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Rho2 Is a Target of the Farnesyltransferase Cpp1 and Acts Upstream of Pmk1 Mitogen-activated Protein Kinase Signaling in Fission Yeast

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We have previously demonstrated that knockout of the calcineurin gene or inhibition of calcineurin activity by immunosuppressants resulted in hypersensitivity to Cl^- in fission yeast. We also demonstrated that knockout of the components of the Pmk1 mitogen-activated protein kinase (MAPK) pathway, such as Pmk1 or Pek1 complemented the hypersensitivity to Cl^-. Using this interaction between calcineurin and Pmk1 MAPK, here we developed a genetic screen that aims to identify new regulators of the Pmk1 signaling and isolated *vic* (*v*iable in the presence of *i*mmunosuppressant and *c*hloride ion) mutants. One of the mutants, *vic1-1*, carried a missense mutation in the *cpp1*$^+$ gene encoding a β subunit of the protein farnesyltransferase, which caused an amino acid substitution of aspartate 155 of Cpp1 to asparagine (Cpp1^{D155N}). Analysis of the mutant strain revealed that Rho2 is a novel target of Cpp1. Moreover, Cpp1 and Rho2 act upstream of Pck2–Pmk1 MAPK signaling pathway, thereby resulting in the *vic* phenotype upon their mutations. Interestingly, compared with other substrates of Cpp1, defects of Rho2 function were more phenotypically manifested by the Cpp1^{D155N} mutation. Together, our results demonstrate that Cpp1 is a key component of the Pck2–Pmk1 signaling through the spatial control of the small GTPase Rho2.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway is one of the most important intracellular signaling that plays a crucial role in cell proliferation, cell differentiation, and cell cycle regulation (Nishida and Gotoh, 1993; Marshall, 1994; Herskowitz, 1995; Levin and Errede, 1995). The Pmk1 MAPK, a homologue of the mammalian extracellular signal-regulated kinase (ERK)/MAPK, regulates cell morphology and cell integrity in fission yeast *Schizosaccharomyces pombe* (Toda *et al*., 1996; Zaitsevskaya-Carter and Cooper, 1997). A functional connection between the Pmk1 pathway and the protein kinase C homologues Pck1 and Pck2 has been suggested, and differential roles of Pck1 and Pck2 in the regulation of cell integrity also have been suggested; however, it is unclear whether these protein kinase C homologues act upstream of the Pmk1 pathway or synergistically regulate independent aspects of cell integrity (Toda *et al*., 1996; Sengar *et al*., 1997; Arellano *et al*., 1999; Calonge *et al*., 2000).

We have previously demonstrated that knockout of the fission yeast calcineurin gene *ppb1*$^+$ or inhibition of calcineurin activity by immunosuppressants results in hypersensitivity to Cl^-, and that calcineurin and Pmk1 MAPK play antagonistic roles in Cl^- homeostasis (Sugiura *et al*., 1998). Based on this genetic interaction between calcineurin and Pmk1 MAPK, we screened for multicopy suppressors of the Cl^--hypersensitive phenotype of the calcineurin knockout and identified genes encoding an MAPK phosphatase Pmp1 (Sugiura *et al*., 1998); MAPK kinase (MAPKK) Pek1 (Sugiura *et al*., 1999), and a novel KH-type RNA-binding protein Rnc1 that binds and stabilizes Pmp1 mRNA (Sugiura *et al*., 2003).

We also demonstrated that knockout of the components of the Pmk1 MAPK pathway, such as Pmk1 or Pek1 complemented the Cl^--hypersensitive phenotype of calcineurin knockout. Notably, these knockout strains are viable in the presence of immunosuppressant FK506 and high concentrations of $MgCl_2$, whereas the wild-type cells are inviable in the same condition (Sugiura *et al*., 1998, 1999). These results prompted us to perform a novel genetic screen to isolate *vic* (*v*iable in the presence of *i*mmunosuppressant and *c*hloride ion) mutants, aiming to identify novel components of the Pmk1 MAPK pathway.

We have identified a *vic1-1/cpp1-v1* mutant, in addition to the mutation allele in the known components of the Pmk1 MAPK pathway, including *pmk1*$^+$ (MAPK), *pek1*$^+$ (MAPKK), *mkh1*$^+$ (MAPKK kinase, MAPKKK) as well as *pck2*$^+$ (protein kinase C). The *cpp1*$^+$ gene encodes a β subunit of the farnesyltransferase (FTase) that is highly conserved through evolution.

In mammals, many proteins, including Ras family small GTPases, nuclear lamins A and B, transducin, rhodopsin kinase, and a peroxisomal protein termed PxF, have been reported as substrates for FTase (Glomset and Farnsworth, 1994; Zhang and Casey, 1996; Casey and Seabra, 1996; Gelb, 1997; Mumby, 1997). There is widespread interest in FTase because Ras proteins are modified by FTase and such a modification is critical for the oncogenic transformation (Hancock *et al*., 1989; Jackson *et al*., 1990; Kato *et al*., 1992).

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Table 1. *S. pombe* strains used in this study

Strain	Genotype	Reference
HM123	*h⁻ leu1-32*	Our stock
HM528	*h⁺ his2*	Our stock
KP928	*h⁺ his2 leu1 ura4-D18*	Our stock
KP119	*h⁺ leu1-32 ura4-D18 ppb1::ura4⁺*	Our stock
KP208	*h⁻ leu1-32 ura4-D18 pmk1::ura4⁺*	Our stock
KP330	*h⁻ leu1-32 pck2::LEU2*	Our stock
KP452	*h⁻ leu1-32 ura4-D18 mkh1::ura4⁺*	Our stock
KP454	*h⁻ leu1-32 ura4-D18 pek1::ura4⁺*	Our stock
KP251	*h⁻ leu1-32 ura4-D18 ppb1::ura4⁺ pmk1::ura4⁺*	Our stock
KP327	*h⁻ leu1-32 ura4-D18 ppb1::ura4⁺ pck2::LEU2*	Our stock
KP468	*h⁻ leu1-32 ura4-D18 ppb1::ura4⁺ mkh1::ura4⁺*	Our stock
KP550	*h⁻ leu1-32 ura4-D18 ppb1::ura4⁺ pek1::LEU2*	Our stock
KP754	*h⁻ leu1-32 cpp1-v1*	This study
KP1855	*h⁻ leu1-32 ura4-D18 cpp1::ura4⁺*	This study
KP2436	*h⁻ leu1-32 rho2::KanMX$_6$*	This study
KP2451	*h⁺ his2 leu1-32 ura4-D18 rho2::KanMX$_6$*	This study
KP2174	*h⁻ leu1-32 ura4-D18 ras1::ura4⁺*	This study
KP1375	*h⁻ leu1-32 ura4-D18 rho3::ura4⁺*	This study
KP1825	*h⁻ leu1-32 ura4-D18 pck1::ura4⁺*	Our stock
KP2462	*h⁻ leu1-32 ura4-D18 mkh1::ura4⁺ rho2::KanMX$_6$*	This study
KP2465	*h⁻ leu1-32 ura4-D18 pck1::ura4⁺ rho2::KanMX$_6$*	This study
KP2466	*h⁻ leu1-32 ura4-D18 pmk1::ura4⁺ rho2::KanMX$_6$*	This study
KP2468	*h⁻ leu1-32 ura4-D18 pck2::LEU2 rho2::KanMX$_6$*	This study

Several FTase inhibitors (FTIs) have been in clinical trials for the treatment of cancer (Johnston, 2001; Ayral-Kaloustian and Salaski, 2002). Also, recent studies have reported that abnormal persistence of the farnesyl modifications on the nuclear prelamin A has been implicated in the pathogenesis of Hutchinson–Gilford progeria syndrome, a devastating premature aging disease (Pollex and Hegele, 2004). Thus, FTIs represent a possible therapeutic option for cancer as well as for individuals with Hutchinson–Gilford progeria syndrome.

In budding yeast *Saccharomyces cerevisiae*, α-factor mating pheromone and Ras2 involved in the cAMP pathway have been identified as substrates of FTase (Goodman *et al*., 1990; He *et al*., 1991). In fission yeast *Schizosaccharomyces pombe*, FTase Cpp1 has been reported to play a critical role in sexual differentiation and morphogenesis through Ras1 (Yang *et al*., 2000) and also has been reported to play a role in cell cycle progression through Rheb (Yang *et al*., 2001).

Here, we show that the small GTPase Rho2 is a novel target of Cpp1 and that Cpp1 and Rho2 act upstream of Pck2-Pmk1 MAPK cell integrity signaling pathway.

MATERIALS AND METHODS

Strains, Media, and Genetic and Molecular Biology Methods

The *S. pombe* strains used in this study are listed in Table 1. The complete medium yeast extract-peptone-dextrose (YPD) and the minimal medium Edinburgh minimal medium (EMM) have been described previously (Toda *et al*., 1996). Standard genetic and recombinant DNA methods (Moreno *et al*., 1991) were used except where noted. FK506 was provided by Fujisawa Pharmaceutical Co. (Osaka, Japan).

Table 2. *S. pombe* primers used in this study

Gene	Primer
Rho1 sense	5′-CGG GAT CCC ATA TGG CGA CAG AAC TTC GC-3′
Rho1 antisense	5′-CGG GAT CCT TAC AAC AAG ATA CAA CGC-3′
Rho2 sense	5′-CGC GGA TCC CAT ATG TTG CAA TCT CAA CC-3′
Rho2 antisense	5′-CGC GGA TCC TTA TGA AAT GAT GCA GC-3′
Rho2CIIL antisense	5′-CGC GGA TCC TTA TAA AAT GAT GCA GC-3′
Rho2SIIS antisense	5′-CGC GGA TCC TTA TGA AAT GAT GGA GCA TTT TGT AG-3′
Rho3 sense	5′-CGC GGA TCC CAT ATG TCA AGC TGT TTC GG-3′
Rho3 antisense	5′-CGG GAT CCT CAA GCA ATG ATA CAT CCG GTA-3′
Ras1 sense	5′-CGG GAT CCC ATA TGA GGG TAA GTC TAA GCA ATG-3′
Ras1 antisense	5′-CGG GAT CCC TAA CAT ATA ACA CAA CAT TTA G-3′

Isolation of vic1-1/cpp1-v1 Mutant

The *vic1-1/cpp1-v1* mutant was isolated in a screen of cells that had been mutagenized with nitrosoguanidine as described previously (Zhang *et al*., 2000). Mutants were spread on YPD plates to give ~1000 cells/plate, and the plates were incubated at 27°C for 4 d. The plates were then replica-plated at 27°C onto plates containing 0.5 μg/ml FK506 and 0.2 M $MgCl_2$. Mutants that grew in the plates were selected and designated as *vic* mutants. The original mutants isolated were backcrossed three times to wild-type strains HM123 and HM528.

Cloning and Knockout of the cpp1⁺ Gene

To clone the *vic1⁺* gene, the temperature sensitivity of *vic1-1* mutants (KP754) was used. The *vic1-1* mutants were grown at 27°C and transformed with an *S. pombe* genomic DNA library constructed in the vector pDB248 (Beach *et al*., 1982). Leu⁺ transformants were replica-plated onto YPD plates at 36°C, and the plasmid DNA was recovered from transformants that showed plasmid-dependent rescue. These plasmids complemented both the temperature sensitivity and *vic* phenotype of the *vic1-1* mutant. By DNA sequencing, the suppressing plasmids were identified to contain the *cpp1⁺* gene (SPAC17G6.04c). To investigate the relationship between the cloned *cpp1⁺* gene and *vic1-1* mutant, linkage analysis was performed as follows. The entire *cpp1⁺* gene was subcloned into the pUC-derived plasmid containing *S. cerevisiae LEU2* gene and integrated by homologous recombination into the genome of the wild-type strain HM123. The integrant was mated with the *vic1-1* mutant. The resulting diploid was sporulated, and tetrads were dissected. In total, 30 tetrads were dissected. In all cases, only parental ditype tetrads were found, indicating allelism between the *cpp1⁺* gene and the *vic1-1* mutation (our unpublished data).

To knockout *cpp1⁺* gene, a one-step gene disruption by homologous recombination was performed as described previously (Rothstein, 1983). The *cpp1::ura4⁺* disruption was constructed as follows. The *Bam*HI fragment containing the *cpp1⁺* gene was subcloned into the *Bam*HI site of BlueScriptSK(+) (Stratagene, La Jolla, CA). Then, a *Pst*I/*Sma*I fragment containing the *ura4⁺* gene was inserted into the *Nsi*I/*Eco*RV site of the previous construct. The fragment containing the disrupted *cpp1⁺* gene was transformed into haploid cells. Stable integrants were selected on medium lacking uracil, and knockout of the gene was checked by genomic Southern hybridization and tetrad analysis (our unpublished data).

Cloning and Tagging of the rho1⁺, rho2⁺, rho3⁺, and ras1⁺ Genes

The *rho1⁺*, *rho2⁺*, *rho3⁺*, and *ras1⁺* genes were amplified by PCR with the genomic DNA of wild-type cells as a template. The primers used were summarized in Table 2. The amplified products containing theses genes were digested with *Bam*HI, and the resulting fragments were subcloned into BlueScriptSK(+).

For ectopic expression of proteins, we used the thiamine-repressible *nmt1* promoter (Maundrell, 1993). Expression was repressed by the addition of 4 μM thiamine to EMM and was induced by washing and then incubating the cells in EMM lacking thiamine. To express green fluorescent protein (GFP)-Rho1, Rho2, Rho3, or Ras1, these genes were tagged at its N terminus with

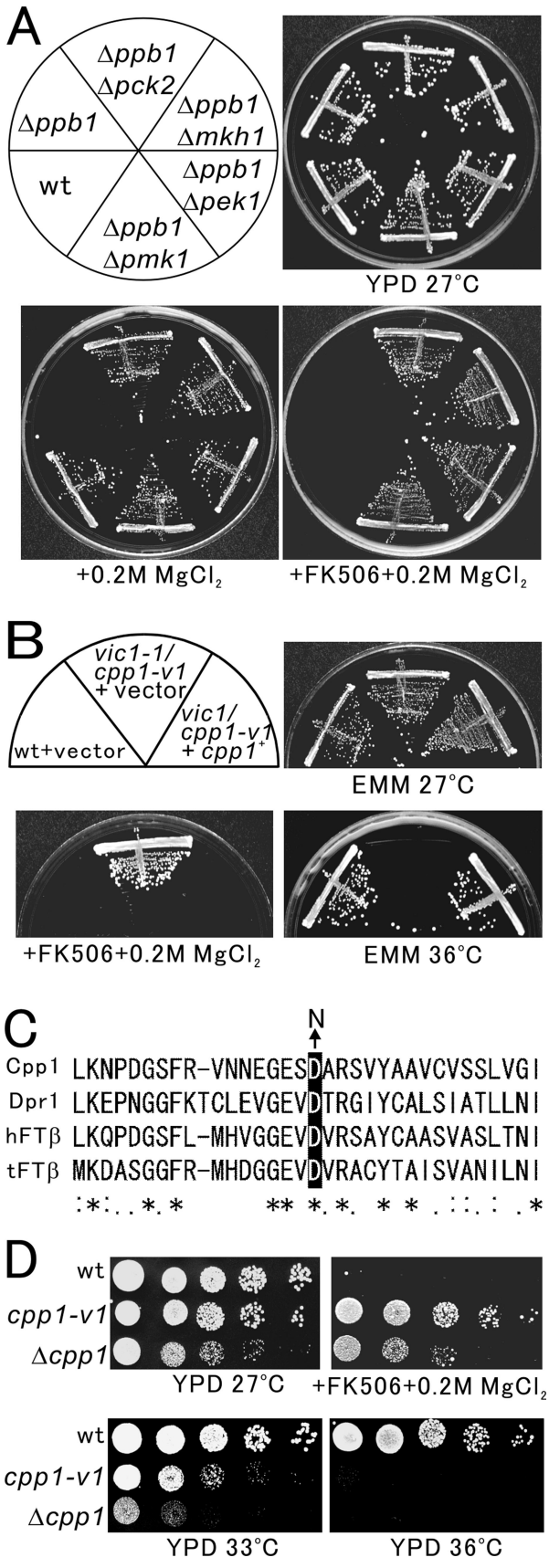

Figure 1. A mutation in the *vic1*$^+$*/cpp1*$^+$ gene causes *vic* and temperature-sensitive phenotypes. (A) Knockout of the components of the Pmk1 MAPK pathway (Δ*mkh1*, Δ*pek1*, or Δ*pmk1*) or the protein kinase C (Δ*pck2*) suppressed the Cl^- hypersensitivity caused by calcineurin knockout (Δ*ppb1*). The cells as shown were streaked onto the plates as indicated, then incubated for 4 d at 27°C. (B) The *vic* and temperature-sensitive phenotypes of *vic1-1/cpp1-v1* mutant cells. Cells transformed with the multicopy vector pDB248 or the vector containing the *cpp1*$^+$ gene were streaked onto the plates as indicated and then incubated for 4 d at 27°C or 3 d at 36°C, respectively. (C) Partial alignment of protein sequences of *S. pombe* Cpp1 with related proteins from *S. cerevisiae* (Dpr1p), human (hFTβ), and tomato (tFTβ). Sequence alignment was performed using the ClustalW program. Asterisks indicate identical amino acids, and colons indicate similar amino acids. Arrow indicates the mutation site in the aspartic acid 155 of Cpp1, which when mutated to asparagine resulted in *vic* and temperature-sensitive phenotypes in Cpp1. (D) The phenotypes of *cpp1-v1* and Δ*cpp1*. Wild-type cells, *cpp1-v1* mutant, and Δ*cpp1* cells were dropped onto the plates as indicated and then incubated for 4 d at 27°C or 3 d at 33 and 36°C, respectively.

GFP carrying the S65T mutation. Rho2CIIL and Rho2SIIS were made in the same way except that the antisense primers were specifically designed for amplifying Rho2CIIL and Rho2SIIS. Similarly, Rho2, Ras1, or Rho3 was tagged at its N terminus with FLAG. These constructs were confirmed to be fully functional as their expression complemented the phenotypes associated with Δ*ras1*, Δ*rho2*, and Δ*rho3*, respectively (our unpublished data).

Microscopy and Miscellaneous Methods

Methods in light microscopy, such as fluorescence microscopy and differential interference contrast microscopy, were performed as described previously (Kita *et al.*, 2004). Cell extract preparation and immunoblot analysis were performed as described previously (Sio *et al.*, 2005).

RESULTS

Isolation of vic Mutants

To identify new components of the protein kinase C-Pmk1 MAPK signaling pathway, we performed a genetic screen in *S. pombe* based on the functional interaction that calcineurin and Pmk1 MAPK play antagonistic roles in Cl^- homeostasis (Sugiura *et al.*, 1998). On inhibition of the Pmk1 MAPK signaling, the Cl^--hypersensitive phenotype of calcineurin knockout was complemented, i.e., the knockout of *pmk1*$^+$, *pek1*$^+$, *mkh1*$^+$, or *pck2*$^+$ all complemented the Cl^--hypersensitive phenotype of calcineurin knockout (Figure 1A, +0.2 M $MgCl_2$). The inhibition of calcineurin signaling is also achieved by the immunosuppressant FK506, a specific inhibitor of calcineurin, because wild-type cells failed to grow in the presence of FK506 and 0.2 M $MgCl_2$ (Figure 1A, wt, +FK506 + 0.2 M $MgCl_2$). Consistently, knockout of the components of protein kinase C-MAPK signaling makes cells grow in the presence of FK506 and $MgCl_2$ (Figure 1A, +FK506 + 0.2 M $MgCl_2$). We therefore hypothesized that if we isolate mutants that can grow in the presence of FK506 and 0.2 M $MgCl_2$, the genes responsible for the mutation are expected to function in the protein kinase C–Pmk1 MAPK signaling. These may include mutations in the unknown factor downstream of Pmk1 or in novel factors required for the activation or function of Pmk1 MAPK. We then performed the isolation of the mutants that are viable in the presence of immunosuppressant and chloride ion; hence, we named these as *vic* mutants. By this genetic approach, we isolated several novel mutants, in addition to *mkh1*, *pek1*, *pmk1*, and *pck2* mutants (our unpublished data). Here, we focus on the characterization of the *vic1-1* mutant.

As shown in Figure 1B, the *vic1-1* mutants grew well in the presence of FK506 and 0.2 M $MgCl_2$ at 27°C wherein wild-type cells failed to grow (Figure 1B, + FK506 + 0.2 M $MgCl_2$). However, *vic1-1* mutant cells could not grow at 36°C whereas wild-type cells grew normally (Figure 1B,

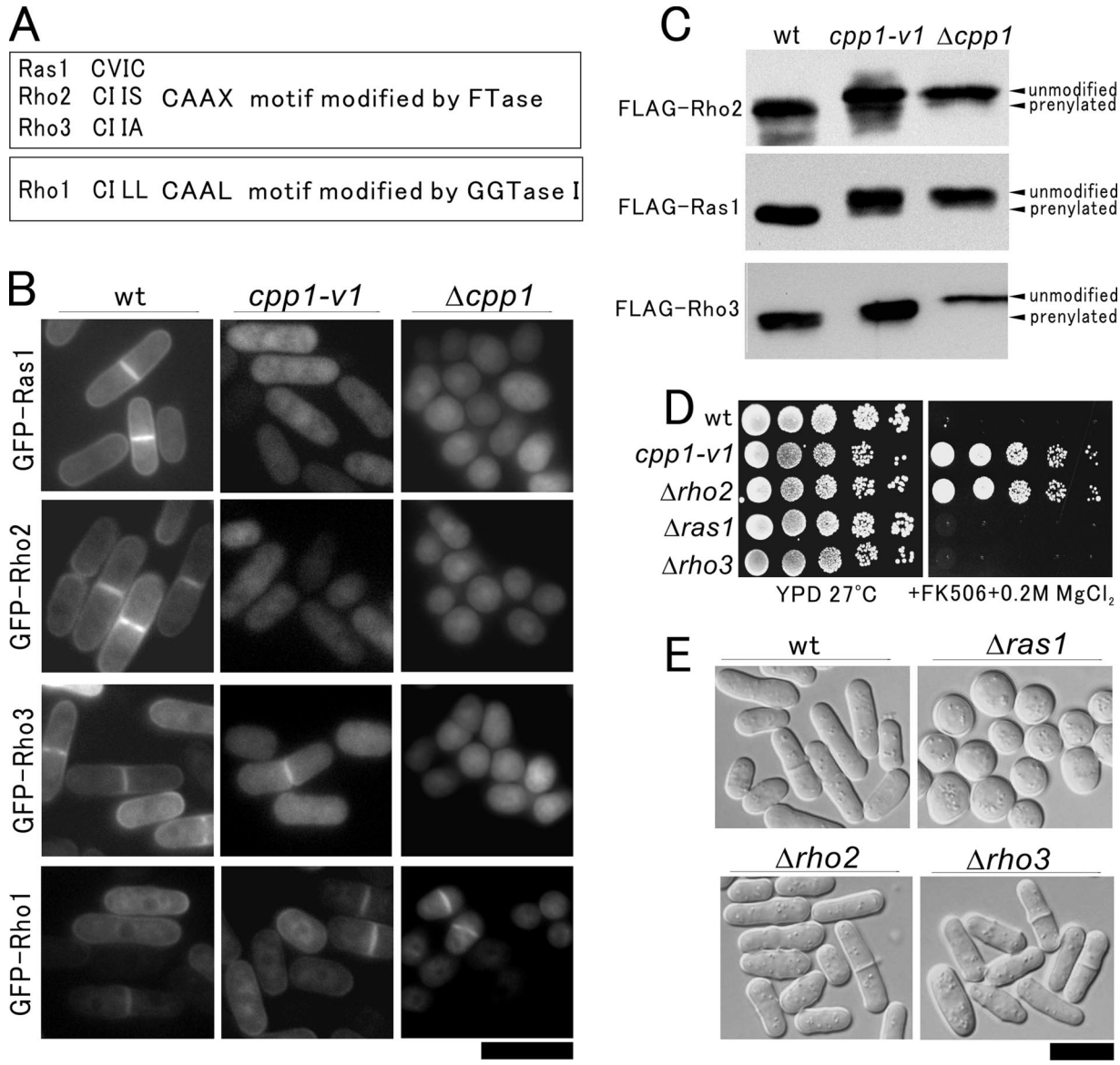

Figure 2. Rho2 is the target of Cpp1 responsible for *vic* phenotype. (A) The C-terminal sequence of Ras1, Rho2, Rho3, and Rho1 in fission yeast. Ras1, Rho2, and Rho3 contain CAAX motif modified by FTase, and Rho1 contains CAAL motif modified by GGTase I. (B) Intracellular localization of various small GTPases in wild-type, *cpp1-v1* mutant, and Δ*cpp1* cells. The GFP-fused Ras1, Rho2, Rho3, or Rho1 was transformed into wild-type, *cpp1-v1* mutant, or Δ*cpp1* cells. The transformants were grown to early log phase in EMM containing 4 μM thiamine and were examined under the fluorescence microscope. Bar, 10 μm. (C) Defective modification of Rho2, Ras1, and Rho3 in *cpp1-v1* mutant and Δ*cpp1* cells. The FLAG-fused Rho2, Ras1, or Rho3 was transformed into wild-type or *cpp1-v1* mutant cells. The transformants were grown in EMM in the absence of thiamine at 27°C for 20 h, and then the cells were collected and lysed. Cell extracts were subjected to 15% polyacrylamide gel and immunoblotted using anti-FLAG antibody. Prenylated and unmodified forms of Rho2, Ras1, or Rho3 are indicated. (D) Δ*rho2* cells showed *vic* phenotype. The cells as shown were dropped onto the plates as indicated and then incubated for 4 d at 27°C. (E) Morphology of Δ*rho2*, Δ*ras1*, and Δ*rho3* cells. Cells were grown to mid-log phase in YPD at 27°C and were observed under the differential interference contrast microscopy. Bar, 10 μm.

EMM 36°C). As predicted, the *vic1-1*Δ*ppb1* double mutant cells were able to grow in the presence of 0.2 M $MgCl_2$ (our unpublished data), indicating that the *vic1-1* mutation suppresses the Cl^- sensitivity of calcineurin knockout and suggesting that the gene product is involved in the regulation of the Pmk1 MAPK pathway.

The vic1-1/cpp1-v1 Is an Allele of the cpp1⁺ Gene That Encodes a β Subunit of Protein FTase

The *vic1⁺* gene was cloned by complementation of the temperature-sensitive growth defect of *vic1-1* mutant cells (Figure 1B, EMM 36°C, +*cpp1⁺*). The *vic1⁺* gene also complemented the *vic* phenotype of *vic1-1* mutant cells, because these cells failed to grow in the presence of FK506 and 0.2 M $MgCl_2$ at 27°C when the *vic1⁺* gene was introduced (Figure 1B, + FK506 + 0.2 M $MgCl_2$, + *cpp1⁺*). Nucleotide sequencing of the cloned DNA fragment revealed that the *vic1⁺* gene is identical to the *cpp1⁺* gene (SPAC17G6.04c), which encodes the β subunit of the protein FTase of 382 amino acids that is highly similar to the human hFTβ and *S. cerevisiae* Dpr1p. Linkage analysis was performed (see *Materials and Methods*), and results indicated the allelism between the

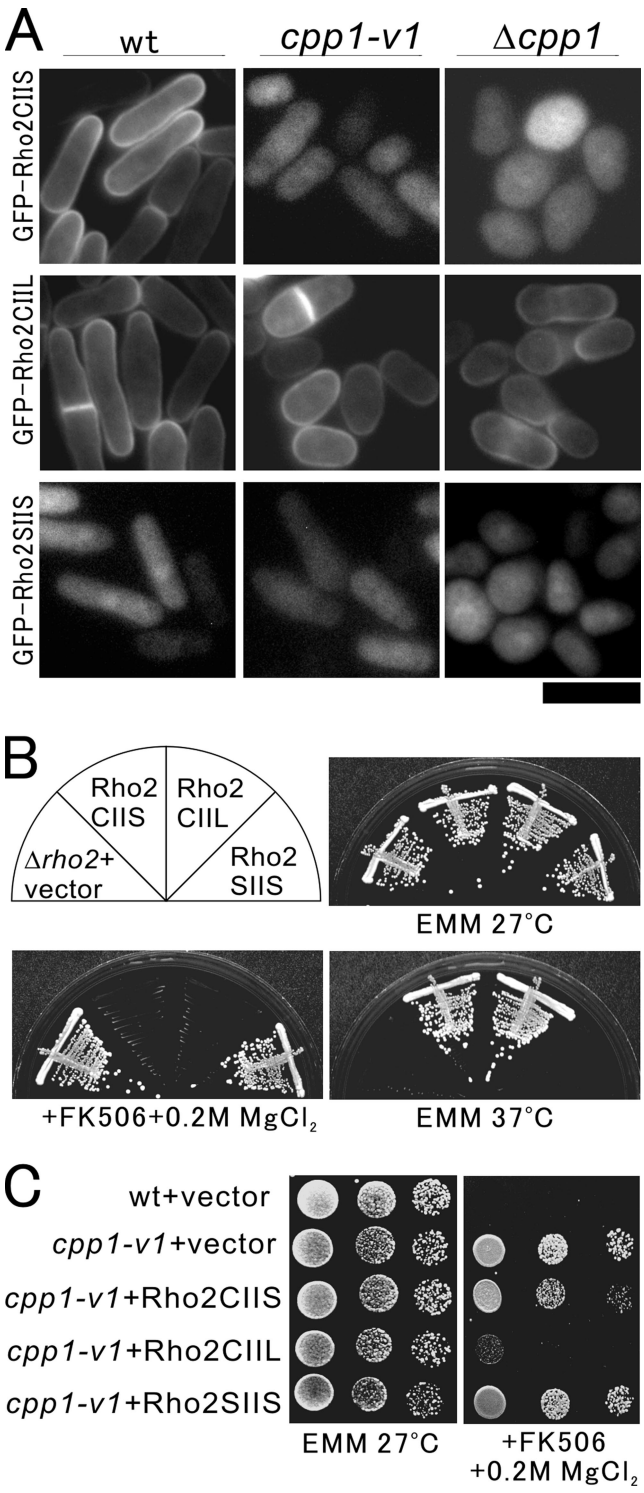

Figure 3. Farnesylation by Cpp1 is essential for the proper membrane localization and function of Rho2. (A) Intracellular localization of GFP-fused wild-type Rho2 (Rho2CIIS), the geranylgeranylated mutant version of Rho2 (Rho2CIIL), or the prenylation-defective mutant version of Rho2 (Rho2SIIS) in wild-type, *cpp1-v1* mutant or Δ*cpp1* cells. The GFP-Rho2CIIS, GFP-Rho2CIIL, or GFP-Rho2SIIS was transformed into wild-type, *cpp1-v1* mutant, or Δ*cpp1* cells. The transformants were cultured and examined as in Figure 2B. (B) The plasma membrane localization is essential for Rho2 function. Wild-type Rho2 (Rho2CIIS) and the geranylgeranylated mutant version of Rho2 (Rho2CIIL), but not the prenylation-defective mutant version of Rho2 (Rho2SIIS), suppressed the phenotypes. The Δ*rho2* cells harboring the control vector, Rho2CIIS, Rho2CIIL, or Rho2SIIS were streaked onto the plates as indicated and then incubated for 4 d at 27°C or 3 d at 37°C, respectively. (C) Geranylgeranylated mutant version of Rho2 (Rho2CIIL) suppressed the *vic* phenotype of *cpp1-v1* mutant cells. The *cpp1-v1* mutant cells harboring the control vector, Rho2CIIS, Rho2CIIL, or Rho2SIIS were dropped onto the plates as indicated and then incubated for 4 d at 27°C.

cpp1$^+$ gene and the *vic1-1* mutation. We therefore renamed *vic1-1* as *cpp1-v1*.

To identify the mutation site in the *cpp1-v1* allele, the genomic DNA from *cpp1-v1* mutant cells was isolated, and the full-length coding region of the *cpp1-v1* gene was sequenced. The G-to-A nucleotide substitution caused a highly conserved aspartic acid to be altered to an asparagine residue at the amino acid position 155 (Figure 1C, black arrow). We therefore refer to the protein product of the *cpp1-v1* gene as Cpp1^{D155N}.

We constructed Cpp1 knockout (see *Materials and Methods*), and Δ*cpp1* cells displayed a slow growth rate even at the permissive temperature of 27°C as reported by Yang *et al*. (2000). In contrast, *cpp1-v1* mutant cells grew normally at the permissive temperature of 27°C (Figure 1D, YPD 27°C). The Δ*cpp1* cells also grew in the presence of FK506 and 0.2 M $MgCl_2$; however, the growth rate was significantly slower compared with that of the *cpp1-v1* mutant cells (Figure 1D, + FK506 + 0.2 M $MgCl_2$). Thus, in contrast to the *cpp1-v1* mutant cells, the Δ*cpp1* cells did not display a markedly clear *vic* phenotype. Notably, the degree of the temperature-sensitive growth defect was more severe in Δ*cpp1* cells, because Δ*cpp1* cells failed to grow even at 33°C, whereas *cpp1-v1* mutant cells grew well (Figure 1D, YPD 33°C). Thus, the *cpp1-v1* mutant and Δ*cpp1* cells displayed similar but distinct phenotypes, suggesting that FTase activity is partially deficient in *cpp1-v1* mutant cells. Consistently, overexpression of Cpp1^{D155N} suppressed the temperature-sensitive phenotype of Δ*cpp1* cells (our unpublished data).

Rho2 Is the Target of Cpp1 Responsible for vic Phenotype

FTase catalyzes the posttranslational modification of Ras and other Ras family proteins. The target proteins have a consensus CAAX motif (where C represents cysteine, A represents aliphatic amino acid, and X preferentially represents methionine, cysteine, serine, alanine, or glutamine) at the C terminus (Maltese, 1990; Cox and Der, 1992; Magee *et al*., 1992).

In fission yeast, Ras1 (a known target of Cpp1), Rho2, and Rho3 small GTPases contain CAAX motif modified by FTase (Figure 2A, FTase), and Rho1 small GTPase contains CAAL motif modified by geranylgeranyltransferase I (Figure 2A, GGTase I). We examined the intracellular localization of Ras1, Rho2, Rho3, and Rho1 in wild-type, *cpp1-v1* mutant, or Δ*cpp1* cells, based on the assumption that a mutation in the *cpp1*$^+$ gene should cause a defect in the farnesylation of the target small GTPases, thereby leading to a defect in their intracellular localization.

As shown in Figure 2B, in wild-type cells all these small GTPases predominantly localized to the plasma membrane. However, in *cpp1-v1* mutant cells and Δ*cpp1* cells, the plasma membrane localization of Ras1 or Rho2 was not clearly observed. In contrast, in *cpp1-v1* mutant cells the plasma membrane localization of Rho3 was clearly observed, and in Δ*cpp1* cells it was abolished. Note that the localization of CAAL-ending GTPase Rho1 was not affected in either *cpp1-v1* mutant or Δ*cpp1* cells. Δ*cpp1* cells displayed the

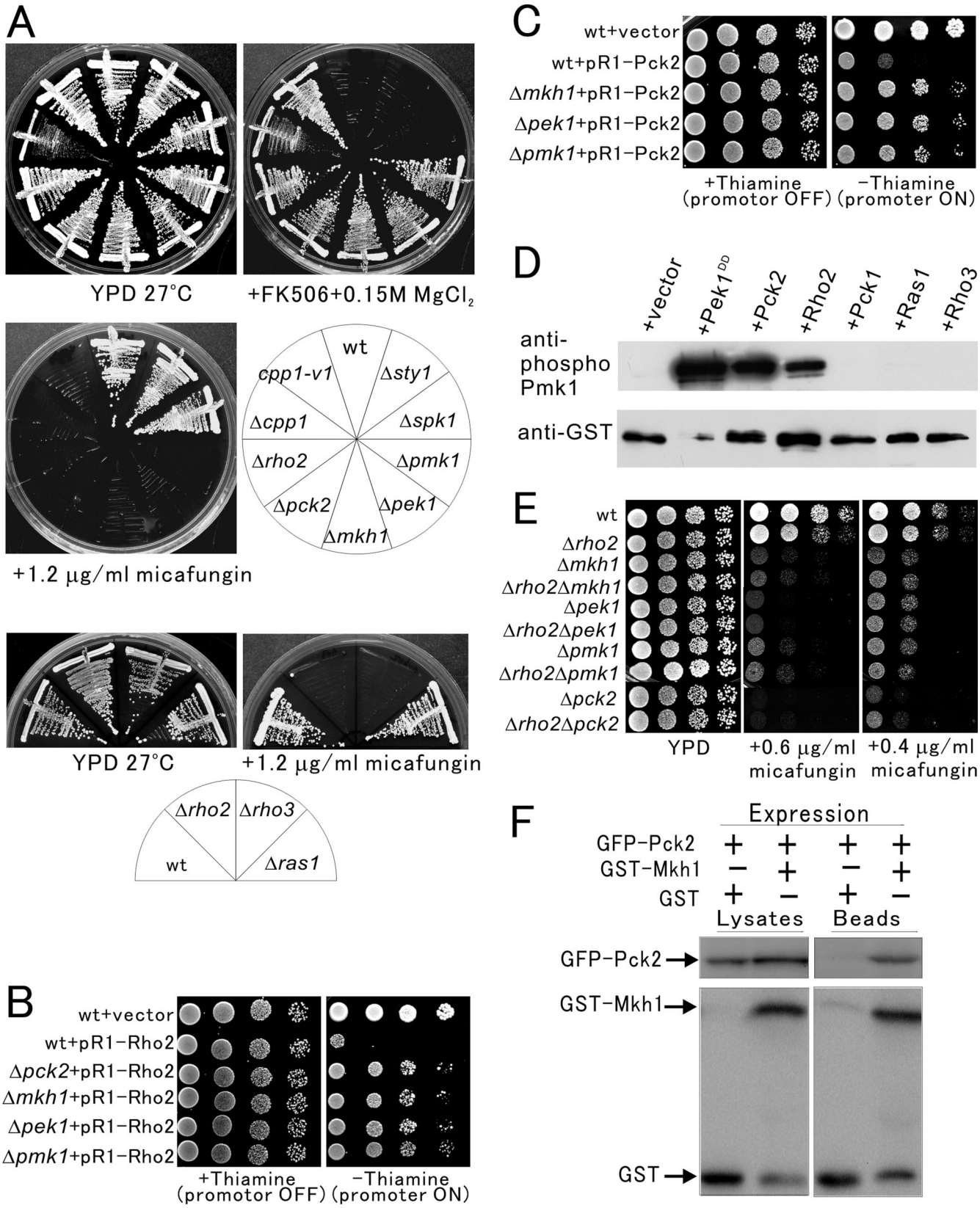

Figure 4. Rho2 functions upstream of the Pck2–Pmk1 MAPK pathway. (A) Knockout of the *rho2*$^+$, *pck2*$^+$, and the component of the Pmk1 MAPK pathway exhibited *vic* phenotype and micafungin sensitivity. The cells as indicated were streaked onto the indicated plates and then incubated for 4 d at 27°C. (B) Overexpression of Rho2 showed toxicity to wild-type cells, but not to Δ*pck2*, Δ*mkh1*, Δ*pek1*, or Δ*pmk1* cells. The cells as indicated transformed with pREP1-GFP-Rho2 were dropped onto EMM plate with or without 4 μM thiamine and then incubated for 4 d at 27°C. (C) Overexpression of Pck2 showed toxicity to wild-type cells but not to Δ*mkh1*, Δ*pek1*, or Δ*pmk1* cells. The cells as shown

round cell shape as reported previously owing to the defective farnesylation of Ras1 (Yang *et al.*, 2000, 2001) (Figure 2B, Δ*cpp1*). In clear contrast, the *cpp1-v1* mutant cells displayed cylindrical cell shape (Figure 2B, *cpp1-v1*), suggesting that farnesylation of Ras1 in *cpp1-v1* mutant cells is not considerably defective so as to affect the cell shape. These results suggest that CAAX-ending Ras1, Rho2, and Rho3 are targets of Cpp1 in fission yeast. More importantly, the partial loss of the farnesyltransferease activity caused by the *cpp1-v1* mutation differentially impacts the function of these small GTPases.

To investigate whether there is a defect in the farnesylation of these three substrates in *cpp1-v1* mutant cells, we constructed FLAG-tagged Rho2, Ras1, and Rho3, and we expressed these in wild-type, *cpp1-v1* mutant, and Δ*cpp1* cells. As shown in Figure 2C, Rho2, Ras1, and Rho3 extracted from Δ*cpp1* cells migrated slower (unmodified), compared with those from wild-type cells (prenylated). Rho2, Ras1, and Rho3 extracted from the *cpp1-v1* mutant cells exhibited significantly different patterns of migration from the wild-type or Δ*cpp1* cells (Figure 2C). Notably, in *cpp1-v1* mutant cells, prenylation of Ras1 and Rho2 was severely impaired, whereas that of Rho3 was modestly impaired (Figure 2C).

Because our intention is to identify components of the protein kinase C–Pmk1 MAPK signaling by isolating *vic* mutants, we next investigated whether the knockout cells of the three targets exhibit *vic* phenotype similar to that of *cpp1-v1/vic1-1* cells. Results clearly showed that Δ*rho2* cells, but not Δ*ras1* or Δ*rho3* cells, were able to grow in the presence of FK506 and 0.2M $MgCl_2$ (Figure 2D). These results raise the possibility that Cpp1-Rho2 is involved in the protein kinase C-Pmk1 MAPK signaling. We also examined the morphology of Δ*rho2*, Δ*ras1*, and Δ*rho3* cells to understand the differential roles of different small GTPases in cellular morphogenesis. Δ*ras1* cells displayed the round cell shape similar to that of Δ*cpp1* cells, whereas neither Δ*rho2* nor Δ*rho3* cells showed obvious defect in morphology (Figure 2E).

Farnesylation by Cpp1 Is Essential for the Membrane Localization and Function of Rho2

To examine whether the membrane localization of Rho2 is critical for its function, we created various mutant versions of Rho2 at its C terminus. As shown in Figure 2B and here, wild-type Rho2 (Rho2CIIS) localized to the plasma membrane in wild-type cells, but not in *cpp1-v1* mutant or Δ*cpp1* cells (Figure 3A, GFP-Rho2CIIS). In contrast, the geranylgeranylated mutant form of Rho2 (Rho2CIIL), which bypasses the farnesylation requirement, localized to the plasma membrane both in wild-type cells and in *cpp1* mutant cells, indicating that geranylgeranylation allows Rho2 to associate with the membrane even in the absence of farnesylation (Figure 3A, GFP-Rho2CIIL). However, the prenylation-defective Rho2 (Rho2SIIS), wherein the cysteine in the CAAX-motif was replaced with serine, failed to localize to the plasma membrane even in wild-type cells (Figure 3A, GFP-Rho2SIIS).

Notably, Rho2CIIL, which localized to the plasma membrane, fully suppressed the temperature-sensitive and *vic* phenotypes of Δ*rho2* cells, suggesting that geranylgeranylated Rho2, which bypasses farnesylation, can function as well as farnesylated Rho2 (Figure 3B, +Rho2CIIL). On the other hand, Rho2SIIS, which failed to localize to the plasma membrane, could not suppress the phenotypes (Figure 3B, +Rho2CIIS), suggesting that the plasma membrane localization of Rho2 is critical for its function.

To address whether the *vic* phenotype of *cpp1-v1* mutant cells is because of the defective farnesylation of Rho2, we overexpressed these mutant forms of Rho2 in *cpp1-v1* mutant cells. As expected, only Rho2CIIL, which can localize to the plasma membrane even in *cpp1-v1* mutant cells, reversed the *vic* phenotype of *cpp1-v1* mutant cells, whereas Rho2CIIS and Rho2SIIS could not (Figure 3C). Thus, Rho2 is the target of Cpp1, that is responsible for the *vic* phenotype of *cpp1-v1* mutant cells, strongly suggesting that Cpp1 functions in Pmk1 MAPK signaling through Rho2 regulation.

Cpp1 and Rho2 Act Upstream of the Pck2–Pmk1 MAPK Pathway

As a first step to examine the functional relationship between Cpp1-Rho2 and Pmk1 signaling, we examined whether the *vic* phenotype is a specific indication for Pmk1 MAPK signaling, or whether it is shared by other MAPK pathways in fission yeast. For this, null cells of $sty1^+$ encoding a stress-activated MAPK or $spk1^+$ encoding a MAPK involved in meiosis was compared with *rho2* null cells, *cpp1* mutant cells, together with null cells of the components of the Pmk1 MAPK pathway. Results clearly showed that Δ*rho2*, *cpp1* mutant cells, as well as Δ*pmk1*, Δ*pek1*, Δ*mkh1*, or Δ*pck2* grew in the presence of FK506 and 0.15M $MgCl_2$, but Δ*sty1* or Δ*spk1* could not grow (Figure 4A, +FK506 + 0.15M $MgCl_2$), indicating that the *vic* phenotype is specifically shared by the mutations in the components of the protein kinase C–Pmk1 MAPK signaling pathway.

Because mutations that perturb the signaling through the protein kinase C–Pmk1 MAPK pathway are known to result in defective cell integrity (Toda *et al.*, 1996), we examined whether knockout of the components of the protein kinase C–Pmk1 MAPK signaling pathway display hypersensitivity to the cell wall-damaging agent micafungin, an inhibitor of (1,3)-β-D-glucan synthase (Carver, 2004). The Δ*rho2*, Δ*cpp1* as well as Δ*pck2* or knockout of the components of Pmk1 MAPK pathway were hypersensitive to micafungin, whereas the growth of Δ*sty1* or Δ*spk1* cells was not inhibited in the presence of 1.2 μg/ml micafungin (Figure 4A, top, + 1.2 μg/ml micafungin). Thus, the *vic* phenotype and the sensitivity to micafungin are specific indications of defective protein kinase C–Pmk1 MAPK signaling. Interestingly, Δ*rho3* cells, but not Δ*ras1* cells, displayed hypersensitivity to micafungin (Figure 4A, bottom), whereas neither of them showed the *vic* phenotype (Figure 2D).

We next examined whether Rho2 acts upstream of the protein kinase C–Pmk1 MAPK signaling. As reported by

Figure 4 (cont). harboring pREP1-GFP-Pck2 were dropped onto the plate as indicated and then incubated as in Figure 4B. (D) Rho2 stimulates the phosphorylation of Pmk1 in vivo. Wild-type cells containing pREP42-GST-Pmk1 and either pREP1-vector (+vector), pREP1-$pek1^{DD}$ (+$Pek1^{DD}$), pREP1-Pck2 (+Pck2), pREP1-Rho2 (+Rho2), pREP1-Pck1 (+Pck1), pREP1-Ras1 (+Ras1), or pREP1-Rho3 (+Rho3) were grown in EMM. Immunoblotting using anti-phospho Pmk1 (top) and anti-GST (bottom) antibodies showed that overproduction of $Pek1^{DD}$, Pck2 as well as Rho2 but not that of Pck1 increased the levels of phosphorylation of Pmk1. (E) Δ*rho2*Δ*mkh1*, Δ*rho2*Δ*pek1*, Δ*rho2*Δ*pmk1*, or Δ*rho2*Δ*pck2* double mutants did not show synergism in the sensitivity to micafungin. The cells as indicated were dropped onto the plate as indicated and then incubated as in Figure 4B. (F) Pck2 associates with Mkh1. Cells integrated with GFP-Pck2 were transformed with the control GST vector or pREP1—GST–Mkh1 and then grown in EMM medium in the absence of thiamine for 20 h. GST-Mkh1 and GST were precipitated by glutathione beads, washed extensively, and subjected to SDS-PAGE and immunoblotted using anti-GFP or anti-GST antibodies.

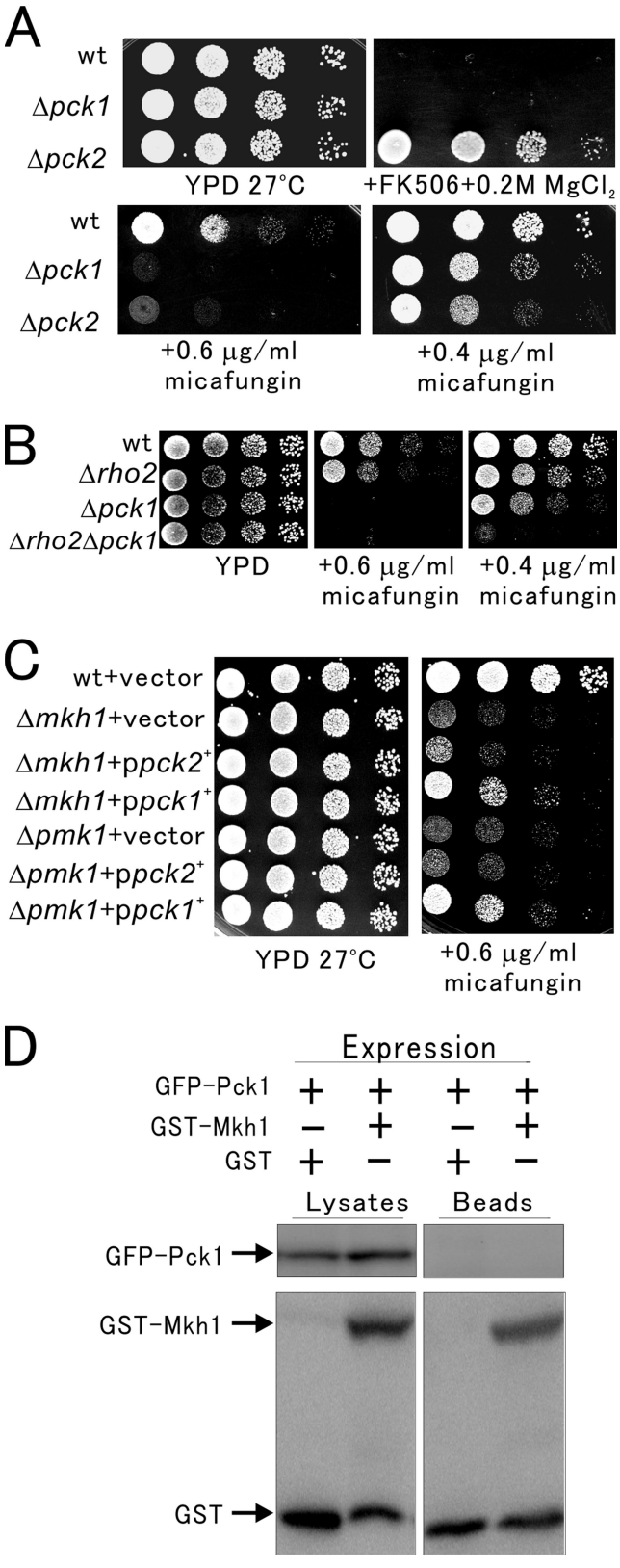

Figure 5. Pck1 is involved in cell integrity signaling and acts independently of the Pmk1 MAPK pathway. (A) The phenotypes of Δ*pck2* and Δ*pck1*. Wild-type, Δ*pck2*, or Δ*pck1* cells were dropped onto the plates as indicated and then incubated for 4 d at 27°C. (B) Δ*rho2*Δ*pck1* double mutants showed synergism in the sensitivity to micafungin. Wild-type, Δ*pck1*, Δ*rho2*, or Δ*rho2*Δ*pck1* cells were dropped onto the plates as indicated and then incubated for 4 d at 27°C. (C) *pck1*$^+$, but not *pck2*$^+$, partially suppressed the micafungin sensitivity of Δ*mkh1* and Δ*pmk1* cells. The Δ*mkh1* or Δ*pmk1* cells were transformed with the control vector, *pck1*$^+$ or *pck2*$^+$ gene. The transformants were dropped onto the plates as indicated and then incubated for 4 d at 27°C. (D) Pck1 does not associate with Mkh1. GFP-Pck1 integrated cells were transformed with the control GST vector or pREP1-GST-Mkh1 and then grown in EMM medium in the absence of thiamine for 20 h. Pull-down assay was performed as described in Figure 4E.

Calonge *et al*. (2000) and as shown here (Figure 4B), overexpression of Rho2 is toxic to wild-type cells, but not to Δ*pck2* cells. If the toxicity of Rho2 overexpression is caused by the hyperactivation of Pmk1 MAPK pathway, it would be expected that this toxicity could be complemented by knockout of the components of Pmk1 signaling pathway. As expected, the overexpression of Rho2 was not toxic to Δ*mkh1*, Δ*pek1*, and Δ*pmk1* cells (Figure 4B), indicating that the toxicity of Rho2 overexpression is mediated by Pck2 and Mkh1–Pek1–Pmk1 signaling pathway. Similarly, overexpression of Pck2 caused the lethality in wild-type cells, but not in Δ*mkh1*, Δ*pek1* and Δ*pmk1* cells (Figure 4C), indicating that the lethality caused by Pck2 overexpression is mediated by Mkh1–Pek1–Pmk1 signaling. Together, these genetic analyses strongly suggest that Rho2 functions upstream of Pck2–Mkh1–Pek1–Pmk1 signaling.

To further confirm that Rho2–Pck2 activates and transmits signaling to Pmk1, we examined the effects of Rho2, Pck2, or Pck1 overexpression on the phosphorylation levels of Pmk1 MAPK by using anti-phospho Pmk1 antibodies (Sugiura *et al*., 1999). As shown in Figure 4D, overexpression of Rho2 and Pck2 dramatically increased the phosphorylation levels of Pmk1 similar to that obtained from the overexpression of Pek1^{DD}, a constitutively active MAPKK for Pmk1 (Figure 4D). In clear contrast, overexpression of Pck1 did not increase the phosphorylation level of Pmk1 (Figure 4D). Thus, Rho2–Pck2 acts upstream of the Pmk1 MAPK pathway and stimulates the Pmk1 signaling in vivo. In addition, overexpression of Ras1 or Rho3 did not increase the phosphorylation level of Pmk1 (Figure 4D). Together with the results of Figures 2D and 4A, this suggests that Rho3 is involved in the regulation of cell wall integrity but independently of the Pmk1 signaling pathway.

Next, we constructed a series of double mutants between Δ*rho2* and knockout of the components of the protein kinase C–Pmk1 MAPK signaling and compared the sensitivity to micafungin with each single mutant. The Δ*rho2* cells showed a relatively weak sensitivity to micafungin, because Δ*rho2* cells grew in the presence of 0.6 μg/ml micafungin, wherein Δ*mkh1*, Δ*pek1*, Δ*pmk1*, or Δ*pck2* mutants failed to grow. However, Δ*rho2*Δ*mkh1*, Δ*rho2*Δ*pek1*, Δ*rho2*Δ*pmk1* or Δ*rho2*Δ*pck2* double knockout mutant cells failed to grow in the presence of 0.4 μg/ml micafungin and do not show synergism in the sensitivity to micafungin compared with the parental single knockout (Figure 4E).

The above-mentioned results strongly suggest that Pck2 functions upstream of Pmk1. We thus examined whether Pck2 associates with the MAPKKK Mkh1. For this, we expressed glutathione *S*-transferase (GST)-fused Mkh1 in strains where GFP-Pck2 is chromosomally integrated. In the GST pull-down assay, GFP–Pck2 associates with GST–Mkh1 but not with control GST vector alone (Figure 4F), indicating a protein–protein interaction between Pck2 and Mkh1.

Pck1 Is Involved in Cell Integrity Signaling and Acts Independently of the Pmk1 MAPK Pathway

In the study by Toda *et al*. (1993), Pck1 and Pck2 have been reported to share an overlapping function for cell viability and to partially complement each other. We then examined whether Pck2 and Pck1 share an overlapping function in cell integrity and chloride ion homeostasis.

For this, we first compared Δ*pck1* and Δ*pck2* cells regarding *vic* phenotype. Results clearly showed that Δ*pck2*, but not Δ*pck1*, grew in the presence of FK506 and 0.2 M $MgCl_2$ (Figure 5A), suggesting that Pck1 is not involved in Cl^- homeostasis. We also compared Δ*pck1* and Δ*pck2* cells regarding the cell integrity defect. As shown in Figure 5B, at a higher concentration of micafungin (0.6 μg/ml), the growth of Δ*pck1* and Δ*pck2* cells were significantly inhibited, but at a lower concentration of micafungin (0.4 μg/ml), the growth of Δ*pck2* cells was markedly inhibited compared with that of Δ*pck1* cells. These results indicate that although Δ*pck1* and Δ*pck2* cells are both sensitive to micafungin, Δ*pck1* cells displayed a weaker sensitivity to micafungin compared with Δ*pck2* cells.

As shown in Figure 4D, Δ*rho2*Δ*pck2* double mutants showed micafungin sensitivity to the same extent as that of Δ*pck2* mutants. Here, we constructed Δ*rho2*Δ*pck1* double mutants and investigated the micafungin sensitivity. Although both Δ*pck1* cells and Δ*rho2* cells can grow in the presence of 0.4 μg/ml micafungin, Δ*rho2*Δ*pck1* double mutants failed to grow in the presence of 0.4 μg/ml micafungin, indicating a synergism in the sensitivity to micafungin compared with that of the parental single knockout (Figure 5B). Thus, Pck1 seems to play a role in cell integrity in parallel to the Rho2–Pck2–Mkh1–Pek1–Pmk1 signaling pathway. Consistently, our recent study showed that Pmk1 is required for the stimulation of calcineurin via Yam8/Cch1-mediated Ca^{2+} influx and that knockout of *pck2*$^+$, but not *pck1*$^+$ gene, markedly diminished the Yam8/Cch1-dependent stimulation of calcineurin activity, suggesting that Pck2 acts upstream of the Pmk1 in this signaling pathway (Deng *et al*., 2006).

Furthermore, overexpression of *pck1*$^+$, but not *pck2*$^+$, partially suppressed the micafungin sensitivity of Δ*mkh1* and Δ*pmk1* cells (Figure 5C). Overexpression of *pck1*$^+$ also partially suppressed the micafungin sensitivity of Δ*pek1* cells (our unpublished data). These results indicate that Pck1 stimulates the cell integrity signaling independently of the Pmk1 MAPK pathway, whereas Pck2 exerts its function on cell integrity through the MAPK pathway. In addition, a pull-down experiment demonstrated that Pck1 does not associate with Mkh1 (Figure 5D), in clear contrast to the association observed between Pck2 and Mkh1 (Figure 4F). These results are in good agreement with the findings obtained with phosphorylation experiments.

DISCUSSION

In the present study, we performed a novel genetic screen that aims to identify new regulators of the Pmk1 signaling, and we identified a missense mutation of the *cpp1*$^+$ gene encoding FTase. Substitution of a highly conserved aspartic acid to an asparagine residue at the amino acid position 155 resulted in the partial but severe impairment of the FTase activity in *cpp1-v1* mutant cells.

Interestingly, the *cpp1-v1* mutation caused the differential effects on the function of its substrates. That is, the membrane distribution of Ras1 and Rho2 was apparently abolished but that of Rho3 was still maintained in *cpp1-v1* mutant cells, indicating that Rho3 farnesylation is not completely abolished in *cpp1-v1* mutant cells. This is in good correlation with the moderate defect of prenylation as shown in Figure 2C. Furthermore, although Ras1 prenylation was severely impaired in *cpp1-v1* mutant cells (Figure 2C), the function of Ras1 seemed to be maintained in the *cpp1-v1* mutant cells because their cell shape was not so much affected compared with that of Δ*cpp1* cells. In clear contrast, the function of Rho2 seemed to be severely impaired in the *cpp1-v1* mutant cells, because the mutant cells showed a clear *vic* phenotype similar to that of the Δ*rho2* cells. These results suggest that Rho2 function is preferentially sensitive to defect of FTase activity in the *cpp1-v1* mutant cells.

Although a numbers of studies showed that functions of Ras proteins depend on its farnesylation, the identities of the relevant farnesylated proteins in human oncogenesis are not fully resolved. FTIs clearly have the potential to inhibit oncogenic Ras signaling, but FTIs are also effective on tumor cell lines that do not contain mutant Ras (Sepp-Lorenzino *et al*., 1995; End *et al*., 2001), suggesting that the pharmacological effects extend outside of the Ras protein (Tamanoi *et al*., 2001). RhoB, a small GTPase of the mammalian Rho family has been suggested as a potential relevant FTI target (Prendergast, 2001; Cox and Der, 2002). RhoB exists as both farnesylated and geranylgeranylated forms, whereas the highly homologous RhoA and RhoC isoforms are solely geranylgeranylated (Adamson *et al*., 1992). Treatment of cells with FTIs causes a loss of farnesylated RhoB and a consequent increase in geranylgeranylated RhoB (Lebowitz *et al*., 1997), and these changes in prenylation have been implicated in the antineoplastic responses to FTIs (Liu *et al*., 2000).

Given the high degree of conservation of FTase, Ras and Rho proteins as well as its involvement in human oncogenesis, the *cpp1-v1* mutant may be a useful model for studying the conserved molecular mechanism of protein farnesylation and the differential effects of FTase inhibition on the various substrates.

In conclusion, the identification and analyses of *cpp1-v1* mutant cells have demonstrated for the first time the functional importance of the posttranslational modification of Rho GTPase protein in the activation of cell integrity signaling through protein kinase C–MAPK pathway. Because of the high similarity between the fission yeast and mammalian MAPK pathway, the screen of *vic* mutants would further provide an excellent opportunity to identify novel components of MAPK cascade and analyze regulatory mechanisms of MAPK signaling in higher eucaryotes.

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