Phosphatidylinositol-4-phosphate 5-Kinase Regulates Fission Yeast Cell Integrity through a Phospholipase C-mediated Protein Kinase C-independent Pathway*

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Fission yeast its3-1 mutant is an allele of the essential gene its3* that encodes a phosphatidylinositol-4-phosphate 5-kinase (PIP5K) that produces phosphatidylinositol 4,5-bisphosphate. We found that the its3-1 mutant is sensitive to micafungin, a (1,3)-β-D-glucan synthase inhibitor, suggesting a cell wall integrity defect. Consistently, its3-1 mutation caused synthetic lethality with a (1,3)-β-D-glucan synthase mutant, bgs1-12, and its3-1 mutant cells showed aberrant localization of green fluorescent protein-Bgs1. Similar aberrant localization of green fluorescent protein-Rgf1, a putative phosphatidylinositol 4,5-bisphosphate-binding guanine nucleotide exchange factor for Rho protein, in its3-1 mutants was observed, suggesting a defective Rgf1/Rho pathway. To unravel the molecular mechanism(s), putative downstream components of PIP5K signaling were analyzed. Unexpectedly, overexpression of phospholipase C (Plc1), but not that of protein kinase C (PKC; Pck1 and Pck2), suppressed the phenotypes of the its3-1 mutant. These findings indicate that PKCs are not involved in the suppression, and further analysis revealed that PKCs are not downstream of Plc1 in fission yeast. Also, the enzymatic activity of Plc1 is essential for the suppression of the phenotypes and for the viability of the its3-1 mutant. These findings suggest that Its3 PIP5K regulates cell integrity through a Plc1-mediated PKC-independent pathway, in addition to the Rho/PKC pathway.

Phosphorylated phosphoinositides play a crucial role in a variety of distinct cellular processes including cell signaling, cell growth, membrane trafficking, transcription, and actin cytoskeletal arrangement (1–5). The major phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP2) is a substrate for phospholipase C, yielding two essential second messengers, IP3 and diacylglycerol (6). In mammalian cells, IP3 binds to specific receptors and induces the release of calcium from intracellular stores, whereas diacylglycerol activates PKC (7). More recently, it has been shown that PIP2 is directly involved in the maintenance of actin cytoskeleton organization and in endocytosis and synaptic vesicle recycling (8, 9).

We have been studying the phosphoinositide signal transduction pathway in fission yeast Schizosaccharomyces pombe because this system is amenable to genetic analysis and has many advantages in terms of its relevance to higher systems. We have developed a genetic screen that utilizes the immunosuppressant drug FK506 and searched for its mutations that display immunosuppressant- and temperature-sensitive phenotypes. By this genetic screen in our previous study, we have isolated its3* gene that encodes a PIP5K (10). The its3-1 mutant had only low levels of PIP2, consistent with the defective PIP5K activity. In addition, overexpression of Its3 in wild-type cells resulted in a significant increase in the level of PIP2 (10). Similar to its budding yeast homolog Mss4, Its3 is encoded by the essential gene its3*, which, when mutated, causes disorganization of the actin cytoskeleton and aberrant cell morphology (10–12). More recently, Its3 has been shown to be required for the synthesis of phosphatidylinositol 3,4,5-trisphosphate in fission yeast (13).

To unravel the signaling pathway regulated by the Its3 PIP5K, we searched for a novel phenotype of the its3-1 mutant and found that the mutant was sensitive to micafungin, a (1,3)-β-D-glucan synthase inhibitor, suggesting a cell wall integrity defect. In this study, we found that there is a lack of GFP-tagged Rgf1 Rho-GTPase exchange factor localization at the cell tips in its3-1 mutant, suggesting a defective Rgf1/Rho pathway. Overexpression of the plc1* gene encoding a phospholipase C, but not that of other genes encoding putative components downstream of PIP5K, suppressed the phenotypes of the its3-1 mutant. Unexpectedly, PKCs are not involved in the suppression. Further analysis revealed that the phospholipase activity of Plc1 is essential for the suppression of the mutant phenotypes and for the viability of the its3-1 mutant. These findings suggest that Its3 regulates cell integrity through a Plc1-mediated PKC-independent pathway, in addition to the Rgf1/Rho pathway.

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1 The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; YPD, yeast extract/peptone/dextrose; EMM, Edinburgh minimum medium; GFP, green fluorescent protein.

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and the localization of GFP-tagged proteins were performed as described previously (24). Actin staining using rhodamine-labeled phalloidin was performed as described previously (10). Tetrad analysis to examine the genetic interaction of its3-1 mutant with other mutants was performed as described previously (10). IP3 receptor binding assay was performed as described previously (25, 26).

RESULTS

The its3-1 Mutant and PKC Mutants Showed Hypersensitivity to Cell Wall-damaging Agents—The its3-1 mutant showed hypersensitivity to micafungin, an inhibitor of (1,3)-β-D-glucan synthase (27) (Fig. 1A), suggesting a defect in cell wall integrity. PIP5K is the enzyme that generates PIP2 (which is a substrate for phospholipase C), yielding IP3 and diacylglycerol, which activates PKC in higher eukaryotes in combination with Ca2+ (7), and PKCs have been shown to be involved in cell integrity regulation in lower eukaryotes (28). In this light, we then examined the effect of micafungin on the mutants of putative downstream components of PIP5K signaling, phospholipase C (Δplc1), and PKCs (Δpck1 and Δpck2) (Fig. 1B). At a low concentration of micafungin (0.3 μg/ml), the growth of Δpck2 and its3-1 mutant cells was markedly inhibited as compared with the growth of Δpck1 cells, which were significantly inhibited, but to a lesser extent. On the other hand, the growth of Δplc1 and wild-type cells was not affected by 0.3 μg/ml micafungin. At higher concentrations of micafungin (0.6 and 1.2 μg/ml), the growth of Δpck1, Δpck2, and its3-1 mutant cells was completely inhibited, whereas the Δplc1 strain evidently grew, but it did so at a slower rate compared with wild-type cells.

Similar results were obtained with aculeacin A, another inhibitor of (1,3)-β-D-glucan synthase (29), and with BE49385A, an inhibitor of Its8 fusion yeast homolog of Mdc4 and Pig-n involved in glycosylphosphatidylinositol anchor synthesis (30) (data not shown).

Sensitivity of its3-1 and Other Mutants of the Putative Downstream Components of PIP5K Signaling to Various Agents—As described above, the mutants of the putative downstream components of PIP5K signaling showed differential sensitivities to micafungin, a (1,3)-β-D-glucan synthase inhibitor. Then we examined the sensitivities of these mutants to various other chemical agents (Table III). One agent tested was the immunosuppressive drug FK506. The its3-1 and bgs1-i2 mutant strains showed sensitivity to FK506, consistent with their original isolation as its mutants (10). Next, the effect of the immunosuppressive drug FK506 plus MgCl2 was examined. As shown in our previous study, wild-type cells were non-viable in the presence of both FK506 and 0.15 mM MgCl2, and gene deletion of the Pmk1 mitogen-activated protein kinase or PKC pathway suppressed the phenotype (31). The Δpck2 cells, but not the Δplc1 strain, grew well in the presence of both FK506 and 0.15 mM MgCl2. Another agent tested was ZnSO4. Various phenotypes of the Δplc1 strain have been reported (16, 32); however, we found that most of these phenotypes were not very evident when the auxotrophic markers were eliminated. Instead, we found that the Δplc1 strain, unlike the other mutants tested, was hypersensitive to ZnSO4.

The above-mentioned results showed that the mutants of the putative downstream components of PIP5K signaling exhibited differential sensitivities to various agents, and among these mutants, only the bgs1-i2 mutant showed phenotypes similar to those of the its3-1 mutant, including temperature sensitivity (Table III).

bgs1-i2, a Mutant Allele of the (1,3)-β-D-Glucan Synthase Gene, Was Synthetically Lethal with its3-1 Mutation—Because its3-1 mutant showed hypersensitivity to several inhibitors of (1,3)-β-D-glucan synthase, as mentioned earlier, and because similar phenotypes were observed in both its3-1 and bgs1-i2

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**Table 1**

| Strain | Genotype               | Ref. or source   |
|--------|------------------------|-----------------|
| HM123  | h′ leu1-32             | Our stock       |
| KP456  | h′ leu1-32 ura4-D18     | Our stock       |
| KP167  | h′ leu1-32 his3-1       | 10              |
| KP722  | h′ leu1-32 ura4-D18 plc1::ura4" | 16 |
| KP1638 | h′ his2 leu1-32 his3-1  | This study      |
| KP185  | h′ leu1-32 bgs1-F2      | This study      |
| KP1825 | h′ leu1-32 ura4-D18 pck1::ura4" | This study |
| KP2286 | h′ leu1-32 ura4-D18 pck2::ura4" | This study |
| KP2495 | h′ leu1-32 ura4-D18 pck1::ura4"他的3-1 | This study |
| KP2496 | h′ leu1-32 ura4-D18 pck2::ura4"他的3-1 | This study |

**EXPERIMENTAL PROCEDURES**

Strains, Media, and Genetic and Molecular Biology Methods—S. pombe strains used in this study are listed in Table I. The complete medium, YPD, and the minimal medium, EMM, have been described previously (14, 15). Standard genetic and recombinant DNA methods (15) were used, except where noted. FK506 was provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Gene disruptions are denoted by a lowercase letter representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, plc1::ura4−). Also, gene disruptions are abbreviated by the gene and preceded by Δ (for example, Δplc1). The Δplc1 cells were a gift from Dr. Toh-e (16). Proteins are denoted by Roman letters, and only the first letter is capitalized (for example, Plc1). Δplc1 and Δpck2 cells were prepared as described previously (17).

**Gene Expression—** For ectopic expression of proteins, we used the thiamine-repressible nmt1 promoter (18). Expression was repressed by the addition of 4 μg/ml thiamine to EMM and induced by washing and incubating the cells in EMM lacking thiamine. Tagged or non-tagged genes were subcloned into pREP1 or pREP41 vectors to express the gene at various levels. Maximum expression of the fused gene was obtained using pREP1, whereas pREP41 contained the attenuated version of the nmt1 promoter (19).

To express green fluorescent protein Rgf1-GFP, the complete open reading frame of rgf1+ (SPCC645.07) was amplified by PCR and ligated to the amino terminus of the GFP carrying the S65T mutation (20). To obtain the chromosome-borne Rgf1-GFP, the fused gene was subcloned into the vector containing the ura4− marker under the authentic rgf1 promoter and integrated into the chromosome at the rgf1+ gene locus of KP456 (h′ leu1-32 ura4-D18). The PH domain of mammalian phospholipase C β1 (21) was overexpressed using pREP1 vector to examine whether the Rgf1-GFP fusion protein localizes in a PIP2-dependent manner. GFP-Bgs1 was expressed using the integrative plasmid pJK-GFP-bgs1−, which was generously given by Dr. Juan C. Ribas (21).

To overexpress the fission yeast homolog of Ipk2, which produces inositol polyphosphates from IP3, the ipk2− gene (SPA0807.04) including its authentic promoter was amplified with PCR primers (forward primer, 5′-CG-GGA-TCC-GAT-TAC-GCA-GAT-GCT-3′; reverse primer, 5′-CG-GGA-TCC-CTT-ATT-CTT-CAG-GAG-CAT-GCG-AAA-AC-3′ and subcloned into the multicopy vector as described previously (10).

**Site-directed Mutagenesis and Generation of Truncated Plc1 Mutants—** We constructed a lipase-deficient form of the Plc1 protein in which the conserved Glu696 in the X domain was mutated to Ala (Plc1 E696A). PlcIE486A was generated using the QuikChange mutagenesis kit (Stratagene). In the amplification reaction, the mutant primers 5′-GTC-AGG-TGT-ATA-GTC-GTA-TTC-GTG-GGA-3′ and 5′-GTC-CCA-GTA-ATG-TCC-GCT-GAT-GTA-AAT-CAA-3′ were used to change Glu696(GQA) into Ala (GCA). This mutation was constructed by analogy to the lipase-deficient mutant Plc1 E641A (22).

A series of amino-terminally truncated Plc1 mutants were generated by the PCR technique. A pair of oligonucleotide primers was used to generate each of the mutants. One primer was derived from the carboxyl-terminal region of the Plc1 open reading frame common to the series of mutants (i.e. AAC-TGG-AGC-GGC-GCG-AAA-TGG-CTT-CTT-TAT-GTC-TC-TGT-CT). The second primer was derived in each mutant from the sequence immediately distal to the region to be deleted, as shown in Table II. BamHI and NotI sites were included in the amino-terminal and carboxyl-terminal oligonucleotide primers, respectively, for cloning of the amplified inserts into the pDS472 plasmid allowing glutathione S-transferase tag to be fused to carboxyl terminus (23).

**Assays and Miscellaneous Methods—** Techniques in light and fluorescence microscopy such as differential interference contrast microscopy
were streaked onto YPD plates containing the indicated concentrations of micafungin.

Cells transformed with the multicopy vector or vector containing the its3 gene showed high temperature sensitivity at 33 °C and could not grow on the plate containing FK506 (0.5 g/ml) or micafungin (0.6 μg/ml) at 27 °C. The overexpression of Plc1, but not that of PKCs, suppressed these phenotypes (Fig. 3B). In its3-1 mutant cells, in contrast, although Rgf1-GFP could be detected in the cytoplasm upon overexpression of the PH domain of mammalian phospholipase C, our previous study showed that Rgf1 shows high homology to S. cerevisiae Rgf1 and examined the localization of Rgf1-GFP in cells overexpressing its PH domain. Results showed that whereas Rgf1-GFP fluorescence was still detected at the ring area (Fig. 2B), in its3-1 mutant showing immunosuppressant-like properties, the localization of Rgf1-GFP suggested that there are PIP2-dependent and -independent interactions that differentially determine tip and ring localization. The aberrant localization of the Rho-GTPase exchange factor is consistent with the aberrant Rom2 localization in budding yeast (33).

To further characterize the role of the its3-1 mutation, we tested the ability of the putative downstream components of PIP5K signaling to suppress phenotypes of its3-1 mutants. As shown in Fig. 3A, its3-1 mutants grew equally well as compared with wild-type cells at 27 °C, but they showed high temperature sensitivity at 33 °C and could not grow on the plate containing FK506 (0.5 μg/ml) or micafungin (0.6 μg/ml) at 27 °C. The overexpression of Plc1, but not that of Pck1 or Pck2, suppressed these phenotypes (Fig. 3A). In addition, the overexpression of Plc1, but not that of PKCs, suppressed the its3-1 Mutant Phenotypes—Previous works in Saccharomyces cerevisiae showed that PIP2 is required for proper localization of Rom2, a GTPase exchange factor for Rho1, which binds PIP2 through its PH domain to establish its normal functions (33). Also, the primary structure of S. pombe Rgf1 shows high homology to S. cerevisiae Rom2, including its PH domain. Our previous study showed that its3-1 mutant cells contained only about 10% of the amount of PIP2 found in wild-type cells (10), thus raising the possibility that the cell integrity defect of its3-1 mutant is caused by the low level of PIP2 resulting in dysregulation of Rho-GTPase signaling pathway. To examine the localization of Rgf1 in its3-1 mutant, GFP-tagged Rgf1 was constructed. In wild-type cells, Rgf1-GFP localized to the septum and cell tips (Fig. 2A). However in its3-1 mutant cells, GFP-Bgs1 was observed only at the ring area and was no longer observed at the cell tips (Fig. 2A).

Lack of Rgf1-GFP Localization at the Cell Tips in its3-1 Mutant—Previous works in Saccharomyces cerevisiae showed that PIP2 is required for proper localization of Rom2, a GTPase exchange factor for Rho1, which binds PIP2 through its PH domain to establish its normal functions (33). Also, the primary structure of S. pombe Rgf1 shows high homology to S. cerevisiae Rom2, including its PH domain. Our previous study showed that its3-1 mutant cells contained only about 10% of the amount of PIP2 found in wild-type cells (10), thus raising the possibility that the cell integrity defect of its3-1 mutant is caused by the low level of PIP2 resulting in dysregulation of Rho-GTPase signaling pathway. To examine the localization of Rgf1 in its3-1 mutant, GFP-tagged Rgf1 was constructed. In wild-type cells, Rgf1-GFP localized to the septum and cell tips (Fig. 2B). In its3-1 mutant cells, in contrast, although Rgf1-GFP could be detected in the ring area, it was hardly observed at the cell tips. The PH domain of the phospholipase C δ1 is known to bind PIP2 (10), so we overexpressed the PH domain of mammalian phospholipase C and examined the localization of Rgf1-GFP in cells overexpressing the PH domain. Results showed that whereas Rgf1-GFP fluorescence was still detected at the ring area (Fig. 2C), the fluorescence at the cell tips disappeared upon overexpression of the PH domain, suggesting that PIP2 levels are sufficiently down-regulated to displace the protein from the tip into the cytoplasm upon overexpression of the PH domain. This also suggests that there are PIP2-dependent and -independent interactions that differentially determine tip and ring localization. The aberrant localization of the Rho-GTPase exchange factor is consistent with the aberrant Rom2 localization in budding yeast (33).

Overexpression of Plc1, but Not That of PKCs, Suppressed the its3-1 Mutant Phenotypes—To further characterize the role of the its3 gene, we tested the ability of the putative downstream components of PIP5K signaling to suppress phenotypes of its3-1 mutants. As shown in Fig. 3A, its3-1 mutants grew equally well as compared with wild-type cells at 27 °C, but they showed high temperature sensitivity at 33 °C and could not grow on the plate containing FK506 (0.5 μg/ml) or micafungin (0.6 μg/ml) at 27 °C. The overexpression of Plc1, but not that of Pck1 or Pck2, suppressed these phenotypes (Fig. 3A). In addition, we examined the genetic interaction between its3-1 mutation and the bgs1 gene that encodes (1,3)-β-D-glucan synthase. Consistently, bgs1Δ, a mutant allele of the bgs1 gene isolated as an its mutant showing immunosuppressant- and temperature-sensitive phenotypes in our genetic screening (10), was synthetically lethal with its3-1 mutation (Table IV).
PKCs Are Not Downstream of Plc1 in Fission Yeast—As described above, overexpression of Plc1, but not that of PKCs, suppressed the mutant phenotypes of its3-1 mutant, suggesting that PKCs are not downstream of Plc1 in fission yeast. To test this hypothesis, we performed the following experiments. First, we examined whether overexpression of Plc1 could suppress the micafungin-sensitive phenotype of Δpck2 and whether overexpression of the PKCs could suppress the zinc-sensitive phenotype of Δplc1 (i.e. whether these two genes interact with each other). Results showed that there was no suppression of the phenotype in each case (Table V). Likewise, in a study by Toda et al. (34) reporting that Δpck2 cells showed aberrant cell polarity as described above, overexpression of Plc1 also failed to suppress the aberrant cell polarity phenotype of Δpck2 as shown in Fig. 4A.

In the study by Toda et al. (34), it was reported that Pck2 hyperactivity causes toxicity in wild-type cells. We then examined whether or not Pck2 overexpression would cause toxicity in Δplc1 and its3-1 mutant cells. Results showed that overexpression of Pck2 (Fig. 4B, Pck2 OP) caused growth defects in Δplc1 as well as in its3-1 mutant cells to the same extent as that observed in wild-type cells.

Lastly, we examined the effect of overexpression of Plc1, Pck1, and Pck1 on the micafungin sensitivity of the Δpck2Δplc1 double mutant. The micafungin sensitivity of the Δpck2 single mutant and that of the Δpck2Δplc1 double mutant were nearly identical (data not shown). As shown in Fig. 4C, the micafungin sensitivity of the Δpck2Δplc1 double mutant was strongly suppressed by overexpression of Pck2 and also significantly suppressed by overexpression of Pck1. However, with overexpression of Plc1, no change in the micafungin sensitivity of the double mutant was observed. These findings support our hypothesis that PKCs are not downstream of Plc1 in fission yeast.

Deletion of Pck1 or Pck2 Could Not Abolish the Effect of Plc1 Overexpression on its3-1 Mutant Phenotypes—Both the its3-1Δpck1 and its3-1Δpck2 double mutants were viable and showed high temperature sensitivity (Fig. 5; Table IV). We then examined the effects of overexpression of Plc1 on the temperature sensitivities of the its3-1Δpck1 and its3-1Δpck2 double mutants. The temperature sensitivity of both double mutants was suppressed by Plc1 overexpression (Fig. 5). Thus, deletion of Pck1 or Pck2 could not abolish the effect of Plc1 overexpression on its3-1 mutant phenotypes. Taken together with the above data showing that PKCs are not downstream of Plc1, the findings suggest that the suppression of the its3-1 mutant phenotype by Plc1 overexpression is not mediated by activation of PKCs.

Plc1 Enzymatic Activity Is Essential for the Suppression of its3-1 Mutant Phenotypes—To study the mechanism of how Plc1 overexpression suppresses the phenotypes of the its3-1 mutant, we performed Plc1 enzymatic activity assay by measuring the cellular level of IP3 (Table VI). In its3-1 mutant cells, overexpression of Plc1 did not significantly affect the IP3 level. In wild-type cells, however, overexpression of Plc1 showed a 55% increase in IP3 level. Notably, IP3 was not detectable in the Δplc1 cells. This suggests that IP3 production is solely dependent on Plc1 in fission yeast. This is in contrast to the study by Stolz et al. (35), which suggested that the budding yeast contains non-Plc1-mediated production of IP3.

To further investigate the role of Plc1, we constructed a lipase-deficient mutant of Plc1 and tested its ability to suppress the its3-1 mutant phenotypes. Lipase-deficient Plc1E486A, which had no enzymatic activity as confirmed by no increasing IP3 level in Δplc1 cells, did not complement the its3-1 mutant phenotypes, indicating that phospholipase activ-
Cell Integrity Regulation by PIP5K

**FIG. 3.** The genetic interaction between the *its3* gene and the *plc1* gene. A and B, overexpression of Plc1, but not that of PKCs, Rho1, Rho2, or Rgf1, suppressed the immunosuppressant and temperature sensitivities of *its3*-1 mutant. Cells transformed with the multicopy vector or vector containing various genes were streaked or spotted onto each plate as indicated. C, actin distribution in the *its3*-1 mutant and Δpck2 cells. The *its3*-1 mutant and Δpck2 cells were grown to exponential phase in YPD at the permissive temperature (27 °C) and shifted to the restrictive temperature (33 °C or 36 °C, respectively). Samples were taken 4 h after temperature upshift, fixed, and stained with rhodamine-conjugated phalloidin to visualize F-actin. Arrowheads indicate polarized actin in the cell tips. Bar, 10 μm. D, the *its3*-1 mutant is synthetically lethal with *plc1* knock-out. Tetrad analysis of progeny derived from crossing KP1638 (h− *his2* leu1-32 ura4-D18 *its3*-1) with KP722 (h− *leu1*-32 ura4-D18 *plc1*:ura4*+*).

**TABLE V**

| Strain | Sensitivity of strain carrying plasmid |
|--------|-------------------------------------|
|        | Vector   | plc1− | pck1− | pck2− |
| Δplc1 (9 mM ZnSO4) | − | + | − | + |
| Δpck2 (0.3 μg/ml micafungin) | − | − | + | + |

**FIG. 4.** PKCs are not downstream of Plc1 in fission yeast. A, overexpression of Plc1 failed to suppress the aberrant cell polarity phenotype of Δpck2 cells. The Δpck2 cells transformed with a multicopy vector or vector containing the *plc1*− gene were grown in EMM at 27 °C and stained with Calcofluor to visualize septum and cell wall. Bar, 10 μm. B, overexpression of Pck2 caused growth defects in Δplc1 and *its3*-1 mutant cells to the same extent as that observed in wild-type cells. Cells transformed with pREP1-pck2′ were streaked onto EMM with or without thiamine and incubated for 4 days at 27 °C. C, overexpression of PKCs, but not that of Plc1, suppressed the micafungin sensitivity of the Δpck2Δplc1 double mutant. Cells were spotted as indicated onto YPD and YPD plus 0.6 μg/ml micafungin and incubated at 27 °C for 4 days.

Furthermore, we prepared a series of truncated forms of Plc1 to examine the relationship between the enzymatic activity and the ability to suppress phenotypes of the *its3*-1 mutant. Structural features of the deletion mutants employed in this study are illustrated in Fig. 6A. In Δplc1 cells, overexpression of full-length Plc1 as well as Plc1 fragment A increased the IP3 level and also suppressed phenotypes of the *its3*-1 mutant (Fig. 6, A and B). However, overexpression of Plc1 fragment B lacking the PH domain-like domain showed a significantly increased level of IP3 in Δplc1 cells but failed to complement the mutant phenotype.

Each transformant, carrying various genes on the multicopy plasmids, was streaked onto YPD plates with or without the agents as indicated and incubated at 30 °C for 3 days. +, not sensitive; −, sensitive.

**FIG. 5.** Sensitivity of the Δplc1 and Δpck2 mutants to ZnSO4 and micafungin, respectively. Each transformant, carrying various genes on the multicopy plasmids, was streaked onto YPD plates with or without the agents as indicated and incubated at 27 °C. Strains indicated were predicted to be wild-type cells (wt). Spores that failed to grow were predicted to be *its3*-1Δplc1 double mutants.
growth defect of its3 mutant at 33 °C (Fig. 6, A and B). These results suggest that the PH domain-like domain of Plc1 plays an important role for the enzymatic activity or is involved in the regulation of its subcellular localization, which is necessary for suppression of the its3-1 mutant phenotypes. Consistently, by the genetic cross experiments between the its3-1 mutant and Δplc1 cells transformed with these amino-terminal truncation mutants, results showed that the its3-1Δplc1 double mutant cells expressing fragment B failed to grow, suggesting that the enzymatic activity of Plc1 is essential for the viability of the its3-1 mutant (Table VII).

Our previous study suggested that Its3, fission yeast PIP5K, functions coordinately with calcineurin and plays a key role in cytokinesis. To determine whether suppression of the phenotypes of its3-1 mutant by overexpression of Plc1 is due to an activation of calcineurin, we overexpressed pph1ΔC, a constitutively active form of calcineurin (31), in its3-1 mutant and Δplc1 cells. As shown in Fig. 6, C and D, overexpression of pph1ΔC failed to suppress the phenotypes of its3-1 mutant or Δplc1 cells.

We also examined whether the suppression depends on inositol polyphosphates, such as inositol tetrakisphosphate, inositol pentakisphosphate, and inositol hexakisphosphate, which are known to act downstream of Plc1 in budding yeast (36). We then overexpressed the ipk2+ gene (SAC607.04) encoding a homolog of budding yeast IPK2/Arg82 that produces IP4 and IP5 from IP3 (36) in its3-1 mutant and Δplc1 cells. As shown in Fig. 6, C and D, overexpression of Ipk2 failed to suppress the phenotypes of its3-1 mutant or Δplc1 cells.

Two Distinct Cell Integrity Signaling Pathways Downstream of Its3 PIP5K—The above-mentioned results indicate that the proper PIP2 level is important for cell integrity. We then examined the effects of overexpression of Its3 PIP5K on hog1Δ2 mutant that showed synthetic lethality with its3-1 mutant

![Image](https://example.com/image.png)
having a low intracellular PIP₂ level. In wild-type cells, overexpression of Its3 resulted in a significant increase in the level of PIP₂, and the increase in the PIP₂ level was dependent on the promoter strength used for overexpression (10). As shown in Fig. 7A, overexpression of Its3 from the attenuated nmt1 promoter using pREP41 vector (19) completely halted the growth of bgs1-i2 cells, suggesting that this cell integrity mutant is sensitive to the increased cellular PIP₂ level. Also, the bgs1-i2 cells overexpressing Its3 PIP5K showed extremely aberrant morphology with elongated, swollen, and branched cells (Fig. 7B). As shown in Table VI, overexpression of Its3 in wild-type cells caused a dramatic increase in IP₃ level, indicating that PIP₂ accumulation and its hydrolysis by Plc1 lead to the increased IP₃ level.

To examine whether or not the growth inhibition and aberrant morphology of bgs1-i2 cells caused by overexpression of Its3 are mediated by Plc1, we then overexpressed Plc1 in bgs1-i2 cells and wild-type cells using the same expression vector. Overexpression of Plc1 had no effect on the growth of both cell types (Fig. 7), suggesting that the growth-inhibitory effect of Its3 PIP5K overexpression is directly mediated by PIP₂ and is not due to Plc1 activation.

**DISCUSSION**

This study demonstrates for the first time the evidence in identifying a novel function of PIP5K that regulates fission yeast cell integrity through a phospholipase C-mediated PKC-independent pathway (Fig. 8).

As described above, the defective cell integrity of the its3-1 mutant seems to be derived from its low PIP₂ level. However, it is puzzling that the elevated dosage of the plc1+ gene that further decreased the PIP₂ level was still able to suppress the phenotype of the its3-1 mutant. Because Plc1 hydrolyzes PIP₂ to IP₃ and diacylglycerol, either metabolite or both metabolites may exert a beneficial effect on cell integrity. Our present study suggests that the effect of diacylglycerol is not due to activation of PKC and that the effect of IP₃ is not mediated by production of inositol polyphosphates.

The presence of PKC-like proteins in fission yeast such as Pck1 and Pck2 suggests that the PKC-mediated phosphorylation pathway, activated by diacylglycerol or Ca²⁺, in mammalian cells, is conserved from yeast to human. However, there has been no evidence for the activation of Pck1 or Pck2 by diacylglycerol or Ca²⁺ in vitro or in vivo. Studies have shown that Pck2 is not activated by Ca²⁺ in vitro (37). Studies have also reported that the activity of Pck1, a budding yeast PKC-like protein that shows high sequence homology to Pck1 and Pck2, is independent of cofactors such as diacylglycerol and Ca²⁺ (38). In addition to these in vitro data, our present in vivo study shows that the fission yeast PKCs are not downstream of Pck1, which is known to generate diacylglycerol, which activates PKC in mammalian cells.

On the other hand, previous genetic studies suggested that the fission yeast PKCs are regulated by Rho protein through interaction with the amino-terminal HR1 domain of PKCs (28, 39). Mammalian Rho protein has been shown to bind to the HR1 domain of protein kinase N/PKC-related protein kinase, another protein kinase subfamily structurally related to PKC, and to activate its kinase activity in vitro (40–42). Taken together, it is suggested that lower eukaryotic PKCs are more functionally similar to the mammalian protein kinase N/PKC-related protein kinase subfamily than to the conventional PKCs that are activated by diacylglycerol and Ca²⁺.

Another puzzling observation in our experiments was the growth phenotype of the Δpck1 cells in micafungin, an inhibitor of (1,3)-β-D-glucan synthase that is a major structural component of the yeast cell wall. Whereas the its3-1 mutant, Δpck1, and Δpck2 cells were significantly hypersensitive to micafungin, the Δpck1 cells, contrary to our expectations, were only slightly more sensitive as compared with the wild-type cells. Notably, the elevated gene dosage of plc1+ suppressed the hypersensitivities of the its3-1 mutants to the cell wall-damaging agents. Thus, it is evident that Plc1 is involved in the

**TABLE VII**

| Cross | Total colony no. | Could not grow on plate lacking uracil | Could grow on plate lacking uracil |
|-------|-----------------|---------------------------------------|----------------------------------|
|       |                 | ts Not ts                             | ts Not ts                        |
| its3-1 × Δpck1 transformed with full-length | 597 (100) | 175 (29) 89 (15) | 129 (22) 204 (34) |
| pREP1-Plc1                              | 526 (100) | 140 (27) 276 (52) | 0 (0) 110 (21) |

**Fig. 7. Overexpression of Its3, but not Plc1, caused severe growth defect and aberrant morphology in the (1,3)-β-D-glucan synthase mutant.** A, overexpression of Its3, but not Plc1, from the attenuated nmt1 promoter significantly inhibited the growth of fission yeast cells and completely inhibited the growth of bgs1-i2 cells. The wild-type or bgs1-i2 cells transformed with pREP41-its3+ (+ Its3) or pREP41-plc1+ (+ Plc1) were streaked onto EM at 27 °C for 5 days. B, aberrant morphology of cells overexpressing Its3. Cells were transformed with pREP41-its3+ (+ Its3) or pREP41-plc1+ (+ Plc1) and streaked onto EM at 27 °C as described above. On the second day, cells as indicated were picked up for differential interference contrast microscopic observation. Bar, 10 μm.
FIG. 8. PIP5K regulates fission yeast cell integrity through a phospholipid C-mediated PKC-independent pathway. PIP, phosphatidylinositol 4-phosphate; DG, diacylglycerol.

regulation of fission yeast cell integrity. One possible explanation for this apparent contradiction is that diacylglycerol is generated by another pathway other than the phospholipase C pathway, thereby maintaining cell integrity. Phospholipase D-catalyzed hydrolysis of phospholipids generates phosphatidic acid that is subsequently metabolized to lysophosphatidic acid and diacylglycerol. Phospholipase D activity is stimulated by an acidic that is subsequently metabolized to lyso-phosphatidic acid.

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