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# *Schizosaccharomyces pombe* Rho2p GTPase Regulates Cell Wall α-Glucan Biosynthesis through the Protein Kinase Pck2p

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*Schizosaccharomyces pombe rho1*$^+$ and *rho2*$^+$ genes are involved in the control of cell morphogenesis, cell integrity, and polarization of the actin cytoskeleton. Although both GTPases interact with each of the two *S. pombe* protein kinase C homologues, Pck1p and Pck2p, their functions are distinct from each other. It is known that Rho1p regulates (1,3)β-D-glucan synthesis both directly and through Pck2p. In this paper, we have investigated Rho2p signaling and show that *pck2*Δ and *rho2*Δ strains display similar defects with regard to cell wall integrity, indicating that they might be in the same signaling pathway. We also show that Rho2 GTPase regulates the synthesis of α-D-glucan, the other main structural polymer of the *S. pombe* cell wall, primarily through Pck2p. Although overexpression of *rho2*$^+$ in wild-type or *pck1*Δ cells is lethal and causes morphological alterations, actin depolarization, and an increase in α-D-glucan biosynthesis, all of these effects are suppressed in a *pck2*Δ strain. In addition, genetic interactions suggest that Rho2p and Pck2p are important for the regulation of Mok1p, the major (1–3)α-D-glucan synthase. Thus, a *rho2*Δ mutation, like *pck2*Δ, is synthetically lethal with *mok1–664*, and the mutant partially fails to localize Mok1p to the growing areas. Moreover, overexpression of *mok1*$^+$ in *rho2*Δ cells causes a lethal phenotype that is completely different from that of *mok1*$^+$ overexpression in wild-type cells, and the increase in α-glucan is considerably lower. Taken together, all of these results indicate the presence of a signaling pathway regulating α-glucan biosynthesis in which the Rho2p GTPase activates Pck2p, and this kinase in turn controls Mok1p.

## INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* undergoes morphogenetic changes during both the vegetative and sexual cell cycles that require asymmetric cell growth and actin cytoskeleton reorganizations (Mata and Nurse, 1997; Arellano *et al.*, 1999a; Verde, 1998). Rho GTPases are critical modulators of these processes as in all other eukaryotes (Schmidt and Hall, 1998; Chant, 1999; Kaibuchi *et al.*, 1999; Pruyne and Bretscher, 2000). In yeasts, these GTPases provide the coordinated regulation of cell wall biosynthetic enzymes and actin organization that is required to maintain cell integrity and polarized growth (Cabib *et al.*, 1998; Arellano *et al.*, 1999a).

The architecture of the fission yeast cell wall differs from that of *Saccharomyces cerevisiae*. The major *S. pombe* cell wall structural components are the (1–3)β-D-glucan (50–54% of total polysaccharides) and (1–3)α-D-glucan (28–32%) (Kopecká *et al.*, 1995; Manners and Meyer, 1977). This latter polymer is not present in *S. cerevisiae*. On the other hand, chitin, a major structural component of the *S. cerevisiae* cell wall, has not been detected in *S. pombe*, although a chitin synthase gene homologue has recently been described that is necessary for the formation of chitin or chitosan during spore cell wall maturation (Arellano *et al.*, 2000). The catalytic subunit of the *S. pombe* (1–3)β-D-glucan synthase is

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**Table 1.** List of strains used in this study

| Strain | Genotype | Reference |
|---|---|---|
| HM123 | *h⁻ leu1-32* | Moreno *et al.*, 1991 |
| TP134-3B | *h⁻ pck1::ura4⁺ ura4-D18 leu1-32* | Toda *et al.*, 1993 |
| TP169-1C | *h⁻ pck2::LEU2 leu1-32 ura4-D18* | Toda *et al.*, 1993 |
| TP179-1A[a] | *h⁻ sts6-8 leu1-32 ura4-D18* | Toda *et al.*, 1993 |
| KN-1 | *h⁻ rho2::ura4⁺ leu1-32 ura4-D18 ade6* | Hirata *et al.*, 1998 |
| MA20 | *h⁻ P81nmt-pck1 leu1-32 kanʳ* | Arellano *et al.*, 1999a, b |
| MA21 | *h⁻ P81nmt-pck2 ura4-D18 kanʳ* | Arellano *et al.*, 1999a, b |
| TMC110[b] | *h⁻ P81nmt-pck1 rho2::ura4⁺ leu1-32 ura4-D18 ade6 kanʳ* | This work |
| TMC111[b] | *h⁻ P81nmt-pck2 rho2::ura4⁺ ura4-D18 leu1-32 ade6 kanʳ* | This work |
| DH664 | *h⁻ mok1-664 leu1-32* | Katayama *et al.*, 1999 |
| SKP100 | *h⁻ mok1-664 pck2::LEU2 leu1-32* | Katayama *et al.*, 1999 |
| TMC112[b] | *h⁻ mok1-664 leu1-32 ura4-D18* | This work |
| TMC113[b] | *h⁻ mok1-664 rho2::ura4⁺ ura4-D18 leu1-32 ade6* | This work |
| SKP103 | *h⁻ Pnmt1-mok1 leu1-32 kanʳ* | Katayama *et al.*, 1999 |
| SKP170 | *h⁻ Pnmt1-mok1 leu1-32 kanʳ pck2::LEU2* | Katayama *et al.*, 1999 |
| TMC114[b] | *h⁻ Pnmt1-mok1 leu1-32, ura4-D18 kanʳ* | This work |
| TMC115[b] | *h⁻ Pnmt1-mok1 rho2::ura4⁺ leu1-32 ura4-D18 ade6 kanʳ* | This work |
| TMC116[b] | *h⁻ Pnmt1-mok1 pck1::ura4⁺ leu1-32 ura4-D18 kanʳ* | This work |

[a] *sts6-8* is a mutant allele of *pck2* identified by its supersensitivity to staurosporine.
[b] TMC strains were obtained by mating other haploid strains (or the $h^+$ relatives) listed in this Table.

encoded by the *cps1⁺* gene, a homologue to *FKS1* and *FKS2* (Ishiguro *et al.*, 1997). *cps1⁺* is important for cytokinesis and cell polarity (Le Goff *et al.*, 1999; Liu *et al.*, 2000) and is part of a Wee1p-dependent septation checkpoint. At least three more genes similar to *cps1⁺* are present in the *S. pombe* genome, but nothing is known yet about their function or regulation. The (1–3)α-D-glucan synthase is encoded by the *mok* gene family, which includes five members. The main one, *ags1⁺*/mok1⁺, is an essential gene (Hoschterbach *et al.*, 1998; Katayama *et al.*, 1999). Mok1p localizes to the growing tips and to the septum during cytokinesis, and it is regulated by Pck2p (Katayama *et al.*, 1999).

*S. pombe* Rho1 GTPase was identified as a regulatory component of the (1–3)β-D-glucan synthase (Arellano *et al.*, 1996) that is also required for maintenance of cell integrity and polarization of the actin cytoskeleton (Arellano *et al.*, 1997; Nakano *et al.*, 1997). We reported recently that GTP-bound Rho1p interacts with the two fission yeast PKC homologues, Pck1p and Pck2p, stabilizing both proteins (Arellano *et al.*, 1999b). The interaction with Rho1p also allows the localization of pck2 to the cell growth areas (Sayers *et al.*, 2000). Pck1p and Pck2p share overlapping roles in cell viability and partially complement each other (Toda *et al.*, 1993, 1996). However, *pck1*Δ and *pck2*Δ cells have different phenotypes and differentially regulate cell wall integrity. Furthermore, GTP-bound Rho1p regulates (1–3)β-D-glucan cell wall biosynthesis and cell polarity mainly through Pck2p (Arellano *et al.*, 1999b).

*S. pombe rho2⁺* was isolated as a gene that causes lethality when overexpressed (Hirata *et al.*, 1998). The Rho2p GTPase is localized to the sites of growth and seems to be involved in the control of cell polarity, reorganization of the actin cytoskeleton, and cell wall biosynthesis. Its disruption is not lethal but renders the cells rounded and hypersensitive to Aculeacin A, a specific (1–3)β-D-glucan synthase inhibitor, or treatment with glucanases (Hirata *et al.*, 1998). This phenotype is only weakly suppressed by *rho1⁺*, indicating a minor functional overlap between the two GTPases (Hirata *et al.*, 1998). *rho2*Δ cells are also hypersensitive to staurosporine, a potent inhibitor of protein kinase C, suggesting that Rho2p might be signaling through the PKC homologues. Moreover, GTP-bound Rho2p interacts with Pck1p and Pck2p in the two-hybrid system (Arellano *et al.*, 1999b). Here we examine the role of Rho2p in cell wall biosynthesis and the function of the Rho2p interaction with Pck1p and Pck2p. We present genetic and biochemical evidence that Rho2p is a positive regulator of Mok1p, stimulating the biosynthesis of α-D-glucan and signaling through Pck2p.

## MATERIALS AND METHODS

### *Strains, Growth Conditions, and Genetic Methods*

Standard *S. pombe* media and genetic manipulations were used (Moreno *et al.*, 1991). All of the strains used were isogenic to wild-type strains $h^-$ 972 and $h^+$ 975 and are described in Table 1.

Yeast cells were grown in YES medium or minimal medium (EMM) supplemented with the necessary requirements. Echinocandin derivative LY280949 was from Lilly (Indianapolis, IN, and Papulacandin B was from Novartis (Basel, Switzerland). Incubations were performed at 28, 32, or 37°C. Growth was monitored by $OD_{600}$ measurements.

*Escherichia coli* DH5α (Life Technologies, Gaithersburg, MD) was used as host for propagation of plasmids. Cells were grown in LB medium supplemented with 50 μg/ml ampicillin or 25 μg/ml kanamycin when appropriate. Solid-medium plates contained 2% agar.

### *Recombinant DNA Methods*

All DNA manipulations were performed by established methods (Sambrook *et al.*, 1989). Enzymes were used according to the recommendation of the suppliers. *S. pombe* was transformed by electroporation (Prentice, 1992) or by the lithium acetate method (Ito *et*

*al.*, 1983). The *nmt* promoter-containing vectors pREP1, pREP3X, pREP4X, pREP41X, and pREP42X (Forsburg, 1993) were used for the overexpression of *pck2*$^+$ and *rho2*$^+$. pREP1-*pck2* has been described previously (Sayers *et al.*, 2000). The *rho2*$^+$ ORF was cloned in the *Sal*I–*Bam*HI sites of the vectors by PCR amplification from an *S. pombe* cDNA library using the following primers: 5′-ATATA*GTCGAC*CATGG TG CAA TCT CAA CCG-3′ (Forward) and 5′-TATAT *GGATCC* TTA TGA AAT GAT GCA TTT TG-3′ (Backward), which contain *Sal*I and *Bam*HI sites (italics), respectively. The entire *rho2*$^+$ ORF was confirmed by automated sequencing.

### Microscopy Techniques

For Calcofluor staining, exponentially growing *S. pombe* cells cultivated at 32°C in minimal medium without thiamine were harvested, washed once, and resuspended in water with Calcofluor at 20 μg/ml final concentration for 5 min at room temperature.

For actin staining, cells were fixed in cold methanol for at least 15 min. Immunofluorescence was performed as described (Hagan and Hyams, 1988). The primary anti-actin antibody was the monoclonal N350 (Amersham, Arlington Heights, IL), and the secondary antibody was a sheep anti-mouse Cy3-conjugated F(ab′)2 fragment (Sigma, St. Louis, MO).

For Mok1p staining, purified rabbit polyclonal anti-mok1 antiserum (1:10) was used as primary antibody (Katayama *et al.*, 1999), and FITC-conjugated goat anti-rabbit was used as secondary antibody.

Cells were immobilized on poly-L-lysine coverslips and observed using a confocal microscope (Zeiss MRC600).

### Electron Microscopy

The procedure for electron microscopy observation was as described previously (Nakano *et al.*, 1997). Briefly, cells were prefixed with 2.5% glutaraldehyde dissolved in 0.1 M sodium phosphate buffer, pH 7.0, at room temperature for 1 h and post-fixed with 1.5% potassium permanganate dissolved in distilled–deionized water at 4°C overnight. After dehydration in a graded series of acetone, samples were embedded in Quetol 812. Thin sections were cut with a Reichert Ultracut S microtome, stained in uranyl acetate and lead citrate, and examined with a JEOL-JEM1200EX electron microscope.

### Glucanase Sensitivity Experiments

Glucanase sensitivity of *S. pombe* cells was evaluated following the procedure described previously (Shiozaki and Russell, 1995). Wild-type (HM123), *rho2*Δ (KN-1), and *rho2*Δ (KN-1) transformed with either pREP1-*rho2* or pREP1-*pck2* strains were grown to midlogarithmic phase in EMM medium with 5 μM thiamine at 30°C. The cells were harvested, washed in TE buffer, and resuspended at an $OD_{600}$ of 1.0 in the same buffer containing 20 μg/ml β-glucanase (Zymolyase 100T; Seikagaku Kogio Co. Ltd., Tokyo, Japan). Cell suspensions were incubated at 30°C with shaking, and cell lysis was monitored by measuring the $OD_{600}$.

### Labeling and Fractionation of Cell Wall Polysaccharides

Labeling and fractionation of cell wall polysaccharides was performed as described (Arellano *et al.*, 1997). Briefly, exponentially growing cultures of *S. pombe* wild-type and transformed cells were supplemented with [U-$^{14}$C]glucose (1 μCi/ml) and incubated for an additional 4 h. Cells were harvested, and unlabeled cells were added to the radioactive samples as carriers. Total glucose incorporation was monitored by measuring the radioactivity in trichloroacetic acid-insoluble material. Mechanical breakage of cells was performed using prechilled glass beads added to the cells, and lysis was achieved in a Fast-Prep System FP120 (Bio 101, Savant, La Jolla, CA) using two 15 s intervals at 5.5 speed. Cell walls were pelleted at 1000 × *g* for 5 min and washed three times with 5% NaCl and three times with 1 mM EDTA. Aliquots (100 μl) of the total wall were incubated with 100 U Zymolyase 100T or Quantazyme (Quantum Biotechnologies Inc., Montreal, Quebec) for 36 h at 30°C. Aliquots without enzyme were included as control. The samples were centrifuged, and the supernatant and washed pellet were counted separately. The supernatants from the Zymolyase 100T reaction were considered β-glucan plus galactomannan, and the pellet was considered α-glucan. The supernatants from the Quantazyme reactions were considered (1–3)β-glucan, and the pellet was considered α-glucan plus galactomannan.

### Immunoblot Analysis

Mok1p expressed in *S. pombe* cells was detected by immunoblotting. Approximately 1 × 10$^8$ cells growing exponentially in minimal medium with or without thiamine were harvested by brief centrifugation, washed once with lysis buffer (20 mM Tris, pH 8.0, 10 mM EDTA, 10% glycerol, 137 mM NaCl, and 1% Nonidet-P40 containing 1 mM *p*-aminophenyl methanesulfonyl fluoride, 2 μg/ml leupeptin, and 10 μg/ml aprotinin), and resuspended in 100 μl of the same buffer. Approximately 1 g of prechilled glass beads was added to the cells, and lysis was achieved in a Fast-Prep system (see above). The resulting homogenates were collected, and glass beads and large debris were removed by centrifugation for 5 min at 750 × *g*. One aliquot was diluted 2× with lysis buffer and subjected to 7.5% SDS-PAGE, and separated proteins were electrophoretically transferred to a nitrocellulose membrane sheet (Schleicher and Schuell, Keene, NH). The blot was processed to detect Mok1p with 1:1000 diluted anti-Mok1p rabbit polyclonal antiserum obtained as described (Katayama *et al.*, 1999) and to detect tubulin with 1:1000 TAT1 anti-α-tubulin monoclonal (kindly provided by K. Gull, University of Manchester, United Kingdom) as primary antibodies. After they were washed several times, the membranes were incubated with HRP-conjugated antibodies, and bands were visualized by the luminol-based ECL detection kit (Amersham).

## RESULTS

### Rho2p Stimulates Cell Wall α-Glucan Biosynthesis

*S. pombe* Rho2p GTPase is involved in cell polarity and morphogenesis, but its function appears to be different from that of Rho1p. Microscopic examination of *rho2*$^+$-overexpressing cells revealed a thicker cell wall than in wild-type cells (Hirata *et al.*, 1998).

To investigate Rho2p function, we analyzed the cell wall composition of *S. pombe rho2*Δ mutants and cells overproducing Rho2p (Table 2). Incorporation of radioactive glucose into the cell wall was slightly lower in the *rho2*Δ cells, with no significant differences in cell wall composition; however, in cells transformed with pREP1-*rho2* and grown in the absence of thiamine for 16 h, *rho2*$^+$ overexpression caused an increase in the total cell wall incorporation (from 29.8 to 38.9%), resulting from a specific increase in α-glucan (from 10.0 to 17.2% of the total $^{14}$C-glucose incorporated into the cells). A similar increase in α-glucan has not been observed in Rho1p-overproducing cells (Arellano *et al.*, 1996), which suggests that Rho2p has a role, distinct from Rho1p, as a positive regulator of α-glucan biosynthesis.

### rho2Δ and pck2Δ Strains Have Similar Cell Wall Defects

*rho2*Δ cells are hypersensitive to staurosporine, a potent inhibitor of protein kinase C (Hirata *et al.*, 1998). We have

**Table 2.** Incorporation of radioactivity from $^{14}$C-glucose into cell wall polysaccharides of different *S. pombe* strains grown in minimal medium without thiamine for 16 h

| Strain | Plasmid | Cell wall | Galactomannan | α-Glucan | β-Glucan |
|---|---|---|---|---|---|
| HM123 (wild-type) | | 29.2 ± 1.8 | 2.3 ± 0.5 (8) | 9.5 ± 0.8 (33) | 17.4 ± 1.8 (60) |
| KN-1 (*rho2*Δ) | | 25.6 ± 0.4 | 2.3 ± 0.7 (9) | 7.6 ± 0.2 (30) | 15.7 ± 1.2 (61) |
| TP179-1A (*sts6–8*) | | 23.3 ± 3.7 | 3.5 ± 1.1 (15) | 6.8 ± 0.7 (29) | 13.0 ± 2.4 (56) |
| TP169-1C (*pck2*Δ) | | 24.7 ± 0.6 | 4.1 ± 0.9 (17) | 6.6 ± 0.4 (27) | 14.0 ± 1.0 (57) |
| TP134-3B (*pck1*Δ) | | 24.3 ± 2.1 | 3.9 ± 1.4 (16) | 8.2 ± 1.0 (34) | 12.2 ± 1.3 (50) |
| HM123 | pREP4X | 29.8 ± 1.6 | 2.1 ± 0.8 (8) | 10.0 ± 0.8 (33) | 17.7 ± 1.5 (59) |
| HM123 | pREP4X-*rho2* | 38.9 ± 3.3 | 1.5 ± 0.7 (4) | 17.2 ± 1.6 (44) | 20.1 ± 1.8 (52) |
| TP169-1C | pREP4X-*rho2* | 23.3 ± 2.1 | 4.5 ± 1.2 (19) | 6.4 ± 0.8 (27) | 12.4 ± 0.9 (53) |
| TP134-3B | pREP3X-*rho2* | 39.8 ± 2.2 | 4.1 ± 1.0 (10) | 16.8 ± 1.2 (42) | 18.9 ± 0.9 (47) |

$^{14}$C-glucose was added 2 h before harvesting the cells.

Values indicate percentage of total $^{14}$C-glucose incorporated and are the mean ± SD calculated from at least four independent experiments. Values in parentheses are the percentage of the corresponding polysaccharide in the cell wall.

also reported that Rho2p physically interacts with Pck1p and Pck2p (Arellano *et al.*, 1999b). To study the possible functional relationship between Rho2p and Pck2p, we first performed electron microscopy examination of *rho2*$^+$-overexpressing cells, *rho2::ura4*$^+$ (KN-1), and *pck2* mutant (*sts6–8*) cells (strain TP179–1A) that show the same phenotype as *pck2* disruptant cells (Toda *et al.*, 1993). The cell wall of *rho2*$^+$-overexpressing cells was thicker than that of wild-type cells (Figure 1, a and b, and Table 2) (Hirata *et al.*, 1998), although the phenotype was moderate in comparison to that of *rho1*$^+$- or *pck2*$^+$-overexpressing cells (Arellano *et al.*, 1996, 1999b; Nakano *et al.*, 1997). In addition, whereas in wild-type cells membranous structures that have been proposed to be endoplasmic reticulum (Osumi, 1998) were seen beneath the cytoplasmic membrane (Figure 1a), in *rho2*$^+$-overexpressing cells these structures were often heavily stacked at the cell ends and in septated regions (Figure 1b). In the *pck2* mutant and the *rho2*Δ cells, the cell wall was thinner than in wild-type cells (Figure 1, c and d, and Table 2), and the outermost layer was less electron-dense. Interestingly, this outer layer was also absent in the α-D-glucan synthase mutant, *ags1–1* (Hochsterbach *et al.*, 1998).

To corroborate the possible connections between Rho2p and Pck2p, we analyzed the sensitivity of mutant strains lacking these proteins to Calcofluor, a drug affecting cell wall integrity, and to the antibiotics Echinocandin and Papulacandin B, inhibitors of β-D-glucan biosynthesis (Perez *et al.*, 1981). We used the previously generated MA20 (*Pnmt81-pck1)* and MA21 (*Pnmt81-pck2)* strains (Arellano *et al.*, 1999a,b), in which each gene is under the control of the thiamine-repressible and reduced-expression–level promoter *nmt81* (Forsburg, 1993). When grown in the presence of thiamine (repressed), MA20 was hypersensitive to Calcofluor, Papulacandin, and Echinocandin, suggesting a general defect in the cell wall. By contrast, neither KN-1 (*rho2::ura4*$^+$) nor MA21 grown with thiamine was hypersensitive to Calcofluor, and both were more resistant to Echinocandin than wild-type cells (Figure 2). KN-1 and MA21 cells grown with thiamine were hypersensitive to Papulacandin B, and this might imply either a cell wall defect that increases the importance of β-glucan in maintaining cell wall integrity, such as a decrease in α-glucan, or an increase in cell wall permeability to the drug. The mutant strain TMC110 (*rho2::ura4*$^+$ Pnmt81-pck1) grown with thiamine was more sensitive to Papulacandin B and Echinocandin than KN-1 or MA20 cells and was as hypersensitive to Calcofluor as MA20 cells, suggesting that Rho2p and Pck1p might act in different signaling pathways. The double-mutant TMC111 (*rho2::ura4*$^+$ Pnmt81-pck2) grown with thiamine was not sensitive to Calcofluor, corroborating that Rho2p is not activating Pck1p, because if that were the case, the lack of Rho2p would cause the same effect as the lack of Pck1p. Interestingly, the TMC111 strain was not resistant to Echinocandin, suggesting that the lack of Rho2p and Pck2p at the same time might cause a different cell wall defect than the lack of Rho2p or Pck2p separately.

### *Overproduction of Pck2p Suppresses the Hypersensitivity to β-Glucanases of rho2Δ Cells*

The cell wall defect of *rho2* disruptant cells was also revealed by testing the resistance to lysis during treatment with a β-glucanase complex that digests the cell wall. KN-1 cells were very sensitive to β-glucanase treatment (Figure 3), corroborating the observed hypersensitivity to the β-D-glucan synthase inhibitor Papulacandin B (Figure 2). We also analyzed whether a mild overexpression of *pck2*$^+$ was able to suppress the *rho2* disruptant phenotype. *rho2*Δ cells bearing pREP1, pREP1-*rho2*, or pREP1-*pck2* were grown in EMM with 5 μM thiamine. In such conditions, the strong *nmt1* promoter is not totally repressed (Forsburg, 1993). As shown in Figure 3, overproduction of either Rho2p or Pck2p largely reduced the lysis rate of KN-1 cells caused by β-glucanase treatment, strongly suggesting a functional relationship between Rho2p and Pck2p.

### *The Morphological Effects Caused by Rho2p Overproduction Require the Presence of Pck2p*

In addition to the formation of a thick cell wall (Figure 1b) and the increase in α-glucan content (Table 2), *rho2*$^+$ overexpression is lethal and causes a rounded morphology and actin depolarization (Hirata *et al.*, 1998). To further analyze whether Pck1p or Pck2p is a functional effector protein of Rho2p, we overproduced the GTPase in HM123, TP134–3B *(pck1*Δ), and TP169–1C *(pck2*Δ) strains. High levels of Rho2p

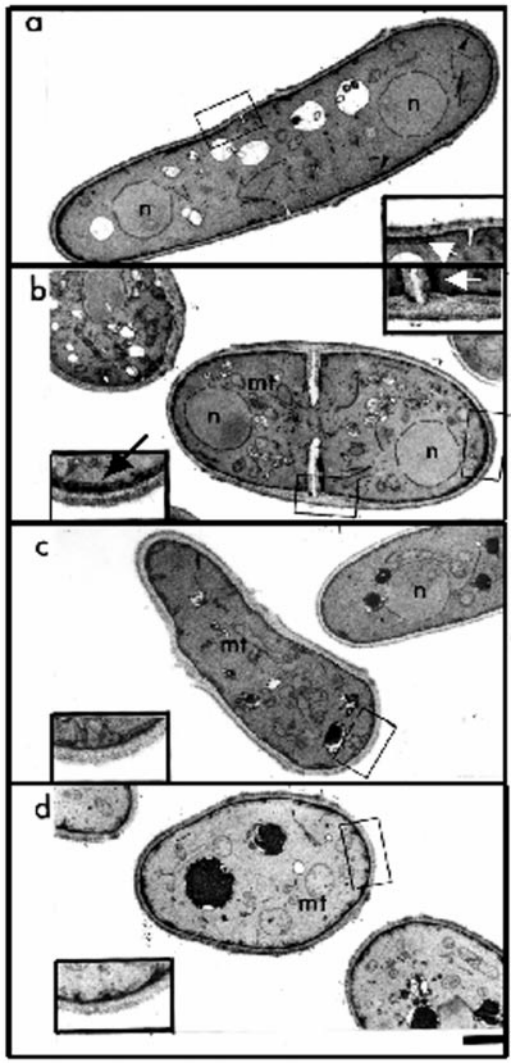


**Figure 1.** rho2Δ and pck2 mutant cells have similar cell wall defects. Shown are electron microscopy images of fission yeast cells expressing different levels of Rho2p. HM123 (wild-type) cells carrying pREP1 (a) or pREP1-*rho2* (b); KN-1 (*rho2*Δ) (c), and TP179–1A (*sts6–8=pck2*) mutant cells (d) were grown at 30°C for 16 h in EMM liquid medium without thiamine before fixation. Arrows indicate membranous structure. N, nucleus, Mt, mitochondria. Twofold-magnified images are shown in insets. More than 30 cells of each sample were observed, and all display a similar phenotype. Bar, 1 μm.

were lethal in HM123 or TP134–3B but not in TP169–1C cells (Figure 4A). Moreover, the actin depolarization and rounded morphology caused by *rho2*$^+$ overexpression in the wild-type strain was suppressed in the *pck2*Δ cells (Figure 4B), suggesting that Rho2p signaling requires Pck2p. Cell wall analysis of these cells revealed that the increase in α-glucan caused by overexpressing *rho2*$^+$ occurred in *pck1*Δ cells but not in *pck2*Δ cells (Table 2), indicating that pck2p kinase might also be required for the stimulating effect of Rho2p on the biosynthesis of cell wall α-glucan. In summary, these results demonstrate that the main effects of *rho2*$^+$ overexpression, lethality, round morphology, actin depolarization, and increase in α-glucan, are mediated by Pck2p but not by Pck1p.

### *Functional Relationships among rho2*$^+$*, pck2*$^+$*, and mok1*$^+$

Mok1p is responsible for the biosynthesis of α-glucan in *S. pombe* and is directly regulated by Pck2p (Katayama *et al.*, 1999). To analyze the functional relationship between *rho2*$^+$ and *mok1*$^+$, we constructed the double-mutant strain TMC113 (*rho2::ura4*$^+$ mok1–664), which grew slowly at 30°C and could not form colonies at 32°C (Figure 5A), whereas each single mutant could grow under these conditions. This synthetic lethal interaction was similar to that observed between *mok1–664* and *pck2*Δ (Figure 5A) (Katayama *et al.*, 1999), and the morphology of the cells was also similar in both double mutants (Figure 5B).

Overexpression of *mok1*$^+$ is lethal in wild-type cells and doubles the amount of cell wall α-glucan. We used strain SKP103, carrying the integrated *Pnmt1–mok1* (Katayama *et al.*, 1999), to construct different mutant strains that allowed the overexpression of *mok1*$^+$ in the absence of Rho2p (strain TMC115), Pck1p (strain TMC116), or Pck2p (strain SKP170) (Katayama *et al.*, 1999). As described previously (Katayama *et al.*, 1999), *mok1*$^+$ overexpression was not lethal in SKP170 cells (Figure 6A). In contrast, a significant toxicity was observed in TMC116 cells, where *Pnmt1*-driven *mok1*$^+$ was deleterious even in the presence of thiamine. Interestingly, overexpression of *mok1*$^+$ in TMC115 cells was toxic, but the observed lethal phenotype was delayed as compared with SKP103 cells (Figure 6A). Under these conditions, the levels of Mok1p, analyzed by immunoblot with anti-Mok1p antiserum, were similar in SKP103, TMC115, and SKP170 (Figure 6B), but the morphologies of the cells were different (Figure 6C). A possible explanation for the observation that strong Mok1p overproduction is still lethal in *rho2*Δ cells could be that in the absence of Rho2p, Rho1p partially substitutes for this protein, and it signals through Pck2p not only to regulate the (1,3)β-glucan-synthase but also to regulate Mok1p.

The different strains carrying *Pnmt1–mok1* were also grown in the presence of 5 μM thiamine in the expectation of producing only a mild increase in the Mok1p levels. Under these conditions, Mok1p caused drastic morphologic defects in the wild-type and *pck1*Δ cells, whereas those defects were not observed in the *pck2*Δ or *rho2*Δ cells (Figure 6D).

Biochemical analysis of cell wall composition was also performed in cells overexpressing *mok1*$^+$ to establish whether Rho2p regulates the function of this protein. As shown in Figure 6E, high levels of Mok1p caused an increase in the α-glucan fraction that was considerably reduced in the *rho2*Δ cells as compared with the wild-type strain. Indeed, the level of α-glucan in the *rho2*Δ strain was close to that in the *pck2*Δ strain.

We therefore analyzed Mok1p localization in *rho2*Δ cells to determine whether there was a defect similar to that observed in *pck2*Δ cells (Katayama *et al.*, 1999). The normal specific localization of Mok1p to the growth areas (Figure 7, left panel) was impaired in 75–80% of the *rho2*Δ cells (Figure 7, center panel). This defect was less pronounced than in *pck2*Δ cells (Figure 7, right panel) in which a dispersed pattern was observed in 100% of the cells.

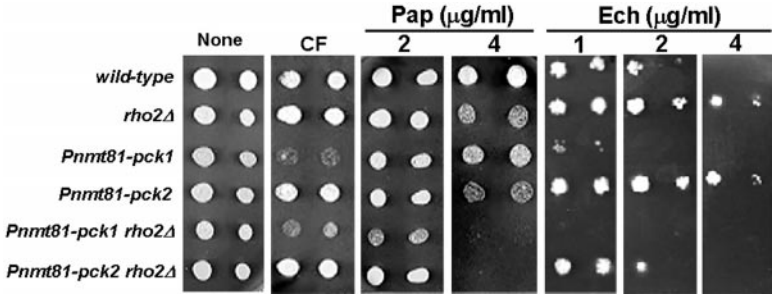


**Figure 2.** Hypersensitivity to cell wall inhibitors of different *S. pombe* strains. Wild-type (HM123), *rho2*Δ (KN-1), *Pnmt81-pck1* (MA20), *Pnmt81-pck2* (MA21), *Pnmt81-pck1 rho2*Δ (TMC110), and *Pnmt81-pck2 rho2*Δ (TMC111) cells were grown at 28°C in EMM with thiamine, spotted on EMM with thiamine plates at $4 \times 10^6$ and $10^6$ cells/ml, and incubated 2–3 d at 28°C. Drug concentrations were as follows: Calcofluor (CF) (0.5 mg/ml), Papulacandin B (Pap) (2 and 4 μg/ml); and Echinocandin (Ech) (1, 2, and 4 μg/ml).

In summary, the results obtained by overexpressing *mok1*$^+$ and localizing Mok1p in different backgrounds indicate that Rho2p and Pck2p, but not Pck1p, regulate Mok1p in a similar manner.

## DISCUSSION

*S. pombe* Rho1p and Rho2p GTPases appear to have different functions. Both affect the morphology of the cells; however, Rho1p is essential and required for the maintenance of (1–3)β-D-glucan synthase activity (Arellano *et al.*, 1997), whereas Rho2p is not essential and does not affect this enzyme (Hirata *et al.*, 1998). As shown in this study, *rho2*Δ cells maintain the normal proportions of all three major cell wall polymers, and the overall incorporation of glucose into this structure is only slightly lower than in a wild-type strain. These results suggest that Rho1p is sufficient to maintain cell wall integrity and can partially substitute for Rho2p (Hirata *et al.*, 1998), whereas Rho2p cannot substitute for Rho1p, because *rho1*Δ cells are inviable. In addition, overexpression of *rho1*$^+$ is deleterious but not lethal and causes a general increase in cell wall biosynthesis, mainly in the β-glucan content (Arellano *et al.*, 1996). In contrast, Rho2p overexpression is lethal and increases the level of α-glucan, whereas it barely changes the β-D-glucan level. Therefore, Rho2p seems mainly to regulate α-D-glucan synthesis. Furthermore, the results suggest that a high increase in the level of this polymer might be lethal for the cells, because overproduction of the α-D-glucan synthase is also lethal (Katayama *et al.*, 1999).

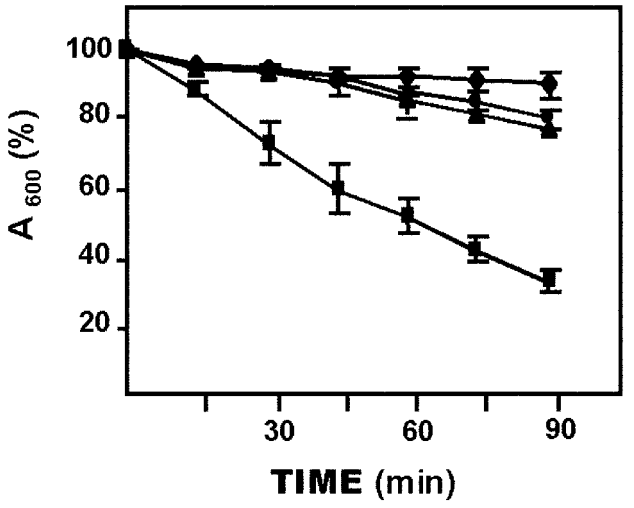


**Figure 3.** Hypersensitivity of *rho2*Δ cells to glucanases is suppressed by *pck2*+ overexpression. *S. pombe* wild-type (◇) and *rho2*Δ (KN-1) cells transformed with the plasmids pREP1 (□), pREP1-*rho2* (○), and pREP1-*pck2* (△) were grown at 32°C in minimal medium with 5 μM thiamine for 20 h and resuspended in TE buffer with 20 mg/ml β-glucanase complex (Zymolyase 100T). Cell lysis was monitored by measuring optical density at 600 nm as a function of time. Values were calculated from at least four independent experiments. Means and SDs are shown.

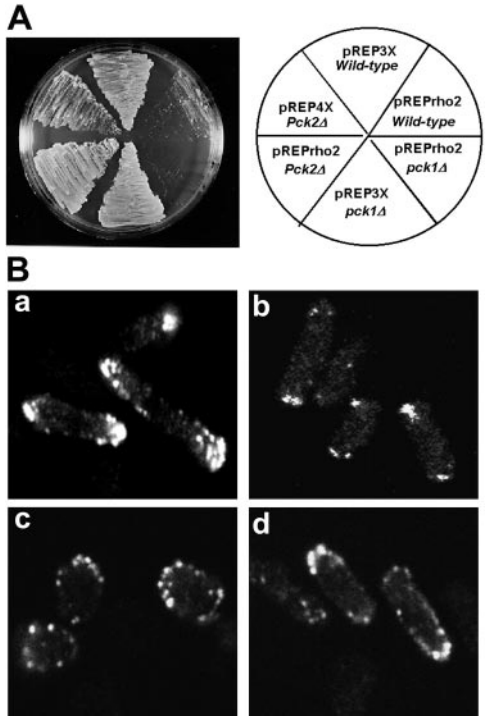


**Figure 4.** *rho2*+ overexpression does not cause any effects in the absence of Pck2p. (A) Growth of wild-type (HM123), *pck1*Δ (TP134–3B), and *pck2*Δ (TP169–1C) cells transformed with pREP3X, pREP4X, pREP3X-*rho2*, or pREP4X-*rho2*. Cells were grown on EMM plates without thiamine. (B) Localization of F-actin in (a) HM123, (b) TP169–1C, (c) HM123 transformed with pREP3X-*rho2*, (d) TP169–1C transformed with pREP3X-*rho2*. Bar, 5 μm.

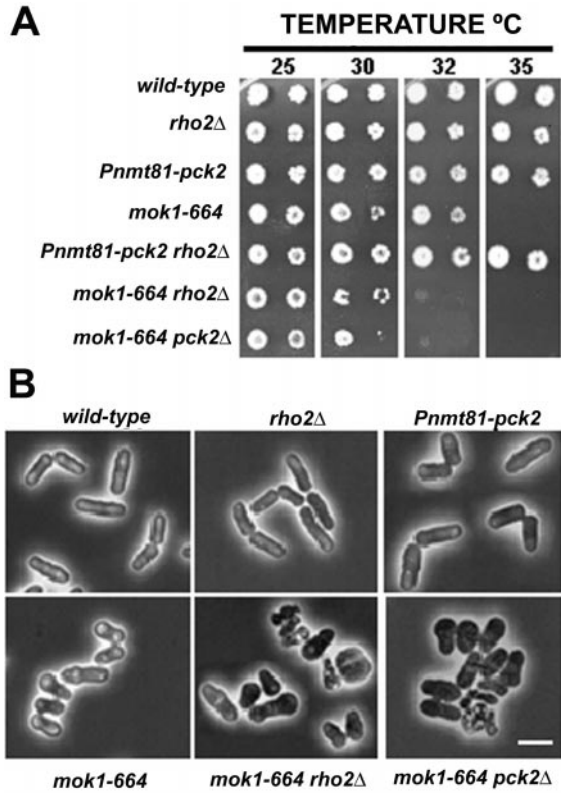


**Figure 5.** Synthetic lethality of *rho2*Δ and *mok1–664* mutations. (A) Wild-type (HM123), *rho2*Δ (KN-1), *Pnmt81-pck2* (MA21), *mok1–664* (DH664), *Pnmt81-pck2 rho2*Δ (TMC111), *mok1–664 rho2*Δ (TMC113), and *mok1–664 pck2*Δ (SKP100) cells were grown in YES medium at 28°C, spotted on YES solid-medium plates, and incubated for 3 d at different temperatures. (B) Phase-contrast micrographs of the same strains grown in YES liquid medium for 20 h at 32°C. Bar, 10 μm.

How does Rho2p signal to stimulate the biosynthesis of α-D-glucan? Our previous results suggested that Pck2p plays crucial roles in the regulation of both (1–3)β-D-glucan synthase and α-D-glucan synthase (Arellano *et al.*, 1999b; Katayama *et al.*, 1999; Sayers *et al.*, 2000). Examination of *rho2*Δ and *pck2*Δ strains by transmission electron microscopy showed similar defects in the cell walls that were indistinguishable in their thickness and staining. The similar phenotypes of *rho2*Δ and *pck2*Δ strains were also revealed using cell wall inhibitors. Although cells lacking Pck1p were hypersensitive to Calcofluor, Papulacandin B, and Echinocandin, both *rho2* and *pck2* null strains were hypersensitive to Papulacandin B but not to Calcofluor, and they were resistant to Echinocandin. Interestingly, *rho2::ura4*$^+$ Pnmt81-pck2 cells grown in the presence of thiamine were not resistant to Echinocandin. If the absence of Rho2p is partially complemented by Rho1p, that might cause a decrease in the available GTP-Rho1p required to directly activate the (1,3)-β-D-glucan synthase. In the absence of Pck2p, that effect would be more dramatic, because this kinase also regulates the (1,3)-β-D-glucan synthase (Arellano *et al.*, 1999b), resulting in a decrease of (1,3)-β-D-glucan that would make the cells more sensitive to Echinocandin. This explanation is consistent with previous data showing that *rho1+* overexpression can partially complement the sensitivity of *rho2*Δ cells to Aculeacin A, an antibiotic with an effect similar to that of Papulacandin B (Hirata *et al.*, 1998). The possibility that Rho2p also signals through another effector is not excluded, but Pck1p is unlikely to be that effector because neither *rho2*Δ cells nor the mutant strain TMC111, lacking both Rho2p and Pck2p when grown in thiamine, is hypersensitive to Calcofluor as would be expected if the Pck1p signaling pathway were impaired. The experiments based on β-glucanase treatment provided confirmatory results for the hypothesis that Rho2p and Pck2p are in the same signaling pathway. Finally, the dramatic changes in actin distribution and cell morphology caused by *rho2*$^+$ overexpression in wild-type or *pck1*Δ cells were not observed in *pck2*Δ cells. Moreover, *rho2*$^+$ overexpression is not lethal and does not have any significant effect on the cell wall of *pck2*Δ cells, supporting the hypothesis that Pck2p is the major Rho2p effector.

The genetic experiments shown in this study also revealed the functional relationship between Rho2p and Mok1p, the major α-glucan synthase. Thus, the double-mutant *rho2*Δ *mok1–664* showed a synthetic lethal interaction in which the cells were round and lysed spontaneously when grown at 32°C, as occurs also in the double-mutant *pck2*Δ *mok1–664* (Katayama *et al.*, 1999). In addition, although a high level of Mok1p was deleterious in *rho2*Δ cells, it caused a different phenotype than overexpression in a wild-type background. The lethality was delayed, the morphology of the cells was different, and the level of α-glucan in the walls was not increased to the same level as when *mok1*$^+$ was overexpressed in a *rho2*$^+$ background.

None of the genetic interactions observed between *mok1 and rho2* or *pck2* could be reproduced with *pck1*, indicating that Pck1p is not involved in α-D-glucan biosynthesis and signals in a different pathway.

Taking together all the available data, we propose that Rho1p regulates (1–3)β-D-glucan biosynthesis in a dual manner, directly and also through Pck2p (Arellano *et al.*, 1999b; Sayers *et al.*, 2000). On the other hand, Rho2p regulates the biosynthesis of α-D-glucan exclusively through Pck2p, because Rho2p overproduction results in an increase of the cell wall α-D-glucan only when Pck2p is present. Rho1p interacts with, stabilizes, and localizes Pck2p to the growth areas (Arellano *et al.*, 1999b, Sayers *et al.*, 2000). Rho2p is also localized to the growth areas, interacts with, and signals to Mok1p through Pck2p. It thus appears that both GTPases use the same kinase to regulate coordinately the biosynthesis of the two main *S. pombe* cell wall polymers.

The regulation of Pck1p and its role in cell wall integrity remain to be established. Besides being able to partially substitute for Pck2p, this kinase seems to be involved in cell integrity through a different pathway, because *pck1*$^+$ also showed genetic interactions with *ras1*$^+$ and *ral1*$^+$ (Arellano *et al.*, 1999b). In addition, at least two more GTPases belonging to the Rho family are present in *S. pombe*, and both are able to interact with Pck1p and Pck2p in the two-hybrid assay (our unpublished results). Clarification of the relationships between these proteins and their direct effectors will require further studies and will help to elucidate the regulation of cell wall assembly and the maintenance of cell integrity.

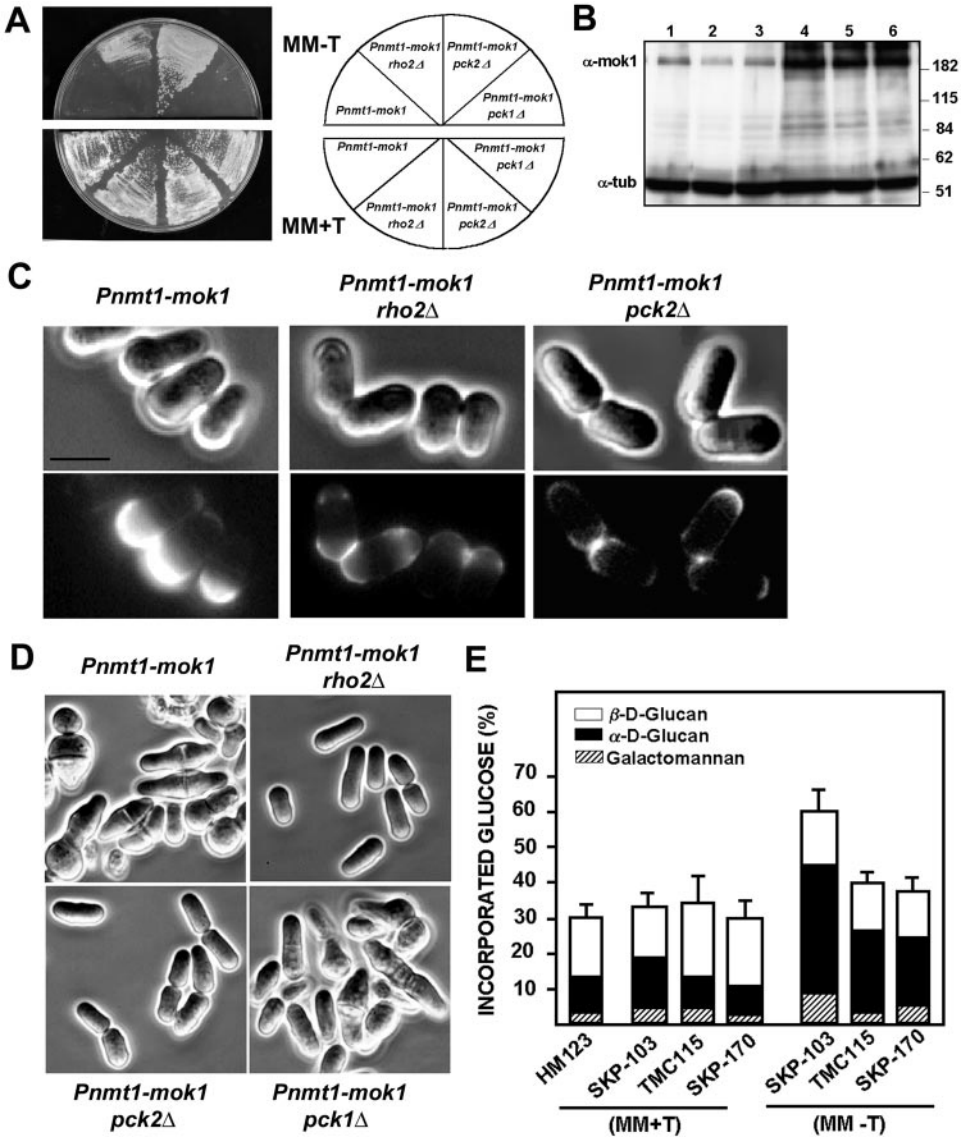


**Figure 6.** Tolerance of *rho2*Δ, *pck2*Δ, and *pck1*Δ strains to *mok1*$^+$ overexpression. (A) Strains SKP103, TMC115, SKP170, and TMC116 (corresponding to wild-type, *rho2*Δ, *pck2*Δ, and *pck1*Δ cells carrying the integrated *Pnmt1–mok1*$^+$ gene, respectively) were streaked on minimal medium in the presence (bottom plate) or absence (top plate) of thiamine and incubated at 28° for 3 d. (B) Mok1p levels in SKP103 (1, 4), TMC115 (2,5), and SKP170 (3,6) cells. Total cell extracts (5 μg) were prepared from cells grown for 14 h in medium with (1–3) or without (4—6) thiamine, run on 7.5% SDS-PAGE, and immunoblotted with anti-Mok1p antiserum and TAT1 anti-tubulin monoclonal antibody. (C) Cell morphologies and Calcofluor staining of the same *S. pombe* strains grown in EMM without thiamine for 14 h. Bar, 8 μm. (D) Morphologies of SKP103, TMC115, SKP170, and TMC116 cells grown in EMM with 5 μM thiamine for 48 h. Bar, 10 μm. (E) Cell wall composition of different *S. pombe* strains analyzed for $^{14}$C-glucose radioactivity incorporated into each cell wall polysaccharide. Strains HM123, SKP103, TMC115, and SKP170 were grown in the presence or absence of thiamine for 18 h, and $^{14}$C-glucose was added 2 h before the cells were harvested. Values are the means of three independent experiments with duplicated samples. SDs for the total carbohydrate values are shown.

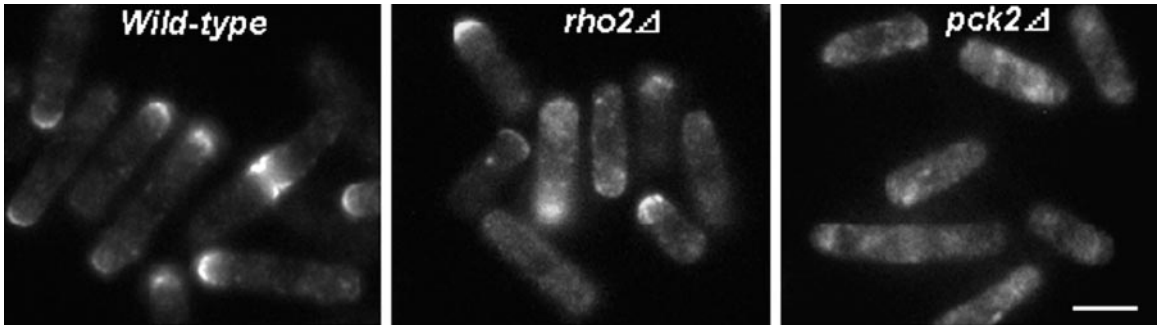


**Figure 7.** Mok1p localization in wild-type, *rho2*Δ, and *pck2*Δ cells. Exponentially growing wild-type (HM123), *rho2*Δ (KN-1), and *pck2*Δ (TP169–1C) cells were fixed and stained for immunofluorescence microscopy using affinity-purified anti-Mok1 antibody. More than 100 cells of each sample were observed. Bar, 5 μm.

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