both strains, a heat-induced disorganization of the actin cytoskeleton. Cables were no longer visible, and actin patches were more randomly distributed after 1 h at 37 °C. However, whereas the heat-induced disorganization of the actin cytoskeleton was transient in wild-type cells, lasting only 1–2 h, this perturbation was irreversible in the *mss4-2^{ts}* cells and became even more severe after 3 or 4 h at 37 °C. Thus, like TOR2, MSS4 is required for organization of the actin cytoskeleton.

Overexpression of RHO2 Suppresses an mss4 Mutation—The actin cytoskeleton is regulated by the Rho family of small GTPases (for review, see Ref. 39). Since overexpression of *RHO2*, encoding a member of this family, restored both organization of the actin and growth in a *tor2^{ts}*, we asked if overexpression of *RHO2* could also suppress an *mss4-2^{ts}* mutation. The *mss4-2^{ts}* mutant (SD102) was transformed with the high copy number plasmid pRHO2, and transformants were monitored for growth at 37 °C. As shown in Fig. 2*A*, overexpression of *RHO2* restored growth of the *mss4-2^{ts}* strain at restrictive temperature. We also examined whether overexpression of *RHO2* could suppress the actin organization defect of the *mss4-2^{ts}* strain. SD102 transformed with pRHO2 or with an empty vector was grown at 24 °C, shifted to 37 °C for 4 h, fixed, and stained with TRITC-phalloidin. Contrary to cells carrying an empty vector, which displayed the actin defect, cells transformed with pRHO2 regained the ability to polarize actin patches and to form actin cables (Fig. 2*B*). The findings that overexpression of *RHO2*, encoding an actin-related function, suppressed both the growth defect and the actin defect of an *mss4-2^{ts}* mutant suggest that the essential function of MSS4 is to control the actin cytoskeleton.

MSS4 Is a PIP Kinase with Dual Substrate Specificity in Vitro—MSS4 shares high sequence homology with the PIP kinases and has therefore been classified as a PI(4)P 5-kinase (15). However, MSS4 has yet to be demonstrated to have enzymatic activity. To determine whether MSS4 is indeed a PI(4)P 5-kinase, we expressed and purified MSS4 as a GST-MSS4 fusion protein. The GST-MSS4 hybrid was functional as determined by the ability of the *GST-MSS4* gene fusion to complement an *MSS4* disruption (data not shown). GST-MSS4 was assayed for PI kinase activity using PI, PI(3)P, PI(4)P, or PI(5)P as substrates. Reaction products were resolved by thin layer chromatography and visualized by autoradiography. As shown in Fig. 3*A*, purified GST-MSS4 had strong kinase activity on PI(4)P and weak activity on PI(3)P; no activity was detected toward PI or PI(5)P. After deacylation and HPLC analysis, the product of the PI(4)P kinase reaction was identified as PI(4,5)P_2, and the product of the PI(3)P reaction was identified as PI(3, 4)P_2 (Fig. 3, *B* and *C*). Thus, as observed

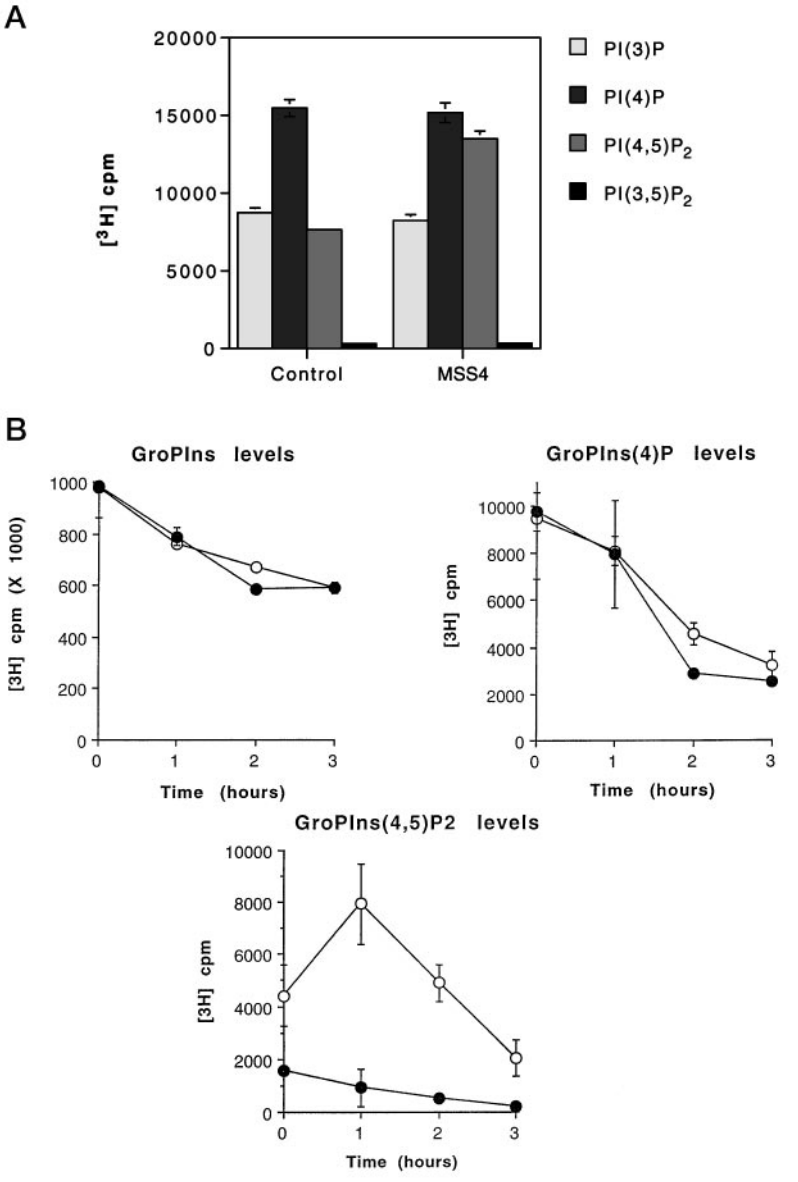

FIG. 4. **MSS4 controls the cellular level of PI(4,5)P_2.** *A*, effects of *MSS4* overexpression on cellular phosphoinositides. Wild-type cells (SD100; control) or cells overexpressing *MSS4* (SD2–5A/pSH22; MSS4) were labeled at 30 °C with [^{3}H]inositol, and [^{3}H]inositol-containing lipids from the cell extracts were deacylated, separated by HPLC, and quantified. *B*, effect of loss of function of MSS4 on cellular phosphoinositides. Wild-type cells (SD100; *open circles*) and cells carrying a temperature-sensitive allele of *mss4* (SD102; *closed circles*) were labeled at 24 °C with [^{3}H]inositol and then shifted to 37 °C. Aliquots were collected at the indicated times; the [^{3}H]inositol-labeled lipids from the cell extracts were deacylated and separated by HPLC, and the radioactivity associated with each lipid was quantified.

previously for at least the mammalian type I PIP kinase, MSS4 phosphorylates PI(4)P on the D-5 position and PI(3)P on the D-4 position *in vitro*. These results indicate that MSS4 is a dual substrate-specific PIP kinase *in vitro*, generating PI(4,5)P_2 and PI(3, 4)P_2 from PI(4)P and PI(3)P, respectively.

MSS4 Is the Major PI(4)P 5-Kinase in S. cerevisiae—To determine the substrate specificity of MSS4 *in vivo*, we first tested the effect of MSS4 overexpression on cellular phosphoinositide levels. A wild-type strain (SD100) and a strain containing *MSS4* on a multicopy plasmid (SD2–5a/pSH22) were labeled with [^{3}H]inositol, and the labeled lipids were deacylated and analyzed by HPLC. Overexpression of MSS4 induced about a 1.7-fold increase in the level of PI(4,5)P_2 compared with a wild-type strain (Fig. 4*A*). Overexpression of MSS4 had no influence on the cellular levels of PI(3)P, PI(4)P, or PI(3, 5)P_2. No PI(3, 4)P_2 was detected in lipid extracts from the control strain, but a small amount of PI(3,4)P_2 (93 ± 6 cpm *versus* 13,487 ± 497 cpm for PI(4,5)P_2) was detected in the strain overexpressing MSS4. Thus, it seems that although MSS4 has the potential to generate PI(3,4)P_2, production of this lipid *in vivo* is minor. To confirm that the increase in cellular PI(4,5)P_2 was due to MSS4, we determined whether the strain containing the temperature-sensitive *mss4-2^{ts}* allele had lowered levels of PI(4,5)P_2. Wild-type (SD100) and *mss4-2^{ts}* (SD102) cells were labeled with [^{3}H]inositol at 24 °C, washed, and shifted to 37 °C. Aliquots were removed at various times, and the tritiated lipid content was analyzed by HPLC. As shown in Fig. 3*B*, the temperature-sensitive strain SD102 contained levels of PI and PI(4)P comparable with those of the wild-type strain, at both permissive and nonpermissive temperatures. However, the level of PI(4,5)P_2 in the mutant strain was moderately reduced already at permissive temperature and severely reduced at nonpermissive temperature. At permissive temperature, SD102 contained about 35% of the amount of PI(4,5)P_2 found in wild-type cells. A shift to the higher temperature induced a transient and mild increase in the level of PI(4,5)P_2 in wild-type cells, whereas in the mutant the level of PI(4,5)P_2 dropped, reaching (within 1 h) approximately 10% of the level found in the wild type. Thus, MSS4 is a PI(4)P 5-kinase both *in vitro* and *in vivo*. Furthermore, MSS4 is the major PI(4)P 5-kinase in *S. cerevisiae* under normal growth conditions.

DISCUSSION

We demonstrate that, under normal growth conditions, MSS4 is the major PI(4)P 5-kinase in *S. cerevisiae*. Furthermore, like mammalian PIP kinases, MSS4 has dual substrate

specificity *in vitro* (17, 18). Comparison of the substrate specificities of MSS4 and mammalian PIP kinases indicates that MSS4 is more closely related to type I than to type II PIP kinases, phosphorylating primarily PI(4)P to generate $PI(4,5)P_2$ and to a lesser extent PI(3)P, yielding $PI(3,4)P_2$. Mammalian type I PIP kinases can, in addition, further phosphorylate $PI(3,4)P_2$ to produce $PI(3,4,5)P_3$ (17). Unlike the studies with mammalian PIP kinases, which demonstrated only the *in vitro* activities of these enzymes, we examined the activity of MSS4 *in vitro* and *in vivo*. We found that MSS4 produces $PI(4,5)P_2$ both *in vitro* and *in vivo*. However, we did not detect $PI(3,4,5)P_3$ in yeast lipid extracts under any of our experimental conditions, and $PI(3,4)P_2$ was detected only weakly and only when MSS4 was overexpressed. This may indicate that, although MSS4 can generate $PI(3,4)P_2$ *in vitro*, the production of this lipid *in vivo* may not be physiologically relevant. Alternatively, synthesis of $PI(3,4)P_2$ might be tightly regulated and might require a very specific stimulus. For example, the synthesis of the recently discovered lipid $PI(3,5)P_2$ (26, 40) is induced by hyperosmotic shock (26). The physiological significance of $PI(3,4)P_2$ remains to be determined.

Several lines of evidence indicate that the essential function of MSS4 is involved in organization of the actin cytoskeleton. First, overexpression of *MSS4* suppresses both the growth defect and the actin defect of a $tor2^{ts}$ mutant that is inviable because of an actin defect (Ref. 21 and data not shown). Second, loss of MSS4 causes a growth arrest that is coincident with a disruption of the actin cytoskeleton. Third, overexpression of *RHO2*, encoding a Rho-type GTPase involved in organization of the actin cytoskeleton (36), suppresses both the growth defect and the actin defect of an $mss4\text{-}2^{ts}$ mutant. Finally, overexpression of a type I PIP kinase in mammalian cells induces massive actin polymerization (41, 42). We could not detect any effect of overexpression of *MSS4* from a high copy plasmid on the actin cytoskeleton (data not shown), but under these conditions, the cellular level of $PI(4,5)P_2$ was less than doubled (Fig. 3*A*). Furthermore, a 65% reduction in the cellular level of $PI(4,5)P_2$, as observed in our $mss4\text{-}2^{ts}$ mutant at permissive temperature, had no visible effect on the organization of the actin cytoskeleton. These results may suggest that the intracellular levels of $PI(4,5)P_2$ can fluctuate significantly without a major effect on the actin cytoskeleton. More severe changes in $PI(4,5)P_2$ levels, like the 90% decrease in the $mss4\text{-}2^{ts}$ mutant at restrictive temperature, might be necessary to affect organization of actin.

How is MSS4 required for organization of the actin cytoskeleton? $PIP(4,5)P_2$ binds and regulates the function of several actin-binding proteins, including profilin (3), gelsolin (4), gCap39 (5), N-WASP (10), ERM proteins (9), α-actinin, and vinculin (7, 8). Thus, a simple model for the role of MSS4 in organization of the actin cytoskeleton is that, by generating $PIP(4,5)P_2$, it directly controls the interactions between actin and the actin-binding proteins. Another possibility is that MSS4 signals to the actin cytoskeleton indirectly, through the Rho-type GTPases (43). Mammalian type I PIP kinases are believed to signal to the actin cytoskeleton as downstream effectors of Rho and Rac GTPases (44–46). Our finding that *RHO2* is a multicopy suppressor of the $mss4\text{-}2^{ts}$ mutation suggests that MSS4 signals to the actin cytoskeleton as part of a signaling pathway involving Rho-type GTPases. This model is strengthened by the fact that *MSS4* is a multicopy suppressor of a *tor2* mutation, and TOR2 signals to the actin cytoskeleton via RHO1 and RHO2 (21, 36). Furthermore, MSS4 may be in a pathway with PKC1 (see below), which is a direct downstream effector of RHO1 (47, 48).

The *S. cerevisiae* protein kinase C (PKC) homologue PKC1 plays a major role in ensuring cell integrity (49, 50) and is activated by heat or hypotonic shock (51, 52). Several lines of evidence suggest that MSS4 and PKC1 are involved in a common signaling pathway. First, the *MSS4* gene was originally identified as a multicopy suppressor of a temperature-sensitive mutation in the PI 4-kinase gene *STT4*, which had previously been implicated in PKC1 signaling (20). Second, both *MSS4* and *PKC1* were isolated as multicopy suppressors of a *tor2* mutation (21). One function of TOR2 is to control organization of the actin cytoskeleton by activating RHO1 (36), and both *MSS4* and *PKC1* can suppress the loss of this function. Third, like a *tor2* mutant, an *mss4* mutant fails to properly localize actin during polarized cell growth. *pkc1* mutants have not been shown to have defects in actin organization, but cells altered in SLT2/MPK1, a mitogen-activated protein kinase activated by PKC1, exhibit delocalized actin (53). Fourth, we observed that high temperature triggers synthesis of $PI(4,5)P_2$, suggesting that MSS4, like PKC1, is activated by heat shock. How MSS4 may be related to PKC1, RHO1, RHO2, and TOR2 in a pathway(s) remains to be determined.

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MSS4, a Phosphatidylinositol-4-phosphate 5-Kinase Required for Organization of the Actin Cytoskeleton in *Saccharomyces cerevisiae*

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