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Rkp1/CPC2, a RACK1 Homolog, Interacts with Pck1 to Regulate PKC-Mediated Signaling in *Schizosaccharomyces pombe*

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Abstract The Rkp1/CPC2, a receptor for activated protein kinase C of *Schizosaccharomyces pombe*, contains seven WD motifs found in the G-protein β-subunit. A 110-kDa protein was identified to interact with Rkp1/CPC2 by immunoprecipitation and following *in vitro* binding assay. To examine its kinase activity and binding ability to Rkp1, the *pck1*$^+$, a PKC homolog of *S. pombe*, was cloned. Pck1 phosphorylated myelin basic protein (MBP) and histone H1 in a phospholipid-dependent and Ca^{2+}-independent manner. It was demonstrated that the N-terminal region of Pck1 was responsible for the binding to Rkp1, thus suggesting that Rkp1 interacted with Pck1 to regulate Pck1-mediated signaling in *S. pombe*.

Key words: RACK1, Pck1, Rkp1, CPC2, *Schizosaccharomyces pombe*

Protein kinase C (PKC) is a family of phospholipids-dependent serine/threonine kinases that are activated by many extracellular signals. All the PKC subspecies contain the kinase catalytic domain in the C-terminal region and the regulatory domain in the N-terminal region. The C1 region mediates diacylglycerol and phorbol ester binding, and the C2 region present only in the cPKC isotype mediates calcium binding [13, 18].

Two PKC homologs of *S. pombe*, Pck1 and Pck2, have been implicated in the control of cell wall synthesis and actin cytoskeleton organization [11, 17, 19]. One structural feature of Pck1 and Pck2 is the N-terminal extension containing HR1 and HR2-like sequences, similar to that found in mammalian PRK protein [13]. It has been reported that Pck1 and Pck2 are targets of Rho1 and Rho2, and both Pck1 and Pck2 coordinately regulate maintenance of cell integrity [3]. Rho1 is required for the maintenance of cell integrity and polarization of the actin cytoskeleton which activated (1-3)-β-D-glucan synthase through the Pck2 or Pck1 signal cascade [2, 8].

RACKs, intracellular receptors for activated protein kinase C, are G-protein β homologs containing seven WD repeats, and they play roles in recruiting PKCs [6, 14, 16]. RACK1 is also a possible adaptor molecule associated with the intracellular domain of cytokine receptors [15], cytoplasmic phosphodiesterase PDE4D5 isoform [21], SH2 domain of src tyrosine kinase [4], and β-intergrin subunit [12]. The CPC2 gene, *S. cerevisiae* RACK1 homolog, is required for repression of GCN4 protein activity in the absence of amino acid [9]. CpcB of *Aspergillus nidulans* is required for the regulation of sexual development under amino acid starvation [10].

Rkp1/Cpc2, a RACK1 homolog of *S. pombe*, located in the membrane, was isolated, and has been demonstrated to directly interact with Pck2 [21]. It has been suggested that Rkp1/Cpc2 forms a complex with PH domain-containing proteins and is coordinated by regulating actin cytoskeleton organization through pck2-mediated signaling pathways [20].

Based on the fact that Rkp1/CPC2 is a receptor for activated PKC, an attempt was made to find a PKC homolog that binds to Rkp1/CPC2, by immunoprecipitation using anti-Rkp1 antibody, followed by an *in vitro* binding assay. It was found that Rkp1/CPC2 interacted with Pck1 to regulate Pck1-mediated signaling.

Materials and Methods

Strains and Media

S. pombe strain ED665 (*h*$^-$, *ade6-M210, leu1-32, ura4-D18*) was used for gene disruption or the overexpression of *pck1*$^+$ or *rkp1*$^+$, and for the preparation of crude cell

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extracts. *E. coli* DH5α was used for the overproduction of GST- or MBP-fusion proteins. The *S. pombe* cells were grown in YEPD (0.5% yeast extract, 0.5% peptone, 3.0% glucose) or EMM (Edinburgh minimal medium), supplemented with adenine, uracil, and/or leucine as previousty described [1]. Transformation of *S. pombe* cells was carried out by the lithium acetate method and standard yeast genetic techniques as described by Moreno *et al.* [15].

Preparation of Crude Cell Extracts

To prepare *E. coli* crude cell extract, cells grown in LB ampicillin medium were resuspended in NETN buffer (20 mM Tris-HCl, pH 8.0, 5 mM Na_2EDTA, 100 mM NaCl, 0.5% NP-40) containing 5 mM PMSF, and then sonicated and centrifuged. To prepare crude cell extracts of *S. pombe*, cells were harvested and resuspended in PBST (1 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 3 mM KCl, pH 7.0, and 1% Triton X-100) containing 5 mM PMSF and 25 μg/ml each of pepstatin, aprotinin, and leupeptin. Cells were broken using a bead beater for 20 sec, for 5–6 times after adding the same volume of acid-treated glass beads (0.2 mm in diameter) to the cell suspension. The lysed cells were centrifuged at 10,000 rpm for 5 min and the supernatant was used as a crude cell extract.

Immunoprecipitation

The crude cell extracts (2–5 mg protein) of *S. pombe* were pretreated with 50 μl of protein A-agarose beads and incubated overnight with anti-Rkp1 polyclonal antibody or a mixture of anti-peptide PKC monoclonal antibodies (PKC-α, PKC-β, PKC-γ, PKC-δ, PKC-ε, and PKC-ζ; purchased from Gibco BRL, Grand Island, NY, U.S.A.) in the immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Tritone-X100, and 1 mM EGTA). After adding Protein A-agarose to the mixture of cell extracts and antibodies, the whole mixture was incubated for 3 h. Immunoprecipitates were then divided into two; one used immediately for a kinase assay and the other for a protein-protein interaction study.

***In vitro* Binding Assay of Proteins Interacting with Rkp1**

To detect any protein interacting with Rkp1 or a PKC homolog, the immunoprecipitates on protein A-agarose beads were washed with PKC binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, 200 mM NaCl, and 1 mM $CaCl_2$) and mixed with *S. pombe* crude cell extracts, and the mixture was incubated for 2 h at 4°C in the PKC binding buffer containing phospholipid micelles formed by sonicating phosphatidylserine (80 μg/ml) and diacylglycerol (20 μg/ml). Proteins bound nonspecifically were removed by washing beads twice with PKC binding buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT). The protein bound to immunoprecipitates on the protein A-agarose beads was detected by Western blot analysis and used for kinase assay. Western blot analysis was carried out with anti-peptide PKC antibodies (PKC-α, PKC-β, PKC-γ, PKC-δ, PKC-ε, and PKC-ζ; purchased from Gibco BRL, Grand Island, NY, U.S.A). To confirm the binding of Rkp1 and PKC homolog, cell extracts prepared from wild-type and Rkp1-deleted cells were used in the same assay using anti-Rkp1 antibody as described above. GST antibody was used as a negative control.

Kinase Assay

The immunoprecipitates were washed with a kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) and kinase activity was measured using myelin basic protein (MBP) or histone H1 as a substrate as described [21]. The immunoprecipitates on the agarose beads were incubated in 30 μl of a kinase buffer containing 100 μg/ml MBP or histone H1, 20 μM ATP, 40 μCi/ml [γ-^{32}P]ATP for 30 min at 30°C in the presence or absence of 1 mM $CaCl_2$ or phospholipid micelles formed by sonicating phosphatidylserine (80 μg/ml) or diacylglycerol (20 μg/ml).

Cloning of *pck1*$^+$ Gene of *S. pombe*

The DNA fragment containing the coding region of *pck1* was cloned by PCR based on the DNA sequence (GenBank accession No. D14337). The DNA fragment, encoding an entire coding region (Pck1F, amino acids 1-988) or a part of Pck1 (Pck1NC, 1-408 and 555-988; Pck1N, 1-408; Pck1CC, 409-988; Pck1C, 555-988), was fused to the 3'-end of *MalE* that encodes a maltose binding protein (M), resulting in M-fusion proteins.

***In vitro* Binding Assay of Rkp1 with M-Pck1 Fusion Protein**

M-Pck1 fusion proteins were purified on amylose agarose as described by the manufacturer (New England Biolab., Beverly, MA, U.S.A.), and then incubated with an *E. coli* crude cell extract containing GST-Rkp1 fusion protein in the PKC binding buffer, in the presence of phospholipid micelles and Ca^{2+}, for 1 h. After washing the agarose beads, the proteins on the beads were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and analyzed with the anti-Rkp1 polyclonal antibody.

Disruption of *rkp1* and *pck1* Genes

The DNA coding region of the *rkp1*$^+$ gene was disrupted using the one-step gene disruption method. The 1.8 kb DNA of the *ura4*$^+$ marker gene was inserted at the 308 base pair (bp) downstream from the translation start site of *rkp1*$^+$. The *pck1*$^+$ gene was disrupted by insertion of the *leu2*$^+$ marker, resulting in truncating the entire ORF of the *pck1*$^+$ gene. The resulting linear DNA fragment, containing the *leu2*$^+$ marker with the 3'- and 5'-UTR regions of *pck1*$^+$,

was used to transform a haploid strain ED665*h*⁻. Disruption of the *rkp1*⁺ and *pck1*⁺ genes was confirmed by Southern and Northern blot analyses (data not shown).

Microscopic Examination of Cells

The shape of *rkp1*- or *pck1*-disruped cells were examined under a microscope (Zeiss Laboval4). Cells were photographed by DIC (differential interference contrast). Cell morphology and septa were examined under a fluorescence microscope after staining cells in calcoflour solution (5 mg/ml) for 15 min.

Results and Discussion

Rkp1/CPC2 Can Bind to the PKC Homolog of *S. pombe* and Enhance Kinase Activity

RACKs, receptors for activated protein kinase C, play a role in the translocation of PKCs from one intracellular location to another [6, 22]. Based on the fact that Rkp1/CPC2, a RACK1 homolog of *S. pombe*, is a receptor for activated PKC, a PKC homolog was screened using immunoprecipitaion with anti-Rkp1 antibody, followed by *in vitro* binding assay in the PKC binding buffer (Fig. 1). Potential PKC homolog in the immunoprecipitation-complex was examined by a kinase assay using myelin basic protein (MBP) as a substrate (Fig. 1A). Only the immunoprecipitate with the anti-peptide PKC antibody showed kinase activity (lane 1), suggesting that antibodies used for immunoprecipitation had cross-reactivity with a potential PKC homolog of *S. pombe*. As expected, the immunoprecipitates with anti-Rkp1 antibody phosphorylated MBP slightly, indicating no kinase activity (lane 1). Surprisingly, when the immunoprecipitates with anti-peptide PKCs (lane 3) or anti-Rkp1 antibody (lane 4) were incubated with crude *S. pombe* cell extract in the PKC binding buffer, in the presence of phospholipid micelles and Ca^{2+}, the kinase activity to phosphorylate MBP increased remarkably. This result indicated that the interaction of Rkp1 with a potential PKC homolog increased the kinase activity of a potential PKC homolog. When anti-GST antibody was used as a negative control, no distinct kinase activity was detected, compared with that of anti-Rkp1 antibody (data not shown).

To confirm the ability of Rkp1 to bind to a PKC homolog, the phospholylation assay of the immunoprecipitate-binding complex was carried out in cell extracts of the wild-type or *rkp1*-deleted cells (Fig. 1A-b). Phosphorylation of MBP was observed when the crude cell extracts of wild-type cells was used for assay (lane 2), whereas little kinase activity was evident in crude cell extracts of Rkp1-deleted cells (lane 3). This result indicated that Rkp1 interacted with a potential PKC homolog of *S. pombe*.

Substrate preference of a potential PKC homolog was examined using MBP or H1. As shown in Fig. 1B, MBP

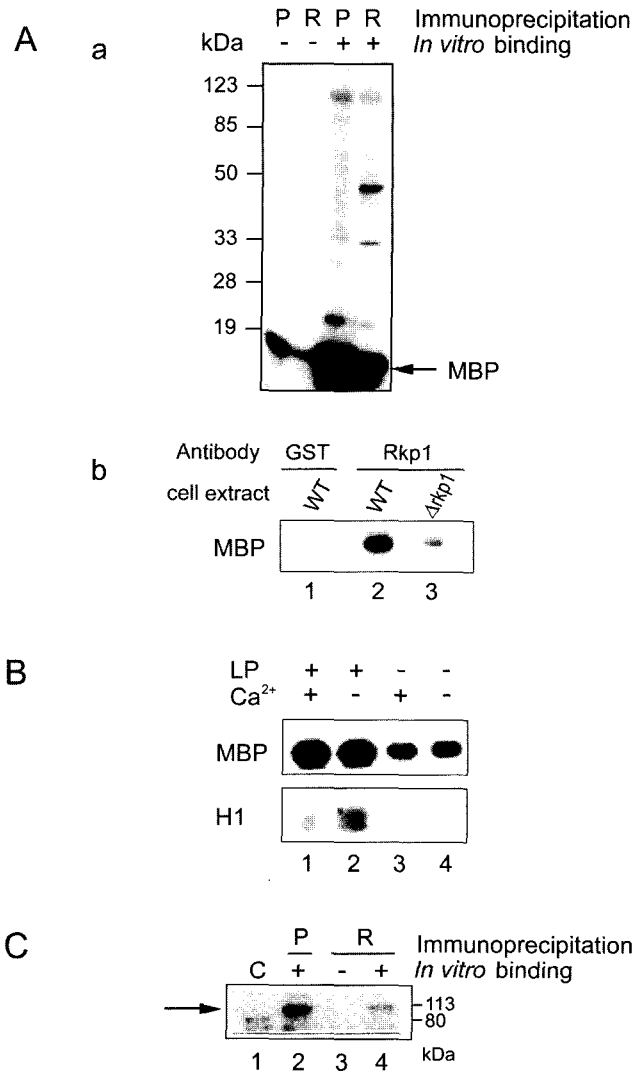

Fig. 1. Interaction of Rkp1/CPC2 with a potential PKC homolog of *S. pombe*.

Immunoprecipitation was carried out with a mixture of mammalian anti-peptide PKC monoclonal antibodies (P), or anti-Rkp1 polyclonal antibody (R), and *in vitro* binding with *S. pombe* crude cell extract. **A. a.** Kinase activity of a potential PKC was measured by phosphorylation of myelin basic protein (MBP) with [γ-^{32}P]ATP in the presence of phospholipids and $CaCl_2$. The arrow indicates MBP. Immunoprecipitates with anti-PKC antibody only (lane 1); anti-Rkp1 antibody only (lane 2); immunoprecipitation with anti-PKC antibodies (lane 3); and anti-Rkp1 antibody (lane 4) followed by *in vitro* binding with crude cell extract of *S. pombe*. **b.** Kinase activities of a potential PKC homolog prepared by immunoprecipitation with anti-Rkp1 antibody of extracts from wild-type (lane 2) and Rkp1-deleted cells (lane 3) were compared. Anti-GST antibody was used as a negative control. **B.** Substrate preference and phospholipid requirement of a potential PKC homolog of *S. pombe*. Kinase activity of immunoprecipitates complex was assayed using MBP or HI (histone I) as a substrate, in the presence or absence of phosphatidylserine (80 μg/ml) and diacylglycerol (20 μg/ml) or Ca^{2+} (1 mM). **C.** The proteins in immunoprecipitates were analyzed by Western blotting with anti-peptide PKCs antibody. A potential PKC homolog of *S. pombe* is indicated by an arrow. Lane 1, partially purified PKC-β (cPKC) from rat brain; lane 2, immunoprecipitate with anti-PKC antibodies; lane 3, immunoprecipitate of anti-Rkp1 antibody only; lane 4, immunoprecipitate of anti-Rkp1 antibody followed by *in vitro* binding with a crude cell extract of *S. pombe*.

(upper panel) was a better substrate for phosphorylation than H1 (lower panel). The phosphorylation increased in the presence of phosphatidylserine and diacylglycerol (lanes 1 vesus 3), while Ca^{2+} did not affect phosphorylation of MBP (lanes 1–2). Thus, it was clear that the potential PKC homolog of *S. pombe* interacting with Rkp1 showed substrate specificity and phosphorylated substrates in a phospholipids-dependent and Ca^{2+}-independent manner.

Western blot analysis was attempted in order to visualize a PKC homolog using the mixture of anti-peptide PKC antibodies (Fig. 1C), because immunoprecipitates of anti-peptide PKC antibodies showed kinase activity (Fig. 1A). The rat PKCβ was used (lane 1) as a control. A 110-kDa protein formed by immunoprecipition with an anti-peptide PKC antibody was detected (lane 2). The same sized 110-kDa protein was also detected, when immunoprecipitate of anti-Rkp1 antibody (lane 4) was allowed to bind to a PKC homolog in the PKC binding buffer containing phospholipid micelles and Ca^{2+}. Considering the possibility that a 110-kDa protein could be a potential PKC homolog of *S. pombe*, common sequences between epitope sequences of peptide antibodies and known PCK homologs of *S. pombe* similar in size to 110 kDa were examined. After comparing the amino acid sequences of peptides used for antibody production and two PKC homologs of *S. pombe*, Pck1 and Pck2, the pentapeptide of PKTPE in the epitope of PKCβ was found to be present in amino acids 604 and 608 of Pck1. Pck2 was excluded, since it does not contain a PKTPE sequence. Immunoprecipitation using anti-PKCβ antibody, followed by an *in vitro* binding assay, was also carried out to confirm that anti-PKCβ antibody had cross-reactivity with a potential PKC homolog and that kinase activity was enhanced after *in vitro* binding (data not shown).

Rkp1 Interacted with Pck1, a PKC Homolog of *S. pombe*

A 110-kDa protein cross-reacted with anti-peptide PKC antibody which had interacted with Rkp1. The fact that the Pck1 and epitope sequence of anti-peptide PKCβ share the common pentapeptide of PKTPE suggests a possibility that the 110 kDa protein might be a Pck1. To examine whether Rkp1 was related to the function of Pck1 or not, the coding region of *pck1* was cloned by PCR, and each domain of Pck1 was constructed to overproduce as M (maltose binding protein)-fusion proteins in *E. coli* (Fig. 2A). The purified M-Pck1F containing the entire polypeptide of Pck1 was then tested for kinase activity using MBP as a substrate (Fig. 2B-a). The phosphorylation of MBP increased in the presence of phosphatidylserine and diacylglycerol (lanes 1–2), while Ca^{2+} did not affect phosphorylation (lanes 1 and 3). In the domain analysis, MBP was phosphorylated with full-length Pck1 (Pck1F) (Fig. 2B-b, lane 1), indicating that whole Pck1 was required for kinase activity.

An *in vitro* binding assay was performed to examine the binding ability of Rkp1 to Pck1 (Fig. 2C). The immobilized

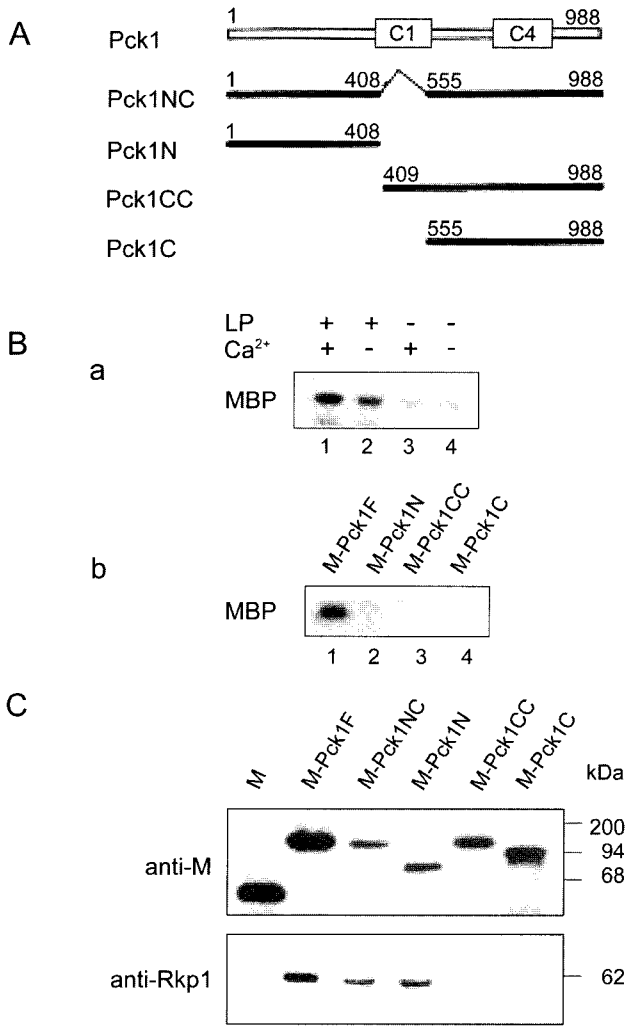

Fig. 2. Interaction of Rkp1/CPC2 with Pck1.
A. M (maltose-bonding protein)-Pck1 fusion proteins were constructed using pMAL-cR1. The Pck1 domains fused to M include entire Pck1 (amino acid 1-988, Pck1F). Each part of Pck1, Pck1NC (amino acid 1-409 and 555-988), Pck1N (1-408), Pck1CC (409-988), and Pck1C (555-988). **B.** Kinase activity of M-Pck1 fusion proteins. **a.** The requirement of phospholipid or Ca^{2+} for kinase assay was examined. Purified M-Pck1F fusion protein was used in kinase assay. **b.** Pck1 domain was analyzed for phosphorylating activity using M-fusion proteins. **C.** Interaction of Rkp1 with M-Pck1 *in vitro*. *In vitro* binding assay of Rkp1 and M (maltose-binding protein) or M-Pck1 fusion protein was performed to find the domain interacting with Rkp1. Different sizes of M and M-Pck1 domain fusion proteins were detected by Western blot with anti-M antibody. The GST-Rkp1 bound to Pck1 domain was detected with anti-Rkp1 polyclonal antibody.

M-Pck1 fusion proteins on amylose agarose beads were incubated with crude *E. coli* cell extracts containing GST-Rkp1, resulting in the bound Rkp1 which was detected with anti-Rkp1 antibody. The full-length Pck1 (M-Pck1F) was shown to bind to GST-Rkp1 *in vitro* (lane 2). When Pck1, lacking the cysteine rich C1 domain sequence (M-Pck1NC) or containing only the N-terminal half sequence lacking the C1 and catalytic domain (M-Pck1N), was used in the binding assay, it interacted with Rkp1 (lanes 3 and

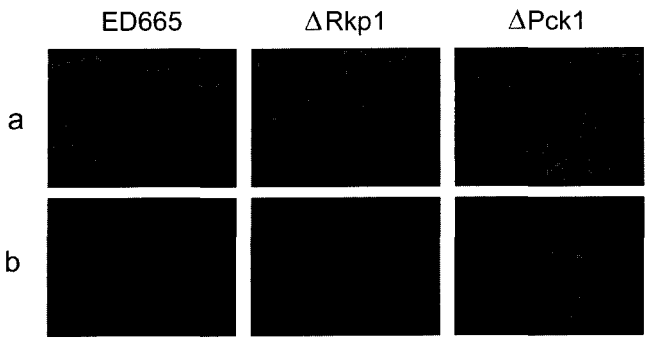

Fig. 3. Cell morphology.
The morphological changes of wild-type cells, rkp1-null mutant (Drkp1), and *pck1*-null mutant (*Δpck1*) were observed under a microscope. Cells were photographed by DIC (differential interference contrast) in **a**. The calcoflour stained cells were examined under a fluorescence microscope, as shown in **b**.

4). However, when Pck1 missing the N-terminal 408 amino acid (M-Pck1CC) or containing the C-terminal with only the catalytic domain (M-Pck1C) was used, Pck1 did not bind to Rkp1/CPC2 *in vitro* (lanes 5 and 6). This result indicated that the N-terminal region of Pck1 is responsible for the interaction with Rkp1/CPC2.

Disruption and Overexpression of *pck1*

In order to elucidate the biological function of Rkp1/CPC2, deletion or overexpression of *rkp1*$^+$ or *pck1*$^+$ was carried out and the mutant phenotype was examined. The *rkp1*$^+$-disrupted cells were viable and had a slightly slender cell shape (Fig. 3), whereas *pck1*$^+$-disrupted cells were viable and did not show distinctive morphological change. This indicated that neither *rkp1*$^+$ nor *pck1*$^+$ was an essential gene for cell viability. When *rkp1*$^+$ or *pck1*$^+$ was overproduced under *nmt1* promoter control in the absence of thiamine, the cells did not show a distinctive phenotype (data not shown). This observation was consistent with a previous report [3]. Overall, Pck1 seemed to be less related to morphogenesis than Pck2.

The factors influencing the activation of Pck1 and Pck2 and the signaling mechanism are poorly characterized. Compared to Pck2, the functional contribution of Pck1 to the biological function, morphology, cell wall formation, and protoplast formation is not evident. This suggests that the function of Pck1 remains to be identified. It should be noted that, in many species, RACK homologs function as scaffolding proteins in different ways, depending on the binding proteins. Rkp1/CPC2 interacts with Pck1 and may mediate signal change in the Pck1-dependent pathway in *S. pombe*.

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