Phosphatidylinositol-4-phosphate 5-Kinase Localized on the Plasma Membrane Is Essential for Yeast Cell Morphogenesis*

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Phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P₂), an important element in eukaryotic signal transduction, is synthesized either by phosphatidylinositol 4-phosphate 5-kinase (PtdIns(4)P 5K) or by phosphatidylinositol 5-phosphate 4-kinase (PtdIns(5)P 4K) from phosphatidylinositol 5-phosphate (PtdIns(5)P). Two Saccharomyces cerevisiae genes, MSS4 and FAB1, are homologous to mammalian PtdIns(4)P 5Ks and PtdIns(5)P 4Ks. We show here that MSS4 is a functional homolog of mammalian PtdIns(4)P 5K but not of PtdIns(5)P 4K in vivo. We constructed a hemagglutinin epitope-tagged form of Mss4p and found that Mss4p has PtdIns(4)P 5K activity. Immunofluorescent and fractionation studies of the epitope-tagged Mss4p suggest that Mss4p is localized on the plasma membrane, whereas Fab1p is reportedly localized on the vacuolar membrane. A temperature-sensitive mss4-1 mutant was isolated, and its phenotypes at restrictive temperatures were found to include increased cell size, round shape, random distribution of actin patches, and delocalized staining of cell wall chitin. Thus, biochemical and genetic analyses on Mss4p indicated that yeast PtdIns(4)P 5K localized on the plasma membrane is required for actin organization.

Phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P₂) has been recognized as an important element in eukaryotic signal transduction. Hydrolysis of PtdIns(4,5)P₂ by phospholipase C produces two second messengers, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol. IP₃ mobilizes Ca²⁺ stores, such as the endoplasmic reticulum in animal cells (1) and vacuoles in plants (2) and yeast (3). It is well known that the elevated intracellular Ca²⁺ stimulates a variety of calcium-modulating signaling enzymes, including calmodulin-dependent protein kinases and calcineurin, a type II B phosphoprotein phosphatase (4). Diacylglycerol, on the other hand, activates the conventional isoforms of protein kinase C, which in turn play a critical role in the regulation of a number of cellular functions in mammalian cells (5). In the budding yeast Saccharomyces cerevisiae, a protein kinase C-homologous gene (PKC1) was isolated (6), whose product was shown to function in cell wall integrity and cell cycle progression (7, 8). In vitro studies of Pkc1p, however, indicated that Pkc1p is strongly activated by phosphatidylinerse in the presence of Rho1p, but not by diacylglycerol (9). The stimulation by phosphatidylinerse alone is characteristic of the atypical ζ isoform of protein kinase C, which is stimulated by phosphatidylinerse alone. Since the biochemical property of Pkc1p is different from that of the conventional isoforms of mammalian protein kinase C, it remains unclear whether and how diacylglycerol acts as an important second messenger in S. cerevisiae.

PtdIns(4,5)P₂ is also known to function as a regulator of actin-binding proteins (10) such as profilin (11), gelsolin (12), and α-actinin of vertebrates (13). Recently, profilin was reported to be localized both in the plasma membrane and cytoplasmic fractions in S. cerevisiae, with the membrane association presumably facilitated by its interaction with phosphatidylinerse metabolites (14). Therefore, it is likely that through its regulation of actin-binding proteins, phosphatidylinositol metabolites affect the cytoskeleton in yeast.

Moreover, PtdIns(4,5)P₂ stimulates GDP to GTP exchange of ADP-ribosylation factor 1 (ARF1) (15). As the GTP-bound form of ARF1 triggers the attachment of the coat proteins (16–18), PtdIns(4,5)P₂ may play a critical role in coat assembly. Interestingly, PtdIns(4,5)P₂ was found to work as a cofactor for brain membrane phospholipase D (PLD) (19). These findings led to the proposal that PLD and phosphatidylinerse 4-phosphate 5-kinase (PtdIns(4)P 5-kinase) with their respective products, PtdIns(4,5)P₂ and phosphatidic acid, form a positive feedback loop that causes a vesicle fusion with the acceptor membrane (19). Since PtdIns(4,5)P₂ as well as phosphatidic acid activates an ARF GTPase-activating protein (20), they further postulated that the positive feedback loop is halted by the conversion of active ARF-GTP to ARF-GDP. Thus, PtdIns(4,5)P₂ may work as a crucial factor in membrane trafficking.

Ins(4,5)P₂ is synthesized either from PtdIns(4)P by the phosphorylation on the fifth hydroxyl group of the myo-inositol ring or from PtdIns(5)P by the phosphorylation on the fourth hydroxyl group (21). Phosphatidylinerse 4-phosphate 5-kinase (PtdIns(4)P 5K) and phosphatidylinerse 5-phosphate 4-kinase (PtdIns(5)P 4K), both of which catalyze PtdIns(4,5)P₂ synthesis, are functionally different (22) but structurally sim-
ilar to each other (23–26). Although mammalian PtdIns(5)P 4K was previously known as type II PtdIns(4)P 5K (23–26), it was reidentified as PtdIns(4)P 5K by careful examination (21). The sequences of mammalian PtdIns(5)P 5K and PtdIns(5)P 4K isoforms have homology to those of two yeast gene products, Fab1p and Mss4p (23–26). Though the Fab1p gene is not essential, the product, localized on the vacuolar membrane, is required for the vacuolar function and morphology (27). Mss4p was originally identified as a multicopy suppressor of the temperature-sensitive mutation in the STT4 gene (28), which encodes an PtdIns-4-kinase, suggesting involvement of Mss4p in PtdIns(4)P metabolism (29). Since a deletion of the Mss4p gene is lethal, characterization of conditional-lethal mutants of mss4 is useful for understanding the function of Mss4p.

We report here that Mss4p has PtdIns(4)P 5K activity in vitro and that expression of murine type I PtdIns(4)P 5K, however, remains to be elucidated. The se-

**Materials and Methods**

**Yeast Strains and Genetic Manipulations**—The yeast strains used are listed in Table I. The complete and minimal yeast media as well as the sporulation medium and procedures of tetrad analyses were as described (30). YPGS medium contains 2% galactose, 0.1% sucrose, 1% sodium acetate, and 2% potassium oxalate, with the exception of lanes 4 and 5 of Fig. 1C, in which similarly treated Whatman 60A plate was utilized. The samples were separated with the solvent system of chloroform/methanol/acetone/acetic acid/water (42:30:12:12:12 by volume), and [3H]Ins(1,4,5)P_3 was visualized by autoradiography except for the product on the Whatman plate, which was processed by BAS2000 Fuji biol Imaging analyzer.

**Isolation of Temperature-sensitive mss4 Mutants**—We first made an mss4 strain carrying the murine gene on a centromer plasmid: the 3.1-kb BamHI-Xhol fragment of pYO1958 carrying the mss4::HIS3 gene was used to transform the diploid strain, YPH501. His^+ transformants were selected, and the disruption of one of the chromosomal MSS4 gene copies was confirmed by Southern hybridization. The MSS4/mss4::HIS3 diploid strain, named YOC801, was transformed with pYO1962 carrying Mss4p and URA3, and the transformants were subjected to tetrad dissection. His^+ ascis were selected and designated YOC802 (mss4::HIS3; pYO1962).

Random mutations were introduced by error-prone polymerase chain reaction mutagenesis (34) in the PtdIns(4)P 5-kinase-conserved region of MSS4 using the two synthetic oligonucleotides, CCTTCTCAGGTTTCGCCAG and CAGATCTCAGGTTTA, as templates. For the PCR reaction, pYO1958, which was designed to aid MSS4 gene disruption, mss4::HIS3, was used as the template for PCR. The PCR reaction was terminated by the addition of 0.4 ml of chloroform/methanol/acetone/acetic acid/water (42:30:12:12:12 by volume), and the samples were separated with the solvent system of chloroform/methanol/acetone/acetic acid/water (42:30:12:12:12 by volume), and [3H]Ins(1,4,5)P_3 was visualized by autoradiography except for the product on the Whatman plate, which was processed by BAS2000 Fuji biol Imaging analyzer.

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Table II
Plasmids used in this study

| Plasmid       | Parent plasmid | Markers       | Ref.           |
|---------------|----------------|---------------|---------------|
| pJY125        | pUC18          | HIS3          |               |
| pRS314        | CEN6, ARSH4, TRP1, f1 origin | Y. Ohya (unpublished) |
| pRS315        | CEN6, ARSH4, LEU2, f1 origin | H. Qadota and Y. Ohya (unpublished) |
| pRS316        | CEN6, ARSH4, URA3, f1 origin | H. Qadota and Y. Ohya (unpublished) |
| pYO324        | TRP1, 2 μ origin | adês::LEU2    | H. Qadota and Y. Ohya (unpublished) |
| pYO761        | TRP1, CEN, pGAL1 |               |               |
| pYO767        | TRP1, 2 μ origin, pGAL1 |               |               |
| pYO885        | pBluescript KS− | adeês::LEU2   | H. Qadota and Y. Ohya (unpublished) |
| pYO1365       | pRS314         | CLS2:3HA      |               |
| pYO1953       | YEp13          | 5.2-kb genomic DNA fragment containing MSS4 | This study |
| pYO1956       | pBluescript SK− | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1958       | pBluescript SK− | mss4::HIS3 | This study |
| pYO1959       | pRS315         | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1960       | pRS314         | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1962       | pRS316         | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1964       | pRS314         | 1.2-kb NdeI-KpnI fragment of YO1960 was replaced with a linker | This study |
| pYO1965       | pRS314         | 3HA:MSS4 | This study |
| pYO1966       | pRS314         | mss4−1        | This study |
| pYO1970       | pRS314         | adeês::MSS4::LEU2 | This study |
| pYO1974       | pRS314         | adeês::MSS4::LEU2 | This study |
| pYO1975       | pRS314         | adeês::MSS4::LEU2 | This study |
| pYO2116       | pGAL1: murine PtdIns(4)P 5K Iβ gene, CEN | This study |
| pYO2117       | pGAL1: human PtdIns(5)P 4K gene, CEN | This study |
| pYO2118       | pGAL1: murine PtdIns(4)P 5K Iβ gene, 2-μ origin | This study |
| pYO2119       | pGAL1: human PtdIns(5)P 4K gene, 2-μ origin | This study |
| pYO2121       | pGAP, CEN | This study |
| pYO2122       | pGAP: murine PtdIns(4)P 5K Iβ gene, CEN | This study |
| pYO2123       | pGAP: human PtdIns(5)P 4K gene, CEN | This study |
| pYO2144       | pGAP, 2-μ origin | This study |
| pYO2145       | pGAP: murine PtdIns(4)P 5K Iβ gene, 2-μ origin | This study |
| pYO2146       | pGAP: human PtdIns(5)P 4K gene, 2-μ origin | This study |
| YEp13         | REF3, LEU2, 2-μ origin | This study |

TABLE II

| Plasmid       | Parent plasmid | Markers       | Ref.           |
|---------------|----------------|---------------|---------------|
| pYO1365       | pRS314         | CLS2:3HA      |               |
| pYO1953       | YEp13          | 5.2-kb genomic DNA fragment containing MSS4 | This study |
| pYO1956       | pBluescript SK− | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1958       | pBluescript SK− | mss4::HIS3 | This study |
| pYO1959       | pRS315         | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1960       | pRS314         | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1962       | pRS316         | 3.9-kb BamHI-Xhol fragment of pYO1953 | This study |
| pYO1964       | pRS314         | 1.2-kb NdeI-KpnI fragment of YO1960 was replaced with a linker | This study |
| pYO1965       | pRS314         | 3HA:MSS4 | This study |
| pYO1966       | pRS314         | mss4−1        | This study |
| pYO1970       | pRS314         | adeês::MSS4::LEU2 | This study |
| pYO1974       | pRS314         | adeês::MSS4::LEU2 | This study |
| pYO1975       | pRS314         | adeês::MSS4::LEU2 | This study |
| pYO2116       | pGAL1: murine PtdIns(4)P 5K Iβ gene, CEN | This study |
| pYO2117       | pGAL1: human PtdIns(5)P 4K gene, CEN | This study |
| pYO2118       | pGAL1: murine PtdIns(4)P 5K Iβ gene, 2-μ origin | This study |
| pYO2119       | pGAL1: human PtdIns(5)P 4K gene, 2-μ origin | This study |
| pYO2121       | pGAP, CEN | This study |
| pYO2122       | pGAP: murine PtdIns(4)P 5K Iβ gene, CEN | This study |
| pYO2123       | pGAP: human PtdIns(5)P 4K gene, CEN | This study |
| pYO2144       | pGAP, 2-μ origin | This study |
| pYO2145       | pGAP: murine PtdIns(4)P 5K Iβ gene, 2-μ origin | This study |
| pYO2146       | pGAP: human PtdIns(5)P 4K gene, 2-μ origin | This study |
| YEp13         | REF3, LEU2, 2-μ origin | This study |

RESULTS

Mss4p Is a Functional Homolog of Mammalian PtdIns(4)P 5-Kinase—A BLAST search of protein sequence data bases revealed that yeast Mss4p has 36, 33, and 31% identity with murine type Iα, type Iβ PtdIns(4)P 5K, and human PtdIns(5)P 4K, respectively, in agreement with previous reports. To examine whether Mss4p is a functional homolog of any of the mammalian phosphatidylinositol phosphokinases (PIPKs) in yeast, we constructed plasmids carrying the genes encoding murine type Iβ PtdIns(4)P 5K and human PtdIns(5)P 4K hooked up to either the constitutive GAP promoter or the galactose-inducible GAL1 promoter. After these expression plasmids were introduced to YC802 strain carrying mss4::HIS3 and a URA3-MSS4 plasmid, the growth on FOA plates was examined. We found that all the transformants expressing the type Iβ PtdIns(4)P 5K gene were capable of growing on FOA plates (Fig. 1A, panels a and b). On the other hand, expression of the PtdIns(5)P 4K gene failed to complement the MSS4 gene disruption, irrespective of copy number or the promoters used (Fig. 1A, panels b and c).

We next made a temperature-sensitive MSS4 mutant and tested the suppression of the temperature sensitivity by mammalian PIPKs. Mutations were introduced into the conserved region for PIPK within the MSS4 gene, and one of the mutants that grew at 23 °C but not at 37.5 °C (mss4−1) was studied (see “Materials and Methods”). The temperature-sensitive mss4−1 strain was transformed with plasmids containing the two mammalian PIPK genes under the control of the two different promoters to test if the temperature sensitivity is suppressed. When murine type Iβ PtdIns(4)P 5K was expressed under the GAL1 promoter on either the single copy or multicopy plasmid, the strain grew on a galactose-containing plate at the restrictive temperature (Fig. 1A, panels e and f). On a glucose-containing plate on which the expression of type Iβ PtdIns(4)P 5K under the GAL1 promoter is reduced, however, suppression of mss4−1 was observed only with the multicopy plasmid, indicating the failure of suppression when expression is greatly reduced. When the same gene was placed on plasmids under the GAP promoter, the plasmid-harboring strains grew at the re-
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The plasmids (2–7) and sucrose (2%). The plates were incubated at either 30 °C (and FOA plates containing 2% glucose (and 2% galactose and 0.1% PA (37)). These results demonstrate that the tagged MSS4 gene product possesses PtdIns(4)P 5K activity and that the amount of the kinase activity is copy number-dependent.

**The Tagged MSS4 Gene Product Has PtdIns(4)P 5-Kinase Activity**—To examine PtdIns(4)P 5K activity of the MSS4 gene product, we immunoprecipitated the 3HA-tagged MSS4 protein expressed in yeast with the anti-HA monoclonal antibody and determined the kinase activity in the immunoprecipitate (Fig. 1B). The immunoprecipitate from the YOC804 cells carrying the tagged MSS4 gene on a multicopy plasmid had the highest PtdIns(4)P 5K activity, followed by that from the YOC803 cells, which harbored the same gene on a single copy plasmid, whereas that from the cells with untagged Mss4p (YOC806) exhibited little activity. Furthermore, the PtdIns(4)P 5K activity in the immunoprecipitates was found to be stimulated by the addition of 50 μM phosphatidic acid (Fig. 1B), a characteristic property of PtdIns(4)P 5K but not of PtdIns(5)P 5K (37). These results demonstrate that the tagged Mss4p possesses PtdIns(4)P 5K activity and that the amount of the kinase activity is copy number-dependent.

**The mss4-1 Protein Has Less PtdIns(4)P 5K Activity When Cultured at the Restrictive Temperature**—We examined whether the PtdIns(4)P 5K activity of the mss4-1 mutant changes at the restrictive temperature. We first made a strain with the MSS4 gene disrupted but harboring a 3HA-tagged mss4-1 gene on a multicopy plasmid and designated it YOC823. The strain was cultured at 23 °C, transferred to 38 °C at early exponential growth phase, and was further cultivated for 0, 2, 4, 6, or 8 h before being harvested. The lysates were made, immunoprecipitated with the anti-HA antibody, and the PtdIns(4)P 5K activities in the immunoprecipitates were assayed. As can be seen in Fig. 1C, the kinase activity starts decreasing immediately upon the temperature shift, and the reduction is complete by 4 h at the restrictive temperature. Western blotting of cell lysates showed that the strain had less amount of the mutant protein when cultured at the restrictive temperature than at the permissive temperature (data not shown). These results indicate a temperature-sensitive defect in the synthesis and/or heat lability of the mutant protein.

**The MSS4 Gene Product Is Localized on the Plasma Membrane**—To examine intracellular localization of the tagged Mss4p, we first investigated the partitioning of 3HA-tagged Mss4p into subcellular fractions by centrifugation of cell lysates. The tagged MSS4 gene product was localized in the particulate fraction, and the membrane fraction was enriched in the 3HA-tagged Mss4p. These results suggest that the 3HA-tagged Mss4p has a membrane association that is necessary for its function.

**Fig. 1. Examination of PtdIns(4)P 5K activity of Mss4p in vitro and in vivo.** A, suppression of mss4 by mammalian PtdIns(4)P 5K. Plasmids containing mammalian PIPK were used to transform YOC802 (Δmss4::HIS3 [URA3-MSS4] (a–c) and YOC808 (mss4-1) (d–f). The transformants were plated onto a YPD plate (a, d, e), a YPGS plate (f), and FOA plates containing 2% glucose (b) or 2% galactose and 0.1% sucrose (c). The plates were incubated at either 30 °C (a, d–f) or 37 °C (b, c). The strains harbored either single copy (1–4, 8–10) or multicopy plasmids (5–7, 11–13). In the plasmids in 2–7 and 8–13, the GAP promoter and the GAL1 promoter were used, respectively. 1, pY01960 (MSS4); 2, pY02141 (vector); 3, pY02142 (PtdIns(4)P 5K); 4, pY02143 (PtdIns(5)P 4K); 5, pY02144 (vector); 6, pY02145 (PtdIns(4)P 5K); 7, pY02146 (PtdIns(5)P 4K); 8, pY0761 (vector); 9, pY02116 (PtdIns(4)P 5K); 10, pY02117 (PtdIns(5)P 4K); 11, pY0767 (vector); 12, pY02118 (PtdIns(4)P 5K); 13, pY02119 (PtdIns(5)P 4K). B, lanes 1–3, YOC804 (Δmss4::HIS3 [YEpT3HA::MSS4]), YOC803 (mss4::HIS3 [YEpT3HA::MSS4]), and YOC806 (Δmss4::HIS3 (YCpTMSS4)) lysates were prepared and immunoprecipitated. The PtdIns(4)P 5K activity in each of the immunoprecipitated samples was assayed at 30 °C with 5.0 μCi of \[^{32}P\]ATP. Lanes 4 and 5, the PtdIns(4)P 5K activity in YOC804 cells was similarly assayed in the presence or absence of 50 μM phosphatidic acid (PA) with 0.5 μCi of \[^{32}P\]ATP per sample. C, lanes 1–5, the YOC823 (Δmss4::HIS3 [YEpSH3::mss4-1]) strain was cultured at 23 °C and then place at 38 °C for indicated times, the lysates prepared and immunoprecipitated, and the PtdIns(4)P 5K assay was carried out at 23 °C with 5.0 μCi of \[^{32}P\]ATP in each sample.
Mss4p was mainly detected on the plasma membrane. A, YOC804 cells expressing 3HA:Mss4p on a multicopy plasmid (multicopy), YOC803 cells expressing 3HA:Mss4p on a single copy plasmid (single-copy), and YOC806 cells expressing untagged Mss4p (vector) were cultured to early exponential phase and used for cell fractionation experiments. 3HA:Mss4p was detected by Western blotting analysis with anti-HA monoclonal antibody (16B12). B, YOC804 cells expressing 3HA:Mss4p on a multicopy plasmid (A, D), YOC803 cells expressing 3HA:Mss4p on a single copy plasmid (B, E), and YOC806 cells expressing untagged Mss4p (C, F) were cultured, fixed, and stained with 16B12, an anti-HA monoclonal antibody. A-C, immunofluorescent pictures; D-F, phase-contrast images.

FIG. 2. Mss4p is mainly localized on the plasma membrane. A, YOC804 cells expressing 3HA:Mss4p on a multicopy plasmid (multicopy), YOC803 cells expressing 3HA:Mss4p on a single copy plasmid (single-copy), and YOC806 cells expressing untagged Mss4p (vector) were cultured to early exponential phase and used for cell fractionation experiments. 3HA:Mss4p was detected by Western blotting analysis with anti-HA monoclonal antibody (16B12). P, 436,000 × g pellet; S, 436,000 × g supernatant. B, YOC804 cells expressing 3HA:Mss4p on a multicopy plasmid (A, D), YOC803 cells expressing 3HA:Mss4p on a single copy plasmid (B, E), and YOC806 cells expressing untagged Mss4p (C, F) were cultured, fixed, and stained with 16B12, an anti-HA monoclonal antibody. A-C, immunofluorescent pictures; D-F, phase-contrast images.

Mss4p by cell fractionation experiments. Mss4p expressed either on the multicopy plasmid or on the single copy plasmid was mainly detected in the membrane fraction (Fig. 2A). Comparison with diluted samples as a standard showed that approximately 80% of the Mss4p was contained in the membrane fraction (data not shown). Next, immunofluorescence microscopy with the anti-HA monoclonal antibody revealed that the tagged Mss4p expressed on a single copy plasmid was almost exclusively localized on the cell surface (Fig. 2B). No polarized localization of the staining was observed during the cell cycle. The tagged Mss4p expressed on a multicopy plasmid gave stronger signals on the cell surface than on a single copy plasmid and occasionally gave a few additional internal punctuated signals (Fig. 2B, panel A), which were distinct from vacuoles. Cells expressing untagged Mss4p on a single copy plasmid did not give a detectable signal (Fig. 2B, panel C), indicating that the staining is not an artifact. Combined with the observation that the tagged Mss4p expressed on a single copy plasmid can fully complement the mss4-1 deletion, these results suggest that the MSS4 gene product is nearly exclusively localized on the plasma membrane.

Phenotypes of the Temperature-sensitive mss4-1 Mutant—Growth of the temperature-sensitive mss4-1 mutant (YOC808) was compared with those of the wild-type strain (YOC807) and the mss4-1::HIS3 strain expressing untagged Mss4p on a single copy plasmid (YOC806) cultured on YPD plates (Fig. 3) and in YPD liquid media (Fig. 4). Judging from the colony size, the mss4-1 strain grew as well as the wild-type strain at 23 °C, grew slowly at 37 °C, and completely failed to grow at 37.5 °C (Fig. 3). The growth defect of the mss4-1 mutant was not suppressed by addition of 100 mM CaCl₂. The doubling time of mss4-1 was 4.3 h at 23 °C, whereas that of the wild-type cells was 4.0 h, indicating that the mss4-1 mutant grows almost as fast as the wild type at the permissive temperature. At 38 °C, however, the growth of the mutant cells stopped within 4 h after the temperature shift (Fig. 4).

When observed under the light microscope, the mss4-1 mutant cells were found to stop growing at 38 °C with an enlarged size and round shape (Fig. 5). There was no indication of cell lysis under the microscope after a 4-h incubation at 38 °C. Most of the mutant cells contain single nuclei or divided two nuclei (Fig. 6, panel I). Even at the permissive temperature (23 °C), the mutant cells were round-shaped, whereas the wild-type cells were all oval-shaped (data not shown).

As similar morphological phenotypes were observed with mutations of actin-binding proteins, such as profilin and capping protein (38, 39), we then examined the actin distribution of the mss4-1 cells by staining with rhodamine-conjugated phalloidin (Fig. 6). In the wild-type cells, the actin staining showed cables running longitudinally and cortical patches, whereas in budding cells, it occurred exclusively on the bud as previously reported (40–42) (Fig. 6, panel F). A 1-h incubation at 38 °C of the wild-type cells made actin cables fainter and altered the localization of actin patches to the bud (Fig. 6, panel G), but further incubation restored polarized distribution of actin patches to the bud and, until 8 h at the restrictive temperature, kept the localization unchanged (Fig. 6, panel H). In contrast, phalloidin staining of mss4-1 mutant cells revealed a random distribution of cortical actin patches. In the mutant cells, actin patches became partially polarized after a 4-h incubation (Fig. 6, panel C), but the polarization was completely lost after 6 and 8 h of incubation at 38 °C (Fig. 6, panels D and E). Even at 23 °C, actin cables were faint, and actin patches were frequently distributed not only at buds but also in mother cells in the temperature-sensitive strain (Fig. 6, panel A). At 38 °C, the staining of actin was obviously brighter in the mutant cells than in the wild-type cells after the 8-h incubation at 38 °C (Fig. 6, panel E), whereas staining of cell wall chitin revealed deposition of chitin all over the cell surface in the mutant cells (Fig. 6, panel K). These results indicate that cell
polarity and actin distribution are significantly impaired in mss4-1 even at 23 °C, and the loss becomes greater at 38 °C.

We further examined a genetic interaction between mss4-1 and cls5-1, the latter of which possesses a missense mutation in the profilin gene. We found that a haploid strain with mss4-1 and cls5-1 mutations harboring a URA3-MSS4 plasmid could not grow on FOA plate at 30 °C (data not shown). This indicates that the mss4-1 cls5-1 double mutant fails to grow, although either an mss4-1 or a cls5-1 single mutant grows well at both 23 and 30 °C. The synthetic lethal interaction between MSS4 and the profilin gene is consistent with our observation that actin cytoskeleton is impaired in the mss4-1 mutant.

DISCUSSION

The first piece of evidence that MSS4 encodes PtdIns(4)P 5K comes from the kinase assay; immunoprecipitates from cells expressing the epitope-tagged form of Mss4p had PtdIns(4)P 5K activity, and the tagged Mss4p is functional as demonstrated by the full complementation of the mss4 deletion by single-copy expression of 3HA-Mss4p. The substrate used, PtdIns(4)P, was purified from bovine brain, is approximately 98% pure on TLC according to the manufacturer, and may contain a small amount of PtdIns(5)P. It is formally possible that MSS4 actually encodes PtdIns(5)P 4K, and the PtdIns(5)P 4K activity produces PtdIns(4,5)P2 from the trace amount of PtdIns(5)P contained in the substrate. However, the following lines of evidence make this alternative explanation unlikely. The kinase activity of the gene product is enhanced in the presence of phosphatidic acid, a characteristic of mammalian PtdIns(4)P 5K isoforms, but not of PtdIns(5)P 4K (37). In addition, the murine PtdIns(4)P 5K gene, but not the human PtdIns(5)P 4K gene, complemented the mss4 gene disruption and, when the expression level was reasonably high, suppressed the temperature sensitivity of the mss4-1 strain. This identification is consistent with the higher homology Mss4p has with mammalian PtdIns(4)P 5K isoforms than with PtdIns(4)P 4K (37).

Our finding that MSS4 encodes PtdIns(4)P 5K explains the previous genetic studies on MSS4 well. Since overexpression of MSS4 suppresses the cell lysis phenotype of Δstt4 at 23 °C and the temperature-sensitive stt4-1 mutation, MSS4 was suggested to function downstream of Stt4p, a PtdIns 4-kinase (29, 59). The present demonstration is in agreement with the idea that Stt4p phosphorylates PtdIns to produce PtdIns(4)P, which in turn is further phosphorylated by the action of Mss4p to yield PtdIns(4,5)P2. In the same paper, it was reported that overproduction of MSS4 did not affect PtdIns 4-kinase activity of wild-type yeast cells and that PtdIns 4-kinase activity of the Δstt4 cells carrying a multicopy MSS4 plasmid was as low as that in the Δstt4 cells. These results are consistent with the notion that Mss4p is a PtdIns(4)P 5K and has little if any PtdIns 4-kinase activity.

Another S. cerevisiae gene, FAB1, whose product has a significant homology to mammalian PIPKs, is not essential (27). Fab1p was suggested to have PtdIns(4)P 5K activity on the vacuolar membrane, and the product of the kinase reaction, PtdIns(4,5)P2, was proposed to function as a regulator of vacuole homeostasis (27). However, the possibility that FAB1 encodes PtdIns(5)P 4K cannot be excluded, especially because no kinase assay has been reported. On the other hand, MSS4 is an essential gene (29) whose product is mainly localized to the plasma membrane (Fig. 2). Thus, it seems that Mss4p functions as PtdIns(4)P 5K on the plasma membrane and the product of the reaction, PtdIns(4,5)P2, plays an essential function at or near the plasma membrane. The idea that the two PIPKs that produce PtdIns(4,5)P2 play different roles at different compart-

FIG. 4. mss4-1 strain stops growing at 38 °C. At zero time, YOC807 (MSS4) and YOC808 (mss4-1) cultures grown in YPD at 23 °C to early exponential phase were diluted with the same medium, and the diluted cultures were grown at either 23 or 38 °C. The cell densities at time zero and subsequent times were determined with a hemocytometer.

FIG. 5. mss4-1 strain shows aberrant cell morphology. YOC808 (mss4-1) cells were cultured at 23 °C, diluted, and further incubated for 8 h at 23 or 38 °C. The cells were observed under the phase-contrast microscope.
ments is supported by our recent observation that MSS4 on a multicopy plasmid suppresses the temperature sensitivity of cmd1–228, a calmodulin mutant with defect in calmodulin localization (43), whereas FAB1 does not (data not shown).

One explanation of the phenotypes of the temperature-sensitive mss4-1 mutant cells is that the phenotypes are caused by a reduced level of PtdIns(4,5)P2, whereas another interpretation is that they are brought about by defects in hitherto unidentified function(s) of Mss4p. We favor the former possibility because it is consistent with our finding that, when the temperature-sensitive mutant cells are shifted to the restrictive temperature, PtdIns(4)P 5K activity decreases before the mutant phenotypes become evident (Figs. 1C and 6).

Through what pathways does PtdIns(4,5)P2 give rise to the mutant phenotypes? The mss4-1 mutant phenotypes are strikingly similar to those of mutants of two actin-binding proteins, i.e. profilin null mutants (38) and the capping protein deletion mutants, Δcap1 and Δcap2 (39). Profilin is a ubiquitous actin- and PtdIns(4,5)P2-binding protein in eukaryotic cells (44) and is required for the proper organization of actin cytoskeleton into actin cables, which occur at regions of active growth and for proper maintenance of cell polarity (38). It was also shown that depletion of PtdIns(4,5)P2 in the plasma membrane leads to profilin translocation to the cytosol (14). The binding of capping protein to the growing end of actin filaments was demonstrated to be prevented by micromolar concentrations of PtdIns(4,5)P2 (39). We show here a synthetic lethal interaction between PtdIns(4)P 5K and profilin. Thus, it is plausible that a lower level of PtdIns(4,5)P2 in mss4-1 cells hinders proper functioning of profilin and capping protein, leading to disorganization of actin cables.

Ins(4,5)P2 is known to be hydrolyzed by phospholipase C, FIG. 6. Staining of actin, nuclear DNA, and cell wall chitin in the mss4-1 mutant cells. YOC808 (mss4-1) cells were cultured at 23 °C (A, J), diluted, and further incubated for 1 h (B), 4 h (C), 6 h (D), and 8 h (E, I, K) at 38 °C. Similarly, YOC807 (wild-type) cells were cultured at 23 °C (F), diluted, and additionally incubated for 1 h (G) and 8 h (H) at 38 °C. After being fixed in formaldehyde, the cells were stained with rhodamine-phalloidin (A–H), 4′,6-diamidino-2-phenylindole dihydrochloride (I), or calcium white M2R new (J, K) and observed under the fluorescent microscope.

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encoded by the PLC1 gene in S. cerevisiae, to produce IP₃ and diacylglycerol. Temperature-sensitive plc1 mutant cells were reported to be swollen with large buds and two nuclei at the restrictive temperature, and the growth defect of the mutant was suppressed by addition of 100 mM CaCl₂ (45). An increased incidence of aberrant chromosomal segregation was also observed with another temperature-sensitive mutant, plc1-1 (46). Since mss4-1 cells at the restrictive temperature do not show these phenotypes, we consider it improbable that the primary effect of Mss4p involves PtdIns(4,5)P₂ hydrolysis. Alternatively, PtdIns(4,5)P₂ may be required to stimulate GDP to GTP exchange of yeast ARF, which is important for secretion (47).

The localization and the mutant phenotypes, however, do not support the idea that the major pathway related to Mss4p involves ARF. PtdIns(4,5)P₂ is also known to work as a cofactor in regulation of actin-binding proteins through generation of PtdIns(4,5)P₂, which is required to stimulate GDP to GTP exchange of yeast ARF, which is important for secretion (47).

In summary, we propose that the MSS4 gene product functions in regulation of actin-binding proteins through generation of PtdIns(4,5)P₂ from PtdIns(4)P in or near the plasma membrane. We believe that novel factors involved in the PtdIns(4,5)P₂ signaling pathway can be identified by genetic approach with the conditional mutant of mss4. Further investigations of the MSS4 gene will greatly elucidate phosphatidylinositol signal transduction cascade.

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