Phosphatidylinositol 4-Phosphate 5-Kinase Its3 and Calcineurin Ppb1 Coordinately Regulate Cytokinesis in Fission Yeast*

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The ppb1† gene encodes a fission yeast homologue of the mammalian calcineurin. We have recently shown that Ppb1 is essential for chloride ion homeostasis, and acts antagonistically with Pmk1 mitogen-activated protein kinase pathway. In an attempt to identify genes that share an essential function with calcineurin, we screened for mutations that confer sensitivity to the calcineurin inhibitor FK506 and high temperature, and isolated a mutant, its3-1. its3‡ was shown to be an essential gene encoding a functional homologue of phosphatidylinositol-4-phosphate 5-kinase (PI(4)P5K). The temperature upshift or addition of FK506 induced marked disorganization of actin patches and dramatic increase in the frequency of septation in the its3-1 mutants but not in the wild-type cells. Expression of a green fluorescent protein-tagged Its3 and the phospholipase C

Calcineurin, Ca2⁺/calmodulin-dependent protein phosphatase, is a molecular target for the specific immunosuppressive drugs, such as cyclosporin A or tacrolimus (FK506),¹ used in organ transplantation. These drugs induce their biological effects by forming an initial complex with cytosolic proteins termed immunophilins. These drug-immunophilin complexes then bind to and inhibit calcineurin (1). Calcineurin is widely distributed in mammalian tissues, and may have additional functions other than its well characterized role in lymphocytes. The inhibition of these functions may contribute to the side effects of these drugs. A better understanding of the biological roles of calcineurin in different cell types should promote the development of improved strategies for immunosuppression.

Calcineurin is conserved from yeast to man (2–4). We have been studying calcineurin signal transduction pathways in fission yeast Schizosaccharomyces pombe because this system is amenable to genetics and has many advantages in terms of relevance to higher systems. S. pombe has a single gene encoding the catalytic subunit of calcineurin, ppb1†, that is essential for cytokinesis (4, 5). We have previously shown that ppb1† plays an essential role in maintaining chloride ion homeostasis, and acts antagonistically with Pmk1 mitogen-activated protein kinase pathway (6–8). To identify new components in the calcineurin signaling pathway, we have developed a simple genetic screen using the immunosuppressive drug FK506 for mutants that depend on calcineurin for growth, and have identified eight complementation groups (its1–8 for immunosuppressant- and temperature-sensitive, to be described elsewhere in detail).

Here we report that its3‡ encodes a protein most similar to Saccharomyces cerevisiae Mss4 PI(4)P5K. The mutant phenotypes were suppressed by the expression of Mss4 or mammalian PI(4)P5K. In addition, its3-1 mutant cells had only low amounts of PI(4,5)P2 and elevated amounts of PI(4)P, indicating that the encoded protein is a PI(4)P5K and that the mutation caused a significant decrease in PI(4)P5K activity. As expected, growth of the its3-1 mutant cells was dependent on calcineurin, strongly suggesting that Its3 PI(4)P5K and Ppb1 calcineurin share an essential overlapping function. Furthermore, analysis of the its3-1 mutant revealed that PI(4)P5K is required for completion of cytokinesis.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Genetic and Molecular Biology Techniques and Nomenclature**—Fission yeast strains used in this study are listed in Table I. The complete medium, YPD, and minimal medium, EMM, have been described previously (8). Standard methods for S. pombe genetics were followed according to Moreno et al. (9). Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, its3::ura4⁺). Also, gene disruptions are abbreviated by the gene preceded by Δ (for example, Δits3). Proteins are denoted by roman letters and only the first letter is capitalized (for example, Its3).

Isolation of its3-1 Mutants—The its3-1 mutant was isolated in a screen of cells that had been mutagenized with nitrosoguanidine. Cells of strain HM123 were mutagenized with 300 μM nitrosoguanidine (Sigma) for 60 min (~10% survival) as described by Moreno et al. (9). Mutants were spread on YPD plates to give ~1,000 cells/plate and incubated at 27 °C for 4 days. The plates were then replica-plated at
36 °C to plates containing 0.5 μg/ml FK506. Mutants that showed both FK506 sensitivity and temperature sensitivity were selected. The original mutants isolated were backcrossed three times to wild-type strains HM123 and HMS28.

Cloning and Deletion of its3-1 Gene—To clone its3-1 gene, its3-1 mutant (KP167) was grown at 27 °C and transformed with an S. pombe genomic DNA library constructed in the vector pDB248 (9). Leu+ transformants were replica-plated onto YPD plates and incubated at 36 °C, and plasmid DNA was recovered from 10 transformants that showed plasmid-dependent rescue. These plasmids had identical or overlapping inserts as judged from restriction digests, and all 10 complemented both the immunosuppressant sensitive and temperature sensitive of the its3-1 mutant.

A one-step gene disruption by homologous recombination (10) was performed. The its3::ura4+ disruption was constructed as follows. The 3.4-kilobase HinIII fragment containing the its3-1 gene was subcloned into the EcoRV site of pGEM-5Zf(+) (Promega) after Klenow fragment exonuclease treatment. Then, a BamHI-EcoRI fragment containing the ura4+ gene was inserted into the BamHI-EcoRI site of the previous construct (Fig. 3B). The HinII fragment containing disrupted its3-1 gene was transformed into diploid cells (5A/1D). Stable integrants were selected on medium lacking uracil, and disruption of the gene was checked by genomic Southern hybridization (Fig. 3C).

Plasmid Constructions—To express various phosphatidylinositol phosphokinases in fission yeast, a genomic fragment containing budding yeast MSS4 gene was subcloned into the multicopy vector pDB248 (9), and the entire coding regions of murine PI(4)P5K (11) and human PI(5)P4K (12) were amplified from cDNAs by polymerase chain reaction (9), and the entire coding regions of murine PI(4)P5K (11) and human PI(5)P4K (12) were amplified from cDNAs by polymerase chain reaction and subcloned into the expression vector pREP1, containing the thiamine-repressible nmt1 promoter (13). To overexpress Its3, the entire coding region of Its3 was amplified from genomic DNA by polymerase chain reaction and subcloned into the expression vector pREP1 and its attenuated version, pREP41 (13).

Its3 was tagged at its N terminus with GFP carrying the S65T mutation (14). To express GFP-Its3 in fission yeast, the coding region for the its3-1 gene was amplified using polymerase chain reaction, ligated to the C terminus of GFP, and the resultant construct was subcloned into the pREP81 vector, containing an attenuated version of the nmt1 promoter (13). The Its3 coding region from its3-1 mutant genome was also amplified, ligated to GFP, and subcloned into pREP81. Similarly, the PH domain of PLCδ (15, 16) was ligated to the C terminus of GFP and the resultant construct was subcloned into the pREP1 vector (13).

Similarly, wild-type and mutant Its3 tagged at their N terminus with GST were constructed and subcloned into the pREP41 vector (13). They were expressed in fission yeast and purified using a glutathione affinity column as described previously (6, 7). Purified samples were processed for lipid kinase assay as described below.

In Vivo Analysis of Phosphoinositides—To label fission yeast strains with [3H]inositol, the cells were grown in EMM. The cells were harvested and disrupted by vigorous vortexing in 4 ml of methanol, 1 N HCl (1:1) with acid-washed glass beads (1.5 g; Sigma). Phospholipids were extracted by the addition of 2 ml of chloroform and subsequent vortexing. The extracted lipids were deacylated with methanol, and resulting glycerophosphoinositides were analyzed by Partisphere 5-SAX column (Whatman Inc.) as described by Serunian et al. (17). Briefly, 1 × 10^10 dpm of labeled glycerophosphoinositides were applied to the column, washed with water (buffer A; 1 ml/min) for 10 min, and eluted by a gradient of 1 × (NH4)2HPO4 (pH 3.8) (buffer B) as follows: 0 min, 0% B; 60 min, 25% B; 110 min, 100% B.

Lipid Kinase Assay—The lipid kinase reaction and detection of phosphorylated products were described previously (18). Briefly, purified GST fusion proteins and substrate lipids (50 μM) were incubated in a kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 0.1 mM ATP, and 1 μCi of [γ-32P]ATP) at room temperature. After the lipids had been extracted by addition of 1 N HCl and chloroform/methanol (2:1), phosphorylated lipids were separated by TLC and observed by autoradiography.

Microscopic Analysis—For actin staining, cells were fixed in 3% formaldehyde in phosphate-buffered saline for 30 min and to 50 μl of fixed cell suspension 1 μl of 100 μg/ml rhodamine-labeled phalloidin (Molecular Probe) was added. After 30 min at room temperature, excess phalloidin was washed away with phosphate-buffered saline. Cells were dried on coverslips treated with poly-L-lysine and counterstained with 4,6-diamidino-2-phenylindole dissolved in phosphate-buffered saline to visualize the DNA. Calcofluor white was used to visualize cell wall and septum. To visualize the medial actomyosin ring in living cells, wild-type or its3-1 mutant cells were transformed with a plasmid encoding GFP-Cdc4 (19).

RESULTS

Isolation of its3-1 Mutant—A genetic screen was performed to identify genes that share an essential overlapping function with calcineurin. For this purpose, we have developed a simple genetic screen using the immunosuppressive drug FK506 for mutants that depend on calcineurin for growth, and have identified eight complementation groups (its1-8 for immunosuppressant- and temperature-sensitive, to be described elsewhere in detail).

As shown in Fig. 1A, its3-1 mutant cells could not grow at 33 °C, 36 °C, or in the YPD plate containing FK506, whereas wild-type cells grew normally. its3-1 mutant cells grew almost normally at the permissive temperature (27 °C), but stopped growing 4, 2, or 8 h after shift to 33 °C, 36 °C, or to the YPD media containing FK506 at 27 °C, respectively (Fig. 1B).

Previous reports showed that calcineurin is an in vivo target of FK506 in fission yeast, and inhibition of calcineurin activity...
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by the addition of FK506 to the culture media resulted in identical phenotypes as those of calcineurin deletion (4). Thus, it could be anticipated that the its3-1 mutant requires functional calcineurin activity for vegetative growth, and tetrad analysis was performed to examine the synthetic lethality with Δppb1 (ΔCN) and the rest of the colonies were predicted to be wild-type (WT). Spores that failed to grow were predicted to be its3-1 Δppb1 double mutants (D).

Isolation and Disruption of its3+ Gene—The its3+ gene was cloned by complementation of the ts growth defect (Fig. 3, A and B). Nucleotide sequencing of the cloned DNA fragment revealed that the its3+ gene was SPAC19G12.14. It encodes a protein of 742 amino acid residues which is most similar to S. cerevisiae Mss4 PI(4)P5K (see below). As a first step to analyze Its3 function, the its3+ gene was knocked out in a diploid by homologous recombination using ura4+ marker gene (Fig. 3B). Southern blotting of the genomic DNA of one such transformant confirmed that the wild-type gene had been replaced by the derivative containing the ura4+ insertion (Fig. 3C). Tetrad analysis of the heterozygous diploid showed two viable (Ura+) and two inviable spores (Fig. 3D), indicating that the its3+ gene is essential for cell growth.

its3+ Encodes a Functional Homologue of PI(4)P5K—As described above, its3+ encodes a PI(4)P5K homologue. A BLAST search of protein sequence data bases revealed that fission yeast Its3 has 44, 34, and 31% identity with budding yeast Mss4 (20, 21), murine PI(4)P5K (11), and human PI(5)P4K (12), respectively (Fig. 4).

To examine whether Its3 is a functional homologue of any of the phosphatidylinositol phosphokinases, we expressed Mss4, murine PI(4)P5K, and human PI(5)P4K in fission yeast. After the expression plasmids were introduced to its3-1 mutant, the cells were grown at the restrictive temperature. All the transformants expressing PI(4)P5K were capable of growing at 36 °C (Fig. 5A). Expression of PI(4)P5Ks also suppressed the FK506-sensitive phenotype of the its3-1 mutant (data not shown). On the other hand, expression of PI(5)P4K failed to complement the its3-1 phenotypes. These results indicate that its3+ encodes a functional homologue of PI(4)P5K.

The Level of PI(4,5)P2 in its3-1 Mutant Is Severely Reduced—Then, we determined whether the its3-1 mutant strain contained lowered levels of PI(4,5)P2. Wild-type and its3-1 mutant cells were labeled with [3H]inositol, and the tritiated lipid content was analyzed. As shown in Fig. 5B, its3-1 mutant cells contained about 10% of the amount of PI(4,5)P2 found in wild-type cells, indicating that the mutation caused a significant decrease in PI(4,5)P2 activity of Its3. Interestingly, the level of PI(4)P was significantly higher than that of the wild-type cells. These results indicate that Its3 is a PI(4)P5K in vivo, and that Its3 is the major PI(4)P5K in S. pombe under normal growth conditions. A shift to the higher temperature induced a further decrease in the level of PI(4,5)P2 (Fig. 5B), strongly suggesting that various phenotypes of its3-1 mutant cells found at the restrictive temperature are due to the lowered level of cellular PI(4,5)P2. It also resulted in a significant increase in the relative peak height of PI(4)P, suggesting that activity of mutant Its3 PI(4)P5K decreases upon the temperature shift.

On the other hand, as shown in Table II, overexpression of Its3 in wild-type cells resulted in a significant increase in the levels of PI(4,5)P2, but not in the levels of PI(3,5)P2. The increase in the PI(4,5)P2 levels were dependent on the promoter strength used for overexpression.

Mutant Its3 Showed Reduced but Detectable PI(4)5K Activity—To examine PI(4)P5K activity of Its3, we expressed a GST-Its3 fusion protein in fission yeast. A GST-tagged version of

![Fig. 2. its3-1 is synthetically lethal with Ppb1 calcineurin null. Tetr...](image-url)

![Fig. 3. Cloning and disruption of the its3+ gene. A, cloning of the its3+ gene. Wild-type (WT) and its3-1 mutant cells transformed with a...](image-url)
Its3 was fully functional as demonstrated by its complementation of its3-1 mutant phenotypes (data not shown). The Its3 coding region from its3-1 mutant genome was also fused with GST and expressed in fission yeast. Purified GST fusion proteins were subjected to in vitro kinase reaction as described under "Experimental Procedures." The individual phosphatidylinositols in wild-type (WT) and its3-1 mutant cells transformed with the multicopy plasmid, pDB248 (vector), pDB248[its3] (pits3), pDB248[MSS4] (pMSS4), pREP1open reading frame of murine PI(4)P5K (pPI(4)P5K), or pREP1(open reading frame of human PI(5)P4K cDNA) (pPI(5)P4K). Transformed cells were streaked onto a plate containing YPD and incubated for 3 days at 36 °C. B, in vivo analysis of phosphoinositides. Wild-type (WT) and its3-1 mutant cells were labeled with myo-[2-3H]inositol, and phospholipids were extracted and analyzed as described under "Experimental Procedures." Fractions were collected and counted for 3H radioactivity. Deacylated PI(3)P, PI(3,5)P2, PI(4,5)P2 are indicated. The data presented are representative of multiple experiments.

**TABLE II**

| Strain | PI(3,5)P2 (%) | PI(4,5)P2 (%) |
|--------|--------------|--------------|
| Wild type + pREP1 | 100 | 100 |
| + pREP41-Its3 | 118 | 157 |
| + pREP1-Its3 | 104 | 249 |

Studies have implicated PI(4,5)P2 in many cell surface events, including regulation of actin cytoskeleton (22). In particular, studies in budding yeast using temperature-sensitive MSS4 mutants have shown that MSS4 PI(4,5)P2 is involved in the organization of the actin cytoskeleton (20, 21). These results prompted us to examine the effects of temperature upshift and FK506 on the actin distribution of the its3-1 mutant. Wild-type and its3-1 cells were grown at 27 °C and then shifted to 33 °C. At different time points, aliquots were removed, and cells were fixed and stained with rhodamine-phalloidin to visualize F-actin (Fig. 7A). When grown at 27 °C, wild-type cells
displayed the normal cell cycle-dependent distribution of actin in two intracellular regions, cell tips (F-actin patches) and the medial region (F-actin ring) (Fig. 7A) (23, 24). In wild-type cells, a shift from 27 to 33 °C caused a transient heat-induced disorganization of actin patches, but further incubation at 33 °C restored normal actin patch distribution (Fig. 7A). Treatment of wild-type cells with FK506 resulted in slight perturbation of cortical patch distribution, but had no effect on the actin ring formation (Fig. 7A). On the other hand, in the its3-1 mutant cells, actin patches were partially polarized at 27 °C, and the polarization was completely lost upon temperature upshift or FK506 treatment (Fig. 7A). Heat- or FK506-induced disorganization of actin patches was irreversible and became more severe with exposure time (data not shown). Interestingly, F-actin ring was observed even when the polarization of actin patches was completely lost (Fig. 7A), indicating that contractile ring formation was not so much affected by the lowered intracellular PI(4,5)P2 level. Visualization of actin contractile ring formation in its3-1 mutant cells expressing GFP-Cdc4 (19) also confirmed that the its3-1 mutant was not defective for actin ring formation, although its abnormality such as an extra ring was often observed (Fig. 7B).

Shift to the Restrictive Temperature or Addition of FK506 to the Culture Media Dramatically Increased the Septation Index of its3-1 Mutant Cells—During the course of experiments studying the organization of the actin cytoskeleton, we noted a high incidence of septated cells associated with the its3-1 mutation (Fig. 7). This prompted us to examine the correlation between the mutation and cell septation in more detail. Microscopic observation revealed that some mutant cells have a thick septum that was brightly stained with Calcofluor and was hardly seen in wild-type cells (Fig. 8A). In addition, its3-1 mutant showed a dramatic increase in the septation index upon shift to the restrictive temperature or by addition of FK506. Saturated cultures of wild-type cells (open circle) or its3-1 mutant cells (closed circle) were diluted with fresh YPD or YPD supplemented with 0.5 μg/ml FK506, and incubated at the indicated temperatures. Percentage of septated cells was measured hourly with Calcofluor staining.

33 °C restored normal actin patch distribution (Fig. 7A). Treatment of wild-type cells with FK506 resulted in slight perturbation of cortical patch distribution, but had no effect on the actin ring formation (Fig. 7A). On the other hand, in the its3-1 mutant cells, actin patches were partially polarized at 27 °C, and the polarization was completely lost upon temperature upshift or FK506 treatment (Fig. 7A). Heat- or FK506-induced disorganization of actin patches was irreversible and became more severe with exposure time (data not shown). Interestingly, F-actin ring was observed even when the polarization of actin patches was completely lost (Fig. 7A), indicating that contractile ring formation was not so much affected by the lowered intracellular PI(4,5)P2 level. Visualization of actin contractile ring formation in its3-1 mutant cells expressing GFP-Cdc4 (19) also confirmed that the its3-1 mutant was not defective for actin ring formation, although its abnormality such as an extra ring was often observed (Fig. 7B).
its3-1 mutant showed a dramatic increase (from 24 to 50%) in the septation index upon shift to the restrictive temperature, while the septation index of wild-type cells remained unchanged (Fig. 8B). These results suggest that the G281D mutation induces a structural defect in Its3 PI(4)P5K that causes a severe impairment of PI(4,5)P2 synthesis, as well as an impairment in cytokinesis.

As described previously (4), treatment of wild-type cells with FK506 had no significant inhibitory effect on the growth rate, but caused a significant increase in the septation index, suggesting a requirement of Ppb1 calcineurin activity in cytokinesis. Consistently, disruption of the ppb1" gene also resulted in a significant increase in the septation index (4). Our present study also showed that addition of FK506 increased the septation index of wild-type cells (Fig. 8B). When the wild-type cells were cultured at 27 °C in the absence of FK506, the septation index was less than 15%. The index was 27% at 6 h after the addition of FK506. It gradually increased and reached its maximum level (45%) at 15 h. On the other hand, upon addition of FK506 to the incubation medium, the its3-1 mutant cells showed a dramatic change as compared with the wild-type cells. Treatment of its3-1 mutant cells with FK506 markedly inhibited the growth (Fig. 1B), and the septation index reached nearly 100% only 6 h after the addition of FK506 (Fig. 8, A and B), suggesting a synergistic control of cytokinesis by PI(4)P5K and calcineurin.

**FIG. 9.** Subcellular localizations of wild-type and mutant (G281D) Its3 and PH domain of PLC6. Mid-log phase wild-type cells expressing GFP-tagged Its3 (A, GFP-Its3), mutant Its3 (B, GFP-mIts3), or PH domain from PLC6 (C, GFP-PH) were examined by differential interference contrast (DIC) and fluorescent microscopy. Representative patterns during each cell cycle stage are shown. Arrow points to an example of strong fluorescence of GFP-Its3 or GFP-PH at septum. The bar indicates 10 μm.

GFP-Its3 and GFP-PH Localized to the Plasma Membrane and Were Concentrated at the Septum of Dividing Cells—To investigate the intracellular distribution of Its3 PI(4)P5K, we generated a GFP-Its3 fusion protein. GFP-tagged version of Its3 was fully functional as demonstrated by its complementation of its3-1 mutant phenotypes (data not shown). GFP-Its3 protein was expressed from a multicopy vector containing an nmt1 promoter. GFP-Its3 localized to the plasma membrane at all stages of the cell cycle (Fig. 9A). Enrichment of Its3 fusion protein in specific locations within the plasma membrane which occurred during the cell cycle was evident. In cells with the secondary septum, the fusion protein is evenly distributed within the membrane but is heavily concentrated in septum as compared with other cell membranes (Fig. 9A). The localization of the Its3-1 mutant (G281D mutation) was also examined. As shown in Fig. 9B, the GFP-Its3-1 mutant protein (GFP-mIts3) was no longer localized to plasma membrane, instead the whole cytoplasm and the nucleus were stained.

In addition, the PLC6 PH domain fused to GFP (GFP-PH) was expressed in fission yeast cells. PH domain from PLC6 has been shown to have a high in vivo binding affinity for plasma membrane PI(4,5)P2 (15). Consistently, GFP-PH also specifically labeled the plasma membrane and was highly concentrated at the septum of dividing cells (Fig. 9C). Expression of GFP-PH induced an increased sensitivity to FK506 in wild-type cells and lowered the restrictive temperature of the its3-1 mutation, suggesting sequestration of plasma membrane PI(4,5)P2 by GFP-PH (data not shown).

**DISCUSSION**

In an attempt to identify genes that may have overlapping function with calcineurin, we isolated a fission yeast gene its3". Five lines of evidence show that Its3 is a functional homologue of PI(4)P5K. First, its3" encodes a protein with high sequence similarity to the budding yeast Mss4 and mammalian PI(4)P5K. Second, the FK506 and temperature sensitivity of its3-1 mutant was complemented by expression of Mss4 and murine PI(4)P5K, but not by expression of human PI(5)P4K. Third, the level of PI(4,5)P2 was severely reduced and the PI(4)P level was significantly elevated in the its3-1 mutant. Fourth, overexpression of Its3 in wild-type cells resulted in a significant increase in the levels of PI(4,5)P2. Fifth, Its3 fused with GST showed in vitro PI(4)P5K activity.

**Synthetic Lethal Interaction between Ppb1 Calcineurin and Its3 PI(4)P5K—**The its3-1 mutant, which exhibits an immunosuppressant- and temperature-sensitive phenotype, carries a single base pair mutation in the PI(4)P5K gene that leads to the production of PI(4,5)P2 at approximately 10% of the normal level. Here, we have shown that the Δppb1 its3-1 double mutant is inviable. These findings suggest that strong genetic interactions between Ppb1 calcineurin and Its3 PI(4)P5K mutation provide support for a functional interaction between calcineurin and PI(4)P5K.

PI(4)P5K catalyzes the production of PI(4,5)P2, which serves as a precursor for diacylglycerol and inositol trisphosphate in signal transduction cascades. Recent studies suggest that there is another signaling mode controlled by PI(4,5)P2, and this novel cascade depends on intact PI(4,5)P2 rather than on the products of its hydrolysis (22). We first examined the possibility that the synthetic lethality is caused by the lowered level of the hydrolysis products. Protein kinase C, a target molecule of diacylglycerol, appeared not to be involved in this genetic interaction because the its3-1 mutant phenotypes were not rescued by overexpression of Pck1 or Pck2. In addition, osmotic stabilization, which suppressed loss of Pck1 function in budding yeast (25, 26), could not suppress both the temperature- and FK506-sensitive phenotypes (data not shown). It is also unlikely that a requirement for inositol triphosphate causes the synthetic lethality, because the plc1 null mutant is not sensitive to FK506 (data not shown). These results suggest that PI(4,5)P2 itself serves as an important regulator of cellular events related to calcineurin. It will be important in future studies to identify downstream components of PI(4,5)P2-mediated signaling pathway responsible for the overlapping function with calcineurin.

**its3-1 Mutant Showed Differential Defects in the Actin-containing Structure**—It has been suggested that PI(4,5)P2 regulates the actin cytoskeleton via its binding to various actin-binding proteins (27–32). In the present study, phalloidin staining of its3-1 mutant cells revealed a random distribution of cortical actin patches at restrictive temperature (Fig. 7A). Notably, disorganized actin cytoskeleton has been previously identified in budding yeast mss4 mutants (20, 21), suggesting...
that Mss4 and Its3 PI(4)P5K are involved in the organization of actin cytoskeleton in a similar regulatory mechanism. Interestingly, marked disorganization of actin patches in its3-1 mutants was also induced by the addition of FK506 (Fig. 7A), suggesting involvement of calcineurin in actin cytoskeleton organization. When its3-1 mutant cells were shifted to the restrictive temperature or to the media containing FK506, the actin ring formation was unaffected, even when actin patches were severely disorganized. Thus, its3-1 mutation is not defective in actin ring formation, but does seem to be defective in the actin patch formation. These results suggest that there may be some intrinsic difference in actin dynamics between actin patches and ring which renders actin patches more sensitive to loss of PI(4,5)P2.

PL(4)P5K Is Involved in Cytokinesis, and Membrane Localization Is Crucial for Its Function—As described in a previous study (4), disruption of Ppb1 calcineurin or incubation with FK506 resulted in the appearance of abnormally septated and branched cells. In the present study, we also showed that the addition of FK506 to the culture media increased the percentage of septated cells up to 45% 15 h after drug addition. From these observations, together with examination of microtubule distribution, it has been suggested that the progression of cytokinesis was delayed and the positioning of septation became abnormal in calcineurin null mutant (4). Likewise, its3-1 mutant cells had a septation index approximately twice that seen in wild-type cells at the permissive temperature, and showed a dramatic increase (from 24 to 50%) in the index upon shift to the restrictive temperature (Fig. 8B). These observations, together with the enriched localization of GFP-Its3 PI(4)P5K and GFP-PH at septum of dividing cells indicate that Its3 PL(4)P5K and PI(4,5)P2 are implicated for efficient cytokinesis. Furthermore, addition of FK506 to the culture media dramatically increased the septation index of its3-1 mutant cells, and the septation index reached nearly 100% only 6 h after drug addition. These results suggest that PL(4)P5K and calcineurin coordinately regulate cytokinesis.

The G281D mutation resulted in a drastic change in its subcellular distribution (Fig. 9B). The mutant Its3 was no longer localized to plasma membrane, indicating that proper localization of Its3 protein is deteriorated in the Its3-1 mutant. This mislocalization may lead to the reduced level of PI(4,5)P2 in the its3-1 mutant, presumably due to substrate unavailability or inaccessibility. Based on these results, we propose that a membrane localization of Its3 is the crucial event in cytokinesis.

Potential Mechanism for PL(4)P5K Regulation—Recently, Vancurova et al. (33) purified and characterized PL(4)P5K from the plasma membranes of S. pombe. They also provided evidence that PL(4)P5K is phosphorylated and inactivated by Cki1, the S. pombe homologue of casein kinase I. Phosphorylation by Cki1 in vitro decreased the activity of PL(4)P5K, and overexpression of Cki1 in S. pombe resulted in a reduced synthesis of PI(4,5)P2 and in a lower activity of PL(4)P5K associated with the plasma membrane. From the results they concluded that PL(4)P5K is a target of Cki1 in S. pombe and that Cki1 is involved in the regulation of PI(4,5)P2 synthesis by phosphorylating and inactivating PL(4)P5K. Robinson et al. (34) have reported that Yck2 casein kinase 1 protein kinase shows cell cycle-specific localization within the plasma membrane and plays distinct roles in morphogenesis and cytokinesis in budding yeast. Taken together, our present results showing the genetic interaction between calcineurin and PL(4)P5K raise the intriguing possibility that Ppb1 calcineurin may directly or indirectly activate Its3 PL(4)P5K through dephosphorylation.

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