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Rkp1/Cpc2, a Fission Yeast rACK1 Homolog, Is Involved in Actin Cytoskeleton Organization through Protein Kinase C, Pck2, Signaling

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The Rkp1/Cpc2, a fission yeast RACK1 homolog, interacted with Pck2, one of the known PKC homologs, *in vivo* and *in vitro*. The *rkp1*-deletion mutants (Δ*rkp1*) are elongated and the *pck2*-deletion mutant (Δ*pck2*) showed abnormal morphology. The double-deletion mutant (Δ*rkp1*Δ*pck2*) showed more aberrant cell shapes and was sensitive to high salt concentration. Both Δ*rkp1* and Δ*pck2* cells were sensitive to latrunculin B (Lat B) which inhibits actin polymerization. The cells expressing the human RACK1 homolog complemented the latrunculin B sensitivity of Δ*rkp1* indicating that human RACK1 is a functional homolog of Rkp1/Cpc2. We propose that Rkp1/Cpc2 may function as a receptor for Pck2 in the regulation of actin cytoskeleton organization during cell wall synthesis and morphogenesis of *Schizosaccharomyces pombe*.

Key Words: **Rkp1; Cpc2; RACK1; Pck2; actin cytoskeleton; *S. pombe*.**

Protein kinase C is a family of phospholipid-dependent serine/threonine kinases, which are activated by many extracellular signals. PKCs are regulated by a variety of lipid secondary messengers and suggested to play a fundamental role in cell signaling mechanism leading to the proliferation and mitogenesis of cells, apoptosis, platelet activation, remodeling of the actin cytoskeleton, modulation of ion channels, and secretion.

The Pkc1p of the budding yeast *Saccharomyces cerevisiae* regulates a MAP kinase-activation cascade comprised of a Bck1p, Mkk1/2p, and Mpk1p (1, 2). Rho1p, which is involved in the regulation of actin cytoskeleton organization, associates with Pkc1p *in vivo* and *in vitro* and stimulates Pkc1p activity (3). Two protein kinase C homologous genes, *pck1*$^+$ and *pck2*$^+$, were isolated from fission yeast *Schizosaccharomyces pombe* (4). The *pck2*$^+$ gene is required for protoplast regeneration and involved in cell wall formation and cell shape control (5). Rho1 protein of *S. pombe* is required for maintenance of cell integrity and polarization of the actin cytoskeleton by activating (1–3)-β-D-glucan synthase through the Pck2 or Pck1 signal cascade (6, 7). Recent study reported that Pck1 and Pck2 are targets of Rho1 and Rho2, and both Pck1 and Pck2 proteins coordinately regulated maintenance of cell integrity (5).

Intracellular receptors for activated protein kinase C, called RACKs, have been identified as the molecules that bind to PKC and play roles in recruiting PKCs from one subcellular site to another (8, 9). They belong to a superfamily of G protein β subunit containing seven WD repeats. A RACK1 homolog of *S. cerevisiae, CPC2,* is required for repression of GCN4 protein activity in the absence of amino acid starvation (10). McLeod *et al.* have recently reported that Cpc2 of *S. pombe* interacted with Ran1 to regulate cell cycle progression and meiotic development (11).

We isolated *rkp1*$^+$ gene that has the same sequences as *cpc2*$^+$ from *S. pombe* separately from McLeod *et al.* and studied its biological role in relation with protein kinase C, Pck1 and Pck2, in *S. pombe*. We report here that Rkp1/Cpc2 may function in the regulation of actin cytoskeleton organization through Pck2-signaling during morphogenesis of *S. pombe*.

MATERIALS AND METHODS

Strains and media. S. pombe strains SP286(h^-/h^+) and ED665(h^-, *ade6, leu2, ura4*) were used for gene disruption, overex-

Abbreviations used: PKC, protein kinase C; RACK, receptor for activated protein kinase C; MBP, maltose binding protein; GST, glutathione *S*-transferase; GFP, green fluorescence protein of jelly fish *Aequorea victoria*.

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pression of *pck2*$^+$ or *rkp1*$^+$, and for preparation of crude cell extracts. *Escherichia coli* DH5α was used for overproduction of GST– or MBP–fusion protein. The *S. pombe* strains 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.

Localization of Rkp1 in S. pombe. To examine the location of Rkp1 inside cells, cell membrane and cytosolic fractions were separated by centrifugation as described (8). Cells were resuspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM EGTA, and 0.25 M sucrose) containing 25 μg/ml each of leupeptin and aprotinin and 1 mM phenylsulfonyl fluoride (PMSF), broken in a beadbeater, and centrifuged at 5000 rpm for 3 min. The supernatant was centrifuged at 60,000 rpm for 1 h and the resulting supernatant was used as a cytosolic fraction. The proteins in the pellet were solubilized by treating with 1% Triton X-100 for 30 min at 4°C and used as the extract of the particulate fraction. The amount of Rkp1 in each fraction was measured by Western blotting with the anti-Rkp1 polyclonal antibody.

The location of Rkp1 was also examined by using GFP–Rkp1 fusion construct. Coding region DNA of *rkp1*$^+$ was cloned into 3′-end of GFP that is under thiamine regulated promoter, *nmt1,* in pREP41–GFP vector. The resulting plasmid was used to transform *rkp1*-deleted strain. For mild expression of GFP–Rkp1 protein, the cells were grown in YEPD medium instead of minimal medium lacking thiamine for 3–7 h. The location of expressed GFP–Rkp1 protein was examined under the fluorescence microscope.

Cloning of fission yeast pck2 and human RACK1 genes. The DNA fragment containing the coding region of *pck2*$^+$ (GenBank Accession No. d14337) or human RACK1 (GenBank Accession No. M24194) was cloned by PCR using cDNAs.

In vitro binding assay of proteins interacting with Rkp1. To generate GST–Rkp1 fusion protein, the coding region DNA of *rkp1*$^+$ was cloned into *E. coli* expression vector pGEX-4T. The coding region DNA of *pck2*$^+$ was cloned into the 3′-end of *MalE* gene of pMAL-cR1 (New England Biolabs Inc., Beverly, MA) resulting in production of MBP–Pck2 fusion protein. The produced MBP–Pck2 fusion protein was purified on amylose agarose beads and incubated with *E. coli* crude cell extracts expressing GST–Rkp1 fusion protein. This reaction was carried out in PKC binding buffer (20 mM Tris–HCl, pH 8.0, 1 mM β-mercaptoethanol, 200 mM NaCl, and 1 mM $CaCl_2$) containing 25 μg/ml of leupeptin and aprotinin, and 1 mM PMSF in the presence of phospholipid micelles and Ca^{2+}. The proteins bound to MBP–Pck2 were subjected to electrophoresis on a SDS–polyacrylamide gel and analyzed with antibodies.

Protein binding assay using copurification of GST-fusion protein overexpressed in S. pombe. The coding region DNA of *pck2*$^+$ was cloned into thiamine regulated GST-fusion vector pESP1 to produce GST–Pck2 fusion protein in *S. pombe.* The GST–Pck2 was overexpressed by growing cells containing plasmid pESP1-*pck2* in minimal medium lacking thiamine. GST–Pck2 produced in *S. pombe* was purified on glutathione agarose beads as described by the manufacturer (Clontech Lab. Inc., Palo Alto, CA). The purified GST–Pck2 on GST–agarose beads was subjected to electrophoresis on SDS–polyacrylamide gel. The proteins associated with Pck2 *in vivo* were detected by using anti-Rkp1 antibody.

Disruption of rkp1 and pck2 genes. The coding region DNA 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 *pck2*$^+$ gene was disrupted by inserting the *leu2*$^+$ marker and by truncating the DNA fragment of nucleotide 350–2720 of gene. The resulting linear DNA fragment containing the *ura4*$^+$ or *leu2*$^+$ marker with the 3′- and 5′-flaking region of *rkp1*$^+$ or *pck2*$^+$ was used to transform a homothallic diploid strain SP286, or a heterothallic haploid strain ED665*h*$^-$. Disruption of the *rkp1*$^+$ and *pck2*$^+$ genes was confirmed by Southern and Northern blotting analyses.

Salt osmosensitivity assay. To test salt osmosensitivity of deletion mutants, the cells in midlogarithmic phase were serially diluted (1:4), spotted on the plate containing 1.4 M sorbitol or 0.9 M NaCl and grew for 3–4 days at 30°C.

Halo assay. Halo assay to determine sensitivity to Lat B (Calbiochem Inc., Darmstadt, Germany) was performed as previously described (12). The cells of midlogarithmic phase were added to 1.8 ml of appropriate selective minimal media and mixed with 1.8 ml of 1% agar solution and poured onto the selective minimal media plates. Lat-B was diluted in DMSO and 30 μl of indicated concentration of Lat-B (0, 1, 2, and 4 mM) was pipetted into 6-mm filter discs placed on the selective minimal plates. Cells were grown for 3–4 days at 30°C and relative sensitivity was calculated by comparing the diameters of halos.

RESULTS AND DISCUSSION

A genomic DNA encoding a RACK1 homolog, a receptor for activated protein kinase C of rat, was isolated from *S. pombe* based on the cDNA sequences previously reported (13) and renamed as *rkp1*$^+$ as receptor for protein kinase of *S. pombe* (GenBank Accession Nos. L37885 and AF320333). Recently, the *cpc2*$^+$ was reported as the same gene as *rkp1*$^+$ (11).

The location of Rkp1/Cpc2 inside cell was examined using fractionated cell extracts (Fig. 1A). When the proteins in each fraction were analyzed with anti-Rkp1 antibody, Rkp1 was detected in the particulate fraction (lane 5) but not in the cytosolic fraction (lane 3) of the wild-type cells. This result is consistent with the fact that the RACKs were purified from the cell particulate fraction of rat heart and brain (16). *In situ* localization of GFP–Rkp1 protein showed that Rkp1 is localized to the cell membrane (Fig. 1B-a).

The ability of Rkp1 to interact with the protein kinase C of *S. pombe,* Pck2, was also examined (Fig. 2). In the *in vitro* binding assay using the purified MBP–Pck2 on amylose beads, Rkp1 fused in GST and expressed in *E. coli* bound to Pck2 (Fig. 2A, lane 3) indicating Rkp1 interacts directly with Pck2. In the assay determining whether Rkp1 was copurified with GST–Pck2 from *S. pombe* or not (Fig. 2B), we observed that Rkp1 in the wild type *S. pombe* cells was copurified with GST–Pck2 (lane 5). However, Rkp1 was absent when cell extracts prepared from Rkp1-deleted *S. pombe* cells (lane 6). The direct interaction of Rkp1 with Pck2 both *in vitro* and *in vivo* implies that Rkp1 may play an important role in Pck2-mediated signaling.

Deletion of *rkp1* or *pck2* alone did not lead cell death indicating that neither *rkp1*$^+$ nor *pck2*$^+$ are essential for cell viability. However, the microscopic observation of these deletion mutants demonstrated that *rkp1*-deleted cells (Δ*rkp1*) were elongated (Fig. 3A-b), and the *pck2*-deleted cells (Δ*pck2*) cells were round, short or bent (Fig. 3A-c). The double-deletion mutant, Δ*rkp1*Δ*pck2,* showed highly elongated, bent or branched amorphous morphology (Fig. 3A-d). These results indicate that both Rkp1

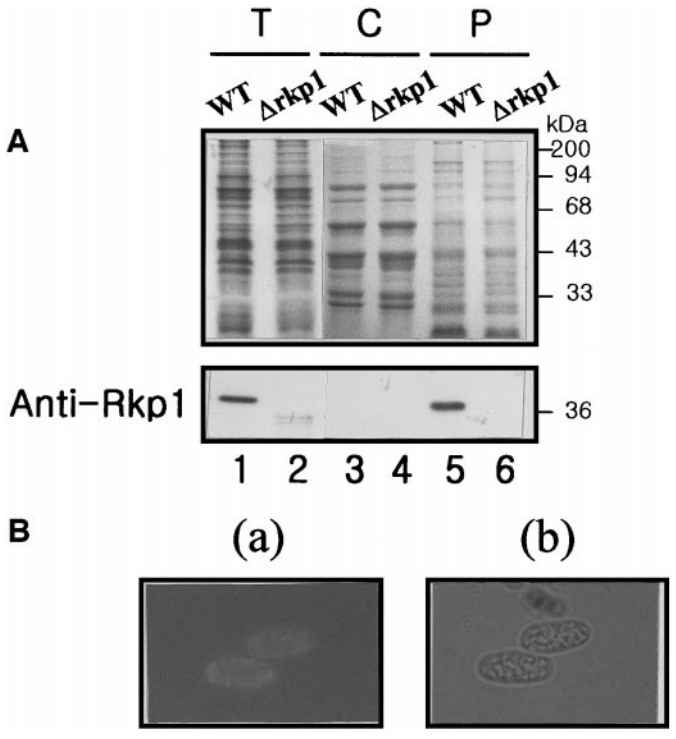

FIG. 1. Localization of Rkp1. (A) The crude cell extracts prepared from wild-type ED665h^- (lanes 1, 3, and 5) and Rkp1-disrupted cell (lanes 2, 4, and 6) were fractionated into cytosol and particulate fraction by centrifugation. The proteins in the crude extract (T, lanes 1 and 2), cytosolic fraction (C, lanes 3 and 4) and particulate fraction (P, lanes 5 and 6) were resolved in a 12% SDS–acrylamide gel (upper panel) and Rkp1 was detected by Western blotting with anti-Rkp1 polyclonal antibody (lower panel). (B) *In situ* localization of Rkp1. The *S. pombe* cells containing GFP–Rkp1 fusion plasmid were grown in YEPD medium lacking thiamine for 3–7 h to induce GFP–Rkp1 protein. Then the cells were visualized under fluorescence (a) and DIC (b) microscope.

and Pck2 are involved in the regulation of cell polarity and morphogenesis.

When growth phenotypes of these deletion mutants were examined in liquid culture (Fig. 3B), the *rkp1*-deleted cells (Δ*rkp1*) grew normally with retarded growth rate. The *pck2*-deleted cells (Δ*pck2*) cells ceased cell growth at lower density than wild type and double-deletion mutant (Δ*rkp1*Δ*pck2*) showed slower growth rate during logarithmic phase suggesting that deletion of both $rkp1^+$ and $pck2^+$ affects vegetative growth. Survivability of the double-deletion mutant dropped dramatically when these cells are transferred to a fresh medium after 100–140 h of cell growth (Fig. 3C).

Thus, the effect of environmental stress on cell growth was examined. Neither glucose nor nitrogen starvation affected the growth of deletion mutants (data not shown). In the experiment examining the salt osmosensitivity, however (Fig. 3D), the growth of neither Δ*pck2* nor Δ*rkp1* cells was affected by the presence of 1.4 M sorbitol. But in the medium containing 0.9 M NaCl, the growth of Δ*rkp1* was slightly inhibited and Δ*pck2* cells did not grow well. In contrast, the double-deletion mutant, Δ*rkp1*Δ*pck2*, cells hardly grew. This phenotype of osmosensitivity to high salt concentration is frequently observed in the strains containing mutations in the genes required for the actin cytoskeleton organization.

Yeast strains carrying mutations in the genes encoding proteins implicated in regulation of actin cytoskeleton showed altered sensitivity to the reagents that disrupt actin polymerization (12). Latrunculin B (LatB), a cell-permeable marine toxin, is known to bind to monomeric G-actin and inhibit actin polymerization. To investigate the sensitivity of the mutant cells to

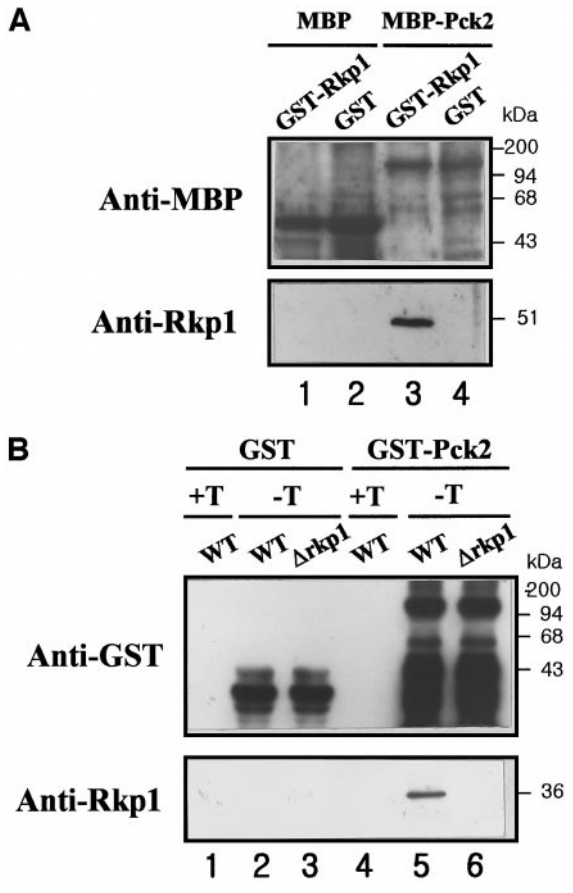

FIG. 2. Binding of Rkp1 with Pck2. (A) *In vitro* binding of GST–Rkp1 to MBP–Pck2 fusion protein. The coding region DNA of $pck2^+$ was fused to the 3′-end of maltose binding protein gene, MalE, in pMAL-cR1 vector. MBP (lanes 1 and 2) and MBP–Pck2 fusion proteins (lanes 3 and 4) on amylose agarose beads were incubated with *E. coli* cell extracts containing GST–Rkp1 fusion protein in the presence of phospholipids and Ca^{2+} for 1 h. GST–Rkp1 and MBP–Pck2 were detected with anti-Rkp1 and anti-MBP polyclonal antibody, respectively. (B) *In vivo* binding of GST–Pck2 to Rkp1. GST (pESP1) or GST–*pck2* fusion plasmid (pESP1-*pck2*) was introduced into the wild-type (lanes 1 and 2 and 4 and 5) and Rkp1-deleted (Δ*rkp1*) *S. pombe* cells (lanes 3 and 6). The transformants were grown in the presence (lanes 1 and 4) or the absence (lanes 2 and 3 and 5 and 6) of thiamine. Expressed GST–Pck2 in *S. pombe* cells was purified on glutathione agarose beads. Presence of Rkp1 in the purified GST–Pck2 was detected with anti-Rkp1 antibody.

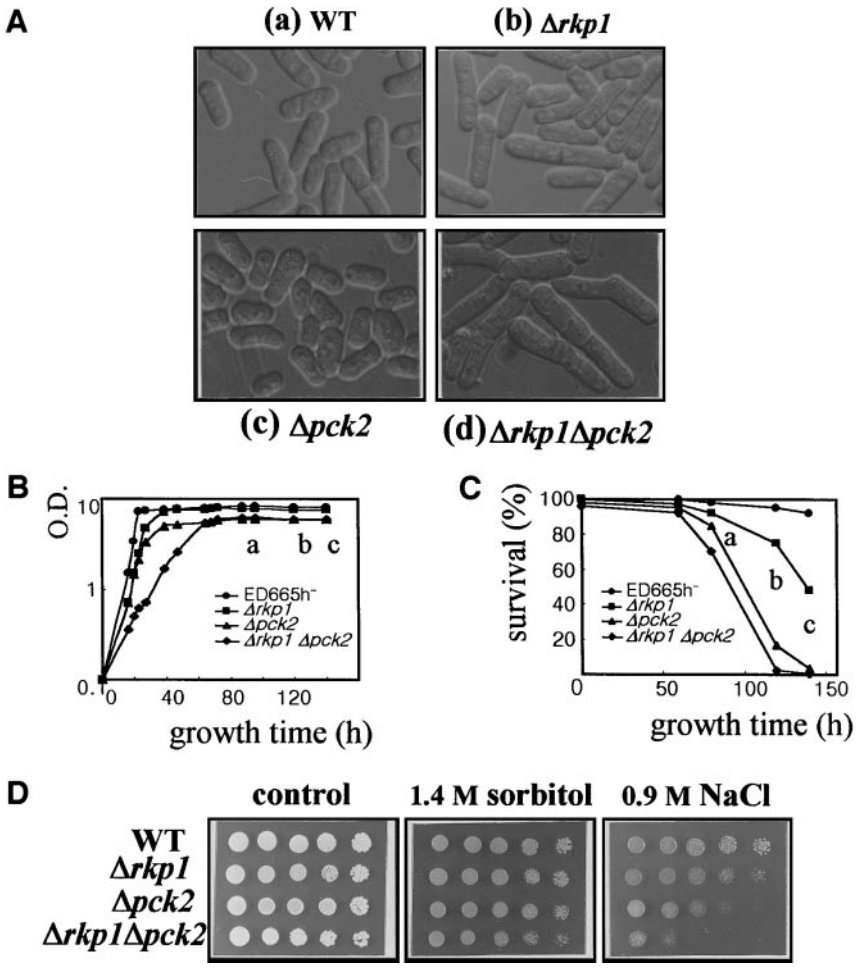

FIG. 3. Phenotypes of deletion mutants of *rkp1* and *pck2*. (A) The morphological changes of the deletion mutants were observed under the microscope. a, wild type cells; b, *rkp1*-null mutant (Δ*rkp1*); c, *pck2*-null mutant (Δ*pck2*); d, double-deletion mutant of both $pck2^{+}$ and $rkp1^{+}$ (Δ*rkp1*Δ*pck2*). (B) Growth curve. The growths of deletion mutants were monitored for 140 h by measuring optical density. (C) Survival test. To examine the survival of deletion mutants, 1×10^{7} cells were plated into fresh rich plates and viable colonies were counted. (D) Salt osmosensitivity assay. To test salt osmosensitivity of deletion mutants, the cells in midlogarithmic phase were serially diluted (1:4), spotted on the plate containing 1.4 M sorbitol or 0.9 M NaCl, and grown for 3–4 days at 30°C.

LatB, a halo sensitivity assay of Δ*rkp1* and Δ*pck2* cells was employed. As shown in Fig. 4, the wild-type cells ED665h^{-} (a) showed a little halo around disc containing 4 mM LatB. The *rkp1*-deleted cells (Δ*rkp1*) (b) showed a clear halo around disc containing 2 mM LatB, which was 2.5 times more sensitive than wild-type cells. The Δ*pck2* cells were 4 times more sensitive than wild-type cells. The Δ*rkp1*Δ*pck2* showed a clear halo around disc containing 2 mM LatB. This hypersensitivity to LatB of mutants of either Δ*rkp1* or Δ*pck2* indicates that both Rkp1 and Pck2 are required for stable filamentous actin network.

To investigate whether or not human RACK1 homolog can complement the function of Rkp1, human RACK1 was expressed in *rkp1*-deleted strain and the sensitivity to LatB was examined. As shown in Fig. 5, growth of the cells expressing human RACK1 was not seriously affected by the presence of LatB indicating human RACK1 is a functional homolog of Rkp1.

Cell morphogenesis of *S. pombe* involves a number of biological processes including cell wall integrity, polarity, actin cytoskeleton organization and cell division. Rho GTPase, which activates (1–3)-D-glucan synthase, is critical modulator of cell wall biosynthesis and the actin organization required to maintain cell integrity and polarized growth. Both Pck1 and Pck2 interact with Rho1 and Rho2 in the presence of GTP (5).

The HOG (high osmolarity glycerol) pathway functions in cellular protection against sudden increases in osmolarity. In *S. cerevisiae,* the HOG pathway has been implicated in the architecture of cell wall through its control over β-glucan synthesis through *PBS2* suggesting an interconnection between PKC and HOG signaling pathways (14). In *S. pombe,* genetic study

has demonstrated that *pck2* is allelic to *sts6*, staurosporine supersensitive gene (4). Also, the Sts5 that is a crucial determinant of polarized growth functionally interacts with Pck1, and an osmosensing MAP-kinase to maintain cell morphology (15). The salt osmosensitivity of either Δ*pck2* or Δ*rkp1* suggests the functional connection of actin cytoskeleton organization and HOG signaling in *S. pombe.*

p21-activated serine/threonine kinases (PAKs) were reported to bind to and be activated by Rho family GTPase (16). Pak1 is an essential protein that affects mating and cell polarity. Pak2 containing the pleckstrin homology domain, p21-binding domain and protein kinase domain suppresses *pak1* deletion by the overexpression. (17). Since Rkp1/Cpc2 localized in the membrane contains WD repeats and provides a rigid anchor for interacting proteins, it can play roles in forming ordered complexes containing macromolecules such as Pck2, Rho GTPase, PAKs, and others to regulate actin cytoskeleton organization during morphogenesis.

Several studies on mammalian RACK1 were reported. RACK1 is a possible adaptor molecule associating with the intracellular domain of cytokine receptors (18), cytoplasmic phosphodiesterase PDE4D5 isoform (19), SH2 domain of src tyrosine kinase (20), β-integrin subunit (21). *CPC2* gene, *S. cerevisiae* RACK1 homolog, is required for repression of GCN4 protein activity in the absence of amino acid starvation (10). CpcB of *Aspergillus nidulans* is required for the regulation of sexual development under amino acid starvation (22). These findings suggest that RACK1 may function as scaffolding protein to regulate various signaling pathways.

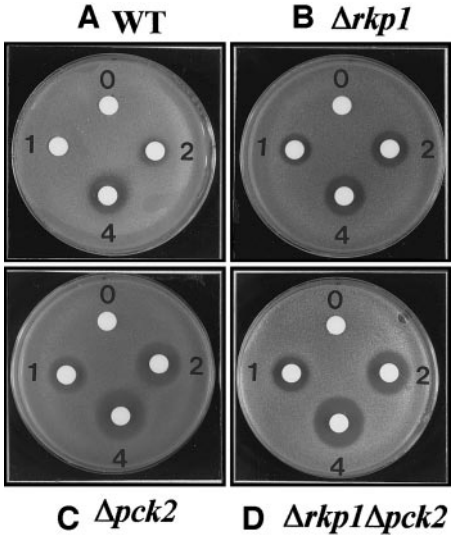

FIG. 4. Latrunculin B assay of deletion mutants. The cells of midlogarithmic phase were added to 1.8 ml of appropriate selective minimal media and mixed with 1.8 ml of 1% agar solution and poured onto the selective minimal media plates. Lat-B was diluted into DMSO and 30 μl of indicated concentration of Lat-B (0, 1, 2, and 4 mM) was pipetted onto 6-mm filter discs placed on the selective minimal plates. The cells were grown for 3–4 days at 30°C.

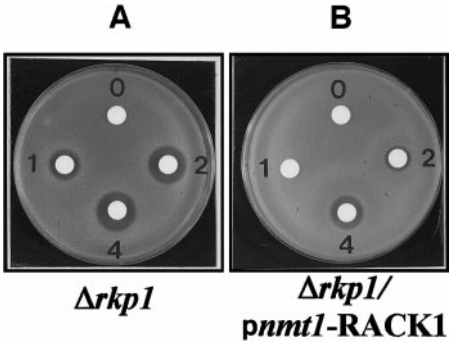

FIG. 5. Functional complementation of human RACK1. The coding region DNA of human RACK1 was cloned to thiamine-regulated pREP81 vector. pREP81-RACK1 was used to transform Δ*rkp1* strain. Lat-B (0, 1, 2, and 4 mM) was diluted into DMSO and pipetted onto 6-mm filter discs placed on the selective minimal plates. The sensitivity of Δ*rkp1* to LatB (A) was compared with that of Δ*rkp1* containing pREP81-RACK1 (B).

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ADDENDUM

While preparing this paper, McLeod *et al.* reported the *cpc2*$^+$ gene which has the same DNA sequence as the *rkp1*$^+$ gene. Cpc2 interacts with Ran1 (Pat1) kinase to regulate cell cycle progression and meiotic development.

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