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Cpc2, a Fission Yeast Homologue of Mammalian RACK1 Protein, Interacts with Ran1 (Pat1) Kinase To Regulate Cell Cycle Progression and Meiotic Development

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The *Schizosaccharomyces pombe ran1*/*pat1* gene regulates the transition between mitosis and meiosis. Inactivation of Ran1 (Pat1) kinase is necessary and sufficient for cells to exit the cell cycle and undergo meiosis. The yeast two-hybrid interaction trap was used to identify protein partners for Ran1/Pat1. Here we report the identification of one of these, Cpc2. Cpc2 encodes a homologue of RACK1, a WD protein with homology to the β subunit of heterotrimeric G proteins. RACK1 is a highly conserved protein, although its function remains undefined. In mammalian cells, RACK1 physically associates with some signal transduction proteins, including Src and protein kinase C. Fission yeast cells containing a *cpc2* null allele are viable but cell cycle delayed. *cpc2*Δ cells fail to accumulate in G_1 when starved of nitrogen. This leads to defects in conjugation and meiosis. Copurification studies show that although *Cpc2* and Ran1 (Pat1) physically associate, Cpc2 does not alter Ran1 (Pat1) kinase activity in vitro. Using a Ran1 (Pat1) fusion to green fluorescent protein, we show that localization of the kinase is impaired in *cpc2*Δ cells. Thus, in parallel with the proposed role of RACK1 in mammalian cells, fission yeast *cpc2* may function as an anchoring protein for Ran1 (Pat1) kinase. All defects associated with loss of *cpc2* are reversed in cells expressing mammalian RACK1, demonstrating that the fission yeast and mammalian gene products are indeed functional homologues.

All living cells integrate signals from the environment to modify the activity of genes required for mitotic division, differentiation, or stationary phase. For the fission yeast *Schizosaccharomyces pombe*, nutritional signals direct life cycle choices. As key nutrients become limited, cells exit the mitotic cycle and enter either G_0 stationary phase or a program of sexual differentiation (6, 10). The choice between G_0 and differentiation is governed by the presence of mating-specific pheromones. Starved cells respond to pheromones produced by cells of the opposite mating type by undergoing transient G_1 arrest, followed by conjugation and meiosis (8, 18, 33, 49). As expected from the need for both nutrient limitation and pheromone signaling, many signal transduction pathways converge to regulate differentiation. These include pathways regulated by cyclic AMP (cAMP), the *ras1*-regulated mitogen-activated protein (MAP) kinase pathway and the stress-activated MAP kinase pathway (see reference 51 for a review). However, a variety of studies indicate that each phase of the fission yeast life cycle can be governed by the activity of Ran1 (Pat1) kinase (referred to as Ran1 hereafter) and its substrates.

Inactivation of Ran1 is necessary and sufficient to divert cells from the mitotic cell cycle into the meiotic developmental program (16, 17, 32). Experiments examining the phenotype of cells carrying a *ran1* temperature-sensitive allele suggest that Ran1 is regulated by stepwise inactivation of the kinase (3, 23). Limiting nutritional conditions trigger partial inactivation of Ran1. This allows cells to accumulate in G_1 (3, 8), the only cell cycle stage permissive for conjugation (10). Following conjugation, continued starvation of the diploid zygote and activation of the mating pheromone pathway lead to full inactivation of Ran1 (24, 49). This promotes meiosis. The complex phenotypes attributed to loss of Ran1 indicate that its activity is likely regulated by a variety of mechanisms.

Attenuation of Ran1 activity provokes expression of genes that function during sexual differentiation as elements of a cascading circuit (31). The most upstream is the product of the *ste11* gene, which encodes a transcription factor required for expression of most meiosis-specific genes (43). Ste11 is an in vitro substrate for Ran1 and a likely physiological target for the kinase. Ran1 phosphorylation negatively regulates the transcription factor, perhaps by hindering its nuclear localization (20). Ste11 binds a specific DNA sequence, the TR box, found upstream of genes it regulates. Ste11 is required for expression of the mating type genes (43), and these regulate meiotic commitment. The mating type locus is not a single genetic entity but includes four genes. $matP_c$ and $matP_m$ are functional in plus cells, and $matM_c$ and $matM_m$ are functional in minus cells. $matP_c$ and $matM_c$ control production of pheromones and pheromone receptors essential for conjugation (see reference 7 for a review). Pheromone signaling is also required for expression of $matM_m$ and $matP_m$, both of which are necessary for meiosis (19, 49). $matP_m$ and $matM_m$ directly provoke transcription of *mei3* (24, 45, 49). As described below, the product of the *mei3* gene is a critical meiotic activator.

mei3 is expressed only in diploid cells competent to undergo meiosis (24). All available data are consistent with the hypothesis that Mei3 activates meiosis by inhibition of Ran1 kinase. The inhibitor contains a region, RKD, which resembles two regions in the Ste11 substrate for Ran1 (20). Structure-function studies indicate that the Mei3 RKD region is critical for

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association with Ran1. Cells containing *mei3* alleles with Mei3 RKD mutations conjugate well but sporulate inefficiently (47). It is hypothesized that during meiosis, inactivation of Ran1 by Mei3 leads to accumulation of hypophosphorylated, and hence active, forms of Ste11. This leads to induction of meiosis-specific genes. One of these, Mei2, is an RNA-binding protein required for premeiotic DNA synthesis (3, 48). Like Ste11, Mei2 is negatively regulated by Ran1 phosphorylation. It has been suggested that the phosphorylation state of Ste11 readies cells for meiosis, while that of Mei2 determines meiotic commitment (48).

As described above, Mei3 does not regulate conjugation but functions only during meiosis to inactivate Ran. The mechanisms used to partially inactivate Ran1 so that G_1 arrest and conjugation can proceed have not been described. To identify other components of the Ran1 signal transduction pathway, we searched for protein partners of Ran1. A fission yeast cDNA library was screened using the two-hybrid interaction trap. We identified RACK1 (Cpc2) as a Ran1-interacting protein. RACK1 functions in mammalian cells to regulate at least two unrelated kinases, protein kinase C (PKC) (27) and Src (5). The fission yeast version of RACK1, named Cpc2 to conform to the *Neurospora crassa* designation (28), is 77% homologous to the human protein. In spite of its strong conservation in many organisms, *cpc2* is not an essential gene in fission yeast. Cells containing a *cpc2* null allele are highly elongated due to a G_2 cell cycle delay. The cells accumulate inefficiently in G_1 when deprived of nitrogen, and conjugation and meiosis are impaired. These phenotypes argue that *cpc2* acts in opposition to *ran1*, perhaps to regulate the activity of the kinase. In support of this hypothesis, *cpc2* functions upstream of *ran1* and a physical association between the two proteins is observed. Fluorescence microscopy using a Ran1-green fluorescent protein (GFP) fusion indicates that Cpc2 may function to alter the cellular localization of Ran1. Consistent with the high level of homology observed between RACK1 and Cpc2, rat RACK1 is capable of functionally complementing a *cpc2* null allele.

MATERIALS AND METHODS

Strains and media. The genotypes of the *S. pombe* strains used in this study are as follows: SP66, h^{90} *leu1-32 ade6-M216*; SP712, h^{+N} *leu1-32 ura4-D18 ade6-M210 ran1*$^{+}$ *OP (OP* refers to genes expressed at high levels under the control of the constitutive *adh* promoter); SP713, h^{+N} *leu1-32 ura4-D18 ade6-M210 ran1*$^{K47R+}$ *OP*; SP870, h^{90} *leu1-32 ura4-D18 ade6-M210 cpc2::pCPC2-24*; SPB5, h^{90} *leu1-32 ade6-M210*; SPB67, h^{+N} *leu1-32 ura4-D18 ade6-M210/h leu1-32 ura4-D18 ade6-M216*; SPB68, h^{90} *mei2::LacZ*; SPB76, h^{+N} *leu1-32 ura4-D18 ade6-M216 cgs1::ura4*; SPB90, h^{-S} *leu1-32 ura4::fbp-lacZ fbp1::ura4 ade6-M216*; SPB93, h^{-S} *leu1-32 cgs1.1 ura4::fbp-lacZ fbp1::ura4 ade6-M210*; SPB173, h^{+N}; SPB182, h^{+N} *ura4-D18 cpc2::ura4*; SPB188, h^{90} *leu1-32 ura4-D18 ade6-M210 cpc2::ura4*; SPB190, h^{90} *leu1-32 ura4-D18 cpc2::pCPC2-24*; SPB191, h^{90} *leu1-32 ura4-D18 ade6-M210 mei2t*^{s} *ran1::pRAN1-90* (*ura*$^{+}$); SPB214, h^{90} *leu1-32 ura4-D18 ade6-M216 ran1-114 cpc2::ura4*; SPB217, h^{90} *leu1-32 ade6-M216 ran1-114*; SPB273, h^{90}; SPB307, h^{90} *ura4-D18 cpc2::ura4 mei2-lacZ*; SPB317, h^{90} *ura4-D18 cpc2::ura4*; SPB318, h^{90} *leu1-32 git2-1*; SPB319, h^{90} *ura4-D18 leu1-32 cpc2::ura4 git2::LEU2*; SPB320, h^{-S} *leu1-32 ura4::fbp-lacZ fbp1::ura4 cpc2::ura4 ade6-M216*.

S. pombe cells were cultured in rich medium (YEA) or minimal medium (PMA) with the required amino acids (1). Strains were constructed using tetrad analysis. Double mutants were identified in a nonparental ditype tetrad, and genotypes were confirmed using PCR. *Escherichia coli* cells were grown in Luria broth supplemented with antibiotics. *Saccharomyces cerevisiae* Y190 (*MAT***a** *leu2-3 ura3-52 trp1-1901 his3-200 ade2-101 gal4*Δ *gal80*Δ *URA3::GAL1-LacZ LYS::GAL1-HIS3 cyh*^{r}) was used for two-hybrid assays (12).

Yeast two-hybrid screen. Plasmids and details of the library screen were essentially as previously described (12). The *ran1* gene was fused in frame to the *GAL4* DNA-binding domain (DBD) in pAS2. Following transformation of this plasmid into Y190, a Western blot using Ran1 antibodies (23) was used to verify that the fusion protein was produced intact. A fission yeast cDNA library fused to the activation domain of *GAL4* in the vector pACT2 (provided by Steve Elledge) was transformed into the Y190/*ran1*-DBD strain. The transformants were selected on plates containing 3-amino triazole (3-AT) at 30°C for 3 to 4 days. Approximately 10^6 transformants were obtained. To eliminate one class of false positives, each library plasmid was tested to identify those capable of activating the *LacZ* reporter gene in the absence of *ran1*-DBD. *ran1*-DBD was reintroduced into those candidate strains and tested for activation of *LacZ*. Measurement of β-galactosidase activity was accomplished using a permeabilized-cell assay. A total of 53 colonies were obtained, and DNA was isolated from each. Limited DNA sequence analysis of each identified 30 clones representing nine unique open reading frames.

Oligonucleotides. The sequences of the oligonucleotides used in this study are as follows: GB-RKDI, 5′CTTCTTTCCGGTTCTGCAGACGCGTCCATCATTTTGTG3′; GB-RKDII, 5′GGTTGTTTCTGGTTCCGCGGACGCGACCATTAAGATTTG3′; CPC2-Xma, 5′TCTTGTCCCGGGAACCAGAAA3′; CPC2-HIII, 5′CGGTACCTTGAAGCTTTGGGA3′; CPC2-35, 5′CTCGAAGGTCACTCTGGATG3′; CPC2-927C, 5′TACTTGGTAACTTGCCAGACAC3′; CPC2-Nde, 5′TACCATATGACCGATGGTGGTCACTC3′; CPC2-Bam, 5′AGAGGATCCACCATCGGTGATAGTGT3′.

Construction of a Cpc2 null allele. Oligonucleotides CPC2-Nde and CPC2-Bam were used in a PCR to obtain the entire *cpc2* cDNA. The linear fragment was cloned into the commercially obtained TA vector (InVitrogen). Inverse PCR of this plasmid using primers CPC2-Xma and CPC2-HIII was used to insert *Hin*dIII and *Xma*I restriction sites into *cpc2*. A *Hin*dIII-to-*Xma*I fragment of *ura4* was cloned into the inverse PCR product. A linear DNA fragment containing *cpc2* disrupted with *ura4* was obtained using primers CPC2-35 and CPC2-927C in a PCR. The linear fragment was purified using a Qiagen column and used to transform SPB67. Stable URA^{+} transformants were identified, and a Southern blot verified the correct replacement. Sporulation of the diploid produced the haploid strain SPB188, which contains *cpc2::ura4* (also referred to as *cpc2*Δ).

Oligonucleotide mutagenesis. The Cpc2 RKD motifs were mutagenized using oligonucleotides GB-RKDI and GBRKDII. All oligonucleotides were designed with the assistance of the OLIGO software package (National Biosciences Inc., Plymouth, Minn.). The identities of the mutations were confirmed using restriction enzyme analysis.

Expression and purification of recombinant proteins. The plasmids used for expression of recombinant proteins were pRAN1.83 (His-hemagglutinin [HA]-Ran1), pRAN1.87 (His-HA-Ran1^{K47R}), and pSTE11.27 (His-p39^{ste11}). The construction of each and expression in bacteria have been described previously (20). pCPC2.2 was used for expression of His-HA-Cpc2. The soluble portion of bacterial whole-cell extract was incubated with 1.0 ml of Ni^{2+}-nitrilotriacetic acid agarose. The mixture was packed into a column and washed sequentially with 10 column volumes each of wash buffer I (300 mM NaCl, 20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 10% glycerol), wash buffer II (wash buffer I with 100 mM NaCl), and wash buffer III (wash buffer II plus 20 mM imidazole). Proteins were eluted from the column in wash buffer III containing 100 mM imidazole. Imidazole was removed by dialysis of the purified protein against wash buffer I.

Copurification of GST-tagged proteins from yeast. Plasmids pALT4-GST and pCPC2-24 were constructed to produce HA epitope-tagged glutathione S-transferase (GST) and HA-GST-tagged Cpc2, respectively. The plasmids were transformed into SP66 (wild type) or SPB191. SPB191 contains an integrated plasmid which produces HA-tagged *ran1* under control of the *nmt1* promoter from plasmid pREP41 (2). The four strains were grown in thiamine-free medium to allow induction of *ran1*. Pelleted cells were washed in HE buffer (50 mM Tris HCl [pH 8.0], 5 mM EDTA, 1 mM dithiothreitol) and resuspended in 0.5 ml of HE buffer containing 1 mM phenylmethylsulfonyl fluoride. Following addition of sterile glass beads, the cells were broken by vortexing for 15 min at 4°C. Lysates were removed to a fresh tube, and the glass beads were washed in 2.0 ml of RIPA buffer (HE plus 0.1% sodium dodecyl sulfate SDS, 1% Triton X-100, 0.5% deoxycholate). Clarified lysate was obtained by centrifugation at 12,000 × *g* for 10 min. Protein concentrations were determined using bovine serum albumin as the standard in the Bio-Rad system. Lysate (5 mg) was purified using glutathione-agarose (Sigma). The beads were resuspended in Laemmli sample buffer prior to separation by SDS–7.5 to 15% polyacrylamide gel electrophoresis (PAGE). Western blotting was performed as previously described (20). The primary antibody used for detection of Ran1 was a mixture of R30, R48, and R99 monoclonal antibodies (23) diluted 1:1,000 in 5% dry milk. For detection of HA fusion proteins, a commercially available antibody was used (12CA5; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Immunoreactive proteins were visualized using the Dupont NEN Research Products chemiluminescence assay kit. Alternatively, the purified proteins were used for a kinase assay. In that case, proteins bound to the agarose beads were washed in RIPA buffer as described above. The beads were resuspended in 50 μl of 1×KAB (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiothreitol) in the final step. Kinase assays utilized 25 μl of bead-bound protein and p39^{Ste11} as the substrate (20).

Plasmids. Steve Elledge provided hybrid plasmids pAS2 and pACT2 and the fission yeast cDNA library. The entire coding region of *ran1* was fused in frame to the DBD of *GAL4* as an *Nde*I-to-*Bam*HI fragment. *cpc2* or *cpc2* RKD was fused to pACT2 as an *Nhe*I-to-*Bam*HI fragment obtained from a pALT4 vector.

The fission yeast expression plasmids used were pALT4 (contains *S. pombe ars1*, *S. cerevisiae LEU2* as a selectable marker, and the *adh* promoter fused to HA1 sequences), pALT2 (described in reference 24), pALT4GST (to express HA-GST; GST was inserted into pALT4 as an *Nde*I-to-*Bam*HI fragment), pCPC2.10 (contains *cpc2* as an *Nde*I-to-*Bam*HI fragment in pALT2), HA-GST-Ran1 (The GST coding sequence was cloned into a yeast expression vector as an *Nde*I-to-*Nhe*I fragment. This allows the insertion of any gene in frame into GST

as an *Nde*I-to-*Bam*HI fragment), pRAN1.81 (expression of *ran1* using the *adh1* promoter [41]), pRAN1.90 (integrative plasmid constructed to express HA-tagged Ran1 from an inducible *nmt* promoter derived from pREP41 [2]), pRAN1.95 (expression of GST-HA1-Ran1 from the *nmt* promoter derived from pREP42 [2]), pCPC2.24 (expression of GST-HA1-Cpc2 using the *adh* promoter), and pCPC2.2 (expression of HA-CPC2 in bacterial expression vector pET15B [NovoLabs]). Exact details of plasmid constructions are available on request.

Flow cytometry. Either wild-type (SPB173) or *cpc2*Δ (SPB182) cells were grown to 10^7/ml and then shifted to medium lacking nitrogen for the designated times. Cells were fixed and stained with propidium iodide as previously described (1). DNA fluorescence was measured using a FACScan (Becton Dickinson).

Measurement of β-galactosidase activity. Total cell extract was prepared from cells containing a *mei2-lacZ* gene after they attained a density of 10^7/ml. A 20-μg protein sample was used to assay β-galactosidase activity (1). Activity is expressed as $A_{420} \times 1.7/0.0045 \times$ protein (micrograms) $\times$ volume $\times$ time. *fbp1-lacZ* activity was measured in 10^7 permeabilized cells.

Measurement of viability in stationary phase. Cells were grown to 10^8/ml at 30°C. At this time (day 0) and each day thereafter (days 1 to 4), a portion of the cells was removed and stained with FUN-1 (Molecular Probes, Eugene, Oreg.) as previously described (25, 53). Cells were immediately examined under a microscope. Viable cells contained red intravacuolar structures, and nonviable cells stained yellow-green.

RESULTS

Isolation of *cpc2* using a two-hybrid library screen. A fission yeast cDNA library was screened by two-hybrid analysis using *ran1*-DBD) as bait. Approximately 10^6 colonies were obtained following cotransformation of *ran1*-DBD and the library plasmids into yeast. Plasmid inserts from *LacZ*-positive transformants were sequenced, and one of these, *cpc2*, is the subject of this paper. *cpc2* was chosen for analysis because the predicted protein has regions of homology with other proteins known to directly interact with Ran1 (see below). The interaction between Ran1 and Cpc2 was quantitatively assessed in the two-hybrid system using *S. cerevisiae* strain Y190. This strain contains a *LacZ* reporter gene controlled by the upstream activating sequence of *GAL4*. We observed that Cpc2 paired with Ran1 produced sixfold higher β-galactosidase activity than Ran1 paired with the ACT2 vector. This interaction is comparable to, but not quite as strong as, the interaction between Ran1 and Ste11, a transcription factor that directly associates with Ran1 (Fig. 1A and reference 20).

The entire nucleotide sequence of the *cpc2* cDNA insert was determined. This analysis showed that the cDNA sequence was deposited under GenBank accession no. gi598437. The predicted protein is 314 amino acids with a molecular mass of 34.85 kDa. Inspection of the sequence revealed that the polypeptide contains two repeated regions (referred to as RKD) which resemble sequences observed in proteins known to directly interact with Ran1 (Fig. 1B). RKD sequences are proposed to function as substrate specificity determinants. They have been identified in Ste11, an in vitro substrate for Ran1, and in Mei3, a pseudosubstrate-like inhibitor of Ran1 (20, 47). Cpc2 contains two regions with spatially conserved basic residues (^{36}R plus ^{38}K and ^{125}R plus ^{127}K; Fig. 1B), followed by a serine or threonine residue (^{39}S and ^{128}T). The basic residues are required for Mei3 RKD function both in vivo and in vitro. ^{39}S and ^{128}T of Cpc2 correspond to the phosphoacceptor residues in the Ste11 substrate. To examine the importance of the putative Cpc2 RKD repeats for association with Ran1, two basic amino acids in each motif (^{36}R plus ^{38}K and ^{125}R plus ^{127}K; Fig. 1B) were mutagenized to alanine residues. The ability of the altered protein (Cpc2 RKD) to bind Ran1 was examined in a two-hybrid assay. We observed that Cpc2 RKD paired with Ran1 reduced expression of the *LacZ* reporter gene to the same level as that measured using Ran1 paired with a control vector (Fig. 1A).

The Cpc2 polypeptide was also compared to sequences deposited in the commonly used databases. Cpc2 is highly homologous to a family of WD repeat proteins. WD repeat proteins have been classified into four subfamilies (29, 30). Cpc2 is a member of the subfamily that includes the heterotrimeric G protein β subunit (41), mammalian RACK1 (37), *S. cerevisiae* Cpc2p (15), and *N. crassa* Cpc2 (28). The fission yeast protein is 64% identical to the mammalian RACK1 protein. The overall level of homology between the two proteins is 77% (Fig. 1C). In spite of the high level of conservation between members of this family, the function of RACK1 proteins is not well understood. Rat RACK1 was initially isolated as a PKC-binding protein (37). Recent reports indicate that RACK1 associates with other signal transduction proteins, including cAMP phosphodiesterase 4D5 (52), Src (5), and β-integrin (21).

Ran1 and Cpc2 associate in cell lysates. The presence of RKD sequences in Cpc2 indicates that if, in fact, Ran1 and Cpc2 are associated, the interaction may be direct. To determine if the two polypeptides are physically connected, we assayed cell lysates for copurification of Ran1 and Cpc2. To accomplish this, cells were transformed with plasmids expressing GST, Cpc2 fused to GST (GST-Cpc2), or Ran1. To permit immunological detection, all proteins were also tagged with HA1 sequences. Soluble proteins were prepared from cells expressing the fusion proteins either individually or in pairs. An immunoblot developed with anti-HA1 antibodies was used to examine the steady-state level of each fusion protein (Fig. 2A). Since Ran1 and GST-Cpc2 migrate close to one another, the immunoblot was also developed using anti-Ran1 antibodies (Fig. 2B). These experiments allowed unequivocal identification of Ran1 and GST-Cpc2 and verified that the proteins were intact. Partial purification of GST fusion proteins was accomplished on glutathione beads. Bead-bound proteins were separated by SDS-PAGE and analyzed in an immunoblot using anti-HA1 antibodies (Fig. 2C) or anti-Ran1 antibodies (Fig. 2D). This experiment showed that Ran1 does not inherently bind glutathione beads or copurify with GST (Fig. 2C and D, lane 3). In contrast, Ran1 was present in partially purified complexes containing GST-Cpc2 (Fig. 2C and D, lane 4).

Loss of cpc2 causes pleiotropic cell cycle defects. Because two independent assays indicate that Ran1 and Cpc2 physically associate, we examined cells for a functional connection between the two proteins. To accomplish this, a *cpc2* null allele was constructed. A portion of the predicted *cpc2* open reading frame was deleted and replaced with the *ura4* gene to form *cpc2::ura4*. A linear DNA fragment containing *cpc2::ura4* was transformed into diploid cells (SPB67). Stable URA4$^+$ transformants were analyzed using a Southern blot to identify a simple replacement of *cpc2* (data not shown). One representative diploid was chosen for further analysis. The cells were allowed to undergo meiosis, and spores were collected. Random spore analysis showed that URA$^+$ and URA$^-$ cells were equally represented. This result indicates that *cpc2* is not an essential gene. During this analysis, it was noted that *cpc2*Δ cells formed small colonies compared to wild-type cells (Fig. 3A). Microscopic examination of the cells showed that they were elongated, a phenotype observed in cells producing activated Ran1 and a hallmark of mitotic delay (Fig. 3B). To examine the role of *cpc2* in division, cell growth was monitored. This experiment revealed that *cpc2*Δ doubled in 3.0 h, as did wild-type cells (Fig. 3C). Thus, loss of *cpc2* allows cells to grow normally but delays cell division. In this and other experiments, it was noted that *cpc2*Δ cells consistently ceased dividing at a two- to fourfold lower density than wild-type cells. These phenotypes are associated with loss of *cpc2* because expression of *cpc2* cDNA completely restores the wild-type phenotype (Fig. 3A and data not shown). Next, we examined the fate of cells exiting the cell cycle.

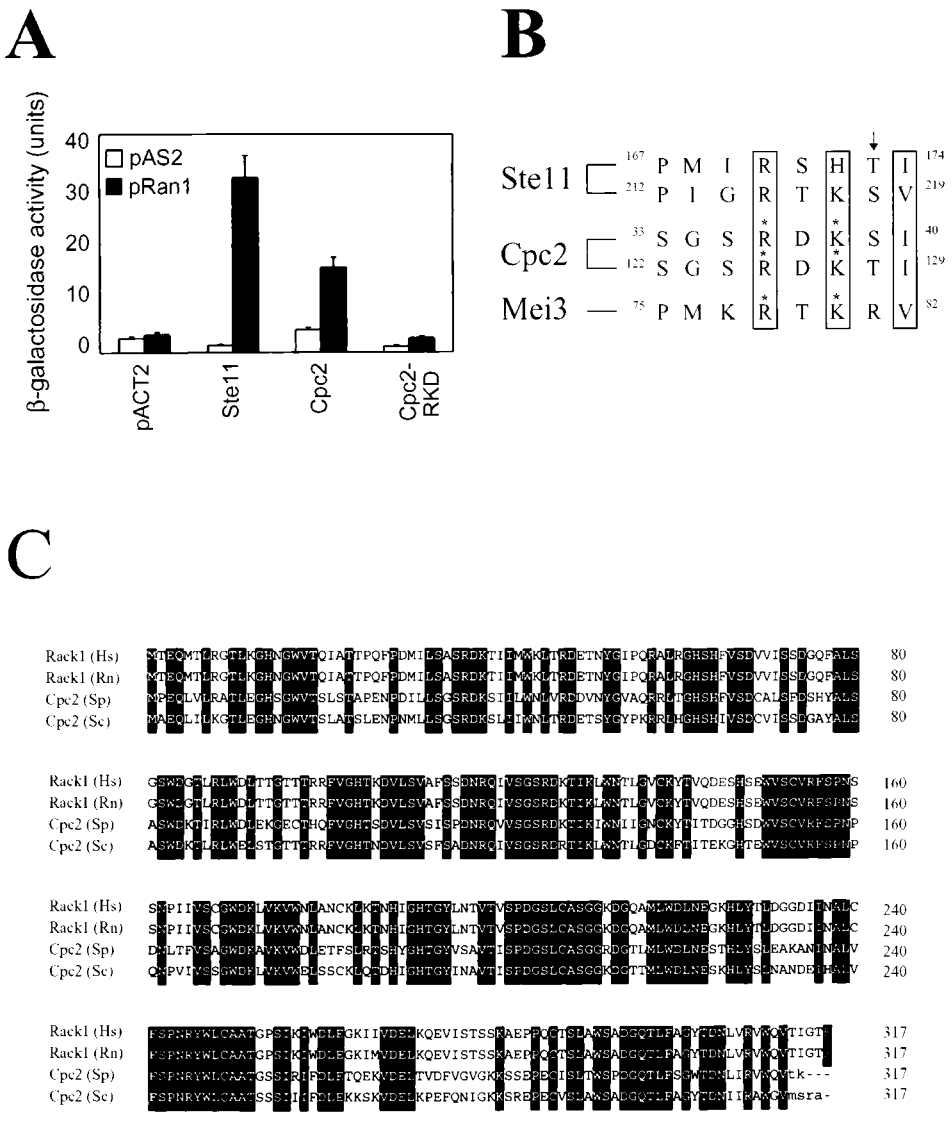

FIG. 1. Ran1 interacts with the highly conserved RACK1 (Cpc2) protein. (A) Two-hybrid analysis of the interaction between Ran1 and Cpc2. The reporter strain (Y190) permits detection of protein-protein interaction through transcriptional activation of a *GAL1-LacZ* gene. Y190 cells were transformed with the indicated plasmids. Ran1 was fused to the *GAL4* DBD. Ste11, Cpc2, and the Cpc2 RKD were each fused to *GAL4* activation domain. Cpc2 RKD contains defined mutations in both Cpc2 RKD domains (see below). A permeabilized-cell assay was used to measure β-galactosidase activity. The results represent the mean values obtained using three independent transformants. (B) Amino acid sequence comparison of regions within Ste11, Cpc2, and Mei3. The arrow marks the phosphoacceptor position occupied by serine or threonine residues in the Ste11 substrate for Ran1 (20). The asterisks over Mei3 residues indicate those required for Mei3 function (47). The amino acids R^{36}, K^{38}, R^{125}, and K^{127} in Cpc2 were each changed to alanine in Cpc2 RKD (indicated with asterisks). The superscript numbers correspond to amino acid positions. (C) Amino acid comparison of *Homo sapiens* (Hs) and *Rattus norvegicus* (Rn; reference 36) RACK1, *S. pombe* (Sp) Cpc2, and *S. cerevisiae* (Sc) Cpc2 (15). Identical amino acids are indicated using a dark box.

The presence of mating pheromones and the absence of nitrogen cause fission yeast cells to undergo transient arrest in G_1 (8, 18, 33), the only phase of the cell cycle permissive for conjugation. The ability of *cpc2*Δ cells to conjugate and sporulate was compared to that of wild-type cells. Following 3 days of growth on minimal medium, nearly 55% of the wild-type cells had either conjugated or sporulated. In contrast, only 10.7% of the *cpc2*Δ cells had undergone sexual differentiation. After 4 days, 89% of the wild-type cells had differentiated, compared to 53.4% of the *cpc2*Δ cells (Fig. 3D). Thus, loss of *cpc2* does not prevent conjugation and sporulation but substantially delays these events. Next, we examined cells for the ability to accumulate in G_1 in response to nitrogen limitation. Cells were transferred to nitrogen-free medium, and the DNA content of individual cells was monitored using flow cytometry. At 6 h following the nutritional shift, 60% of the wild-type cells had a G_1 DNA content and at 8 h, 90% of the cells were in G_1. In contrast, most (95%) *cpc2*Δ cells exhibited a G_2 DNA content after incubation in nitrogen-free medium for 6 h. At 8 h, only 20% of the *cpc2*Δ cells were observed to be in G_1 (Fig. 3E). The wild-type cell number increased 2.5-fold 6 h following the nutritional shift. In contrast, the *cpc2*Δ cell number increased 1.5-fold. Thus, the failure of *cpc2*Δ cells to accumulate in G_1 is most likely due to an inability to advance into mitosis when nitrogen is limiting.

The exact phenotypes observed in *cpc2*Δ cells have not, to the best of our knowledge, been described for any other mutant. However, some (such as mitotic delay and decreased

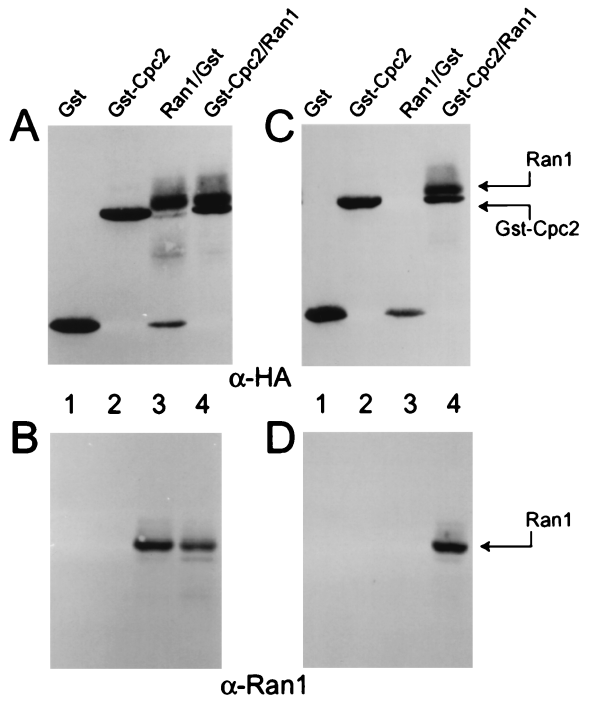

FIG. 2. Ran1 and Cpc2 physically interact. Wild-type cells (SP66, lanes 1 and 2) or cells producing high levels of Ran1 (SPB191, lanes 3 and 4) were transformed with a plasmid expressing either GST (lanes 1 and 3) or GST-Cpc2 (lanes 2 and 4). All expressed proteins were epitope tagged with HA1 sequences. Cells were grown to 10^7/ml in complete medium and shifted to thiamine-free medium for protein induction. Soluble extract was prepared after 18 h of growth. Proteins were separated by SDS–7.5 to 15.0% PAGE and immunoblotted with anti-HA (A) or anti-Ran1 (B) antibody. A portion (2 mg) of each soluble extract was applied to glutathione-agarose beads. Bead-bound material was examined in an immunoblot assay using anti-HA (C) or anti-Ran1 (D) antibody. The positions of HA-Ran1, HA-GST-Cpc2, and HA-GST are marked.

conjugation and sporulation) are observed in cells with defects in the stress-activated MAP kinase pathway (see references 41, 42, and 44, for example). However, unlike loss of the *spc1* MAP kinase, loss of *cpc2* does not prevent growth on medium containing 1.4 M sorbitol or 0.9 M KCl. Nor are *cpc2* cells sensitive to hydrogen peroxide, cycloheximide, or staurosporine (data not shown). Thus, *cpc2*Δ cells display only a limited subset of phenotypes associated with the stress-activated MAP kinase pathway. We did observe that *cpc2*Δ cells are more sensitive to high temperature compared with wild-type cells (Fig. 3F). In view of this observation, we tested cells producing high levels of Ran1 for the ability to grow at 37°C. Like *cpc2*Δ, *ran1OP* cells exhibit temperature-dependent synthetic lethality. In contrast, cells producing high levels of an inactive version of the kinase, *ran1*^{K47R}*OP* cells, grow as well as wild-type cells at 37°C (Fig. 3F).

***cpc2* functions independently of the PKA pathway.** Cell cycle delay, failure to differentiate, and stationary-phase defects are phenotypes associated with activation of the cAMP-dependent protein kinase (PKA) pathway (3, 8, 9, 26). This, and the observation that RACK1 interacts with cAMP phosphodiesterase in mammalian cells (52), led us to test the hypothesis that *cpc2* downregulates cAMP levels. In fission yeast, alteration of cAMP levels or of PKA activity causes pleiotropic defects. Transcription of genes regulated by nitrogen or glucose availability is impaired when cAMP levels are altered. The fructose-1,6-bisphosphatase gene (*fbp1*) is repressed in glucose-grown cells. Shifting the cells to glycerol-containing medium leads to derepression of *fbp1* (14, 17). Addition of cAMP to cells (13) or mutations that increase PKA activity repress *fbp1* transcription, even following a shift to glycerol medium. We used cells containing an *fbp1-lacZ* reporter gene to examine glucose repression in *cpc2*Δ cells. Either wild-type, *cgs1*Δ (*cgs1* encodes the regulatory subunit of PKA), or *cpc2*Δ cells were shifted from glucose- to glycerol-containing medium. *cgs1* cells fail to derepress the fusion gene immediately following the shift to glycerol (Fig. 4A and reference 50). In contrast, *fbp1-lacZ* expression in both wild-type and *cpc2*Δ cells increased to the same extent within 3 h of the nutritional shift and reached maximal expression at 6 h (Fig. 4A). Thus, *cpc2* does not perform a major function in cAMP-regulated transcription of glucose-sensitive genes.

Nitrogen limitation leads to a 50% decrease in the level of intracellular cAMP (26) and promotes expression of differentiation genes, most notably, *ste11* and *mei2*. In contrast, high cAMP levels prevent expression of *ste11* (43) and *mei2* (9). To test the involvement of *cpc2* in nitrogen-regulated transcription, we used cells containing a *mei2-lacZ* reporter gene. *cgs1*Δ cells express no detectable *mei2-lacZ* following nitrogen starvation (9). In contrast, an increase in *mei2-lacZ* expression was observed 3 h following the shift of either wild-type or *cpc2*Δ cells to nitrogen-free medium. Accumulation of β-galactosidase activity continued in both samples until the experiment was terminated at 9 h. β-Galactosidase activity was lower in *cpc2*Δ cells than in wild-type cells. This decrease may reflect a difference in cAMP levels. Alternatively, it may be due to the inability to inactivate Ran1 in *cpc2*Δ cells. Recently, we determined that *mei2* expression is regulated by Ran1 activity, as well as by cAMP levels (J. Qin and M. McLeod, unpublished data).

cAMP levels also regulate sexual differentiation and survival in stationary phase (9). Formally, the possibility remains that *cpc2* functions to repress cAMP levels, but only to the extent that cell cycle events, and not nutrient-regulated transcription, are altered. The ability of *cpc2*Δ cells to survive stationary-phase conditions was examined. As shown previously (9) and in Fig. 4C, *cgs1*Δ cells die after 3 days at G_0. At day 4, less than 0.3% of the cells are viable. Conversely, *cpc2*Δ and wild-type cells survived equally well at G_0. Next, we determined if the sexual differentiation defect observed in *cpc2*Δ cells results from a failure to downregulate cAMP levels. The *git2* (*cyr1*) gene encodes adenylate cyclase, and cells containing a loss-of-function *git2* allele have no measurable cAMP (14, 22). Conjugation and sporulation of *H*^{90} *git2* cells are accelerated relative to those of wild-type cells. If conjugation and meiosis are delayed in *cpc2*Δ cells because cAMP levels cannot be downregulated, then *git2* cells ought to be insensitive to *cpc2* function. The ability of *git2* cells to conjugate and sporulate was compared with that of *git2 cpc2* cells. This experiment revealed that conditions permitting 60% of the *git2* cells to conjugate or sporulate allowed only 8.0% of the *git2 cpc2* cells to differentiate (Fig. 4D). Taken together, the above-described experiments indicate that *cpc2* is not a central regulator of cAMP levels.

Genetic interactions between Ran1 and Cpc2. If *cpc2* and *ran1* function on the same genetic pathway, *cpc2* could potentially function as an upstream regulator or a downstream effector for the kinase. An epistasis test was used to discriminate between these possibilities. Cells containing the *ran1-114* allele undergo haploid sporulation when the cells are incubated under restrictive conditions. Loss of *cpc2* does not abolish conjugation and meiosis but retards the process (Fig. 3D). Conditions that partially inactivate *ran1-114* were used to determine if the sporulation rate of *ran1* cells is dependent on

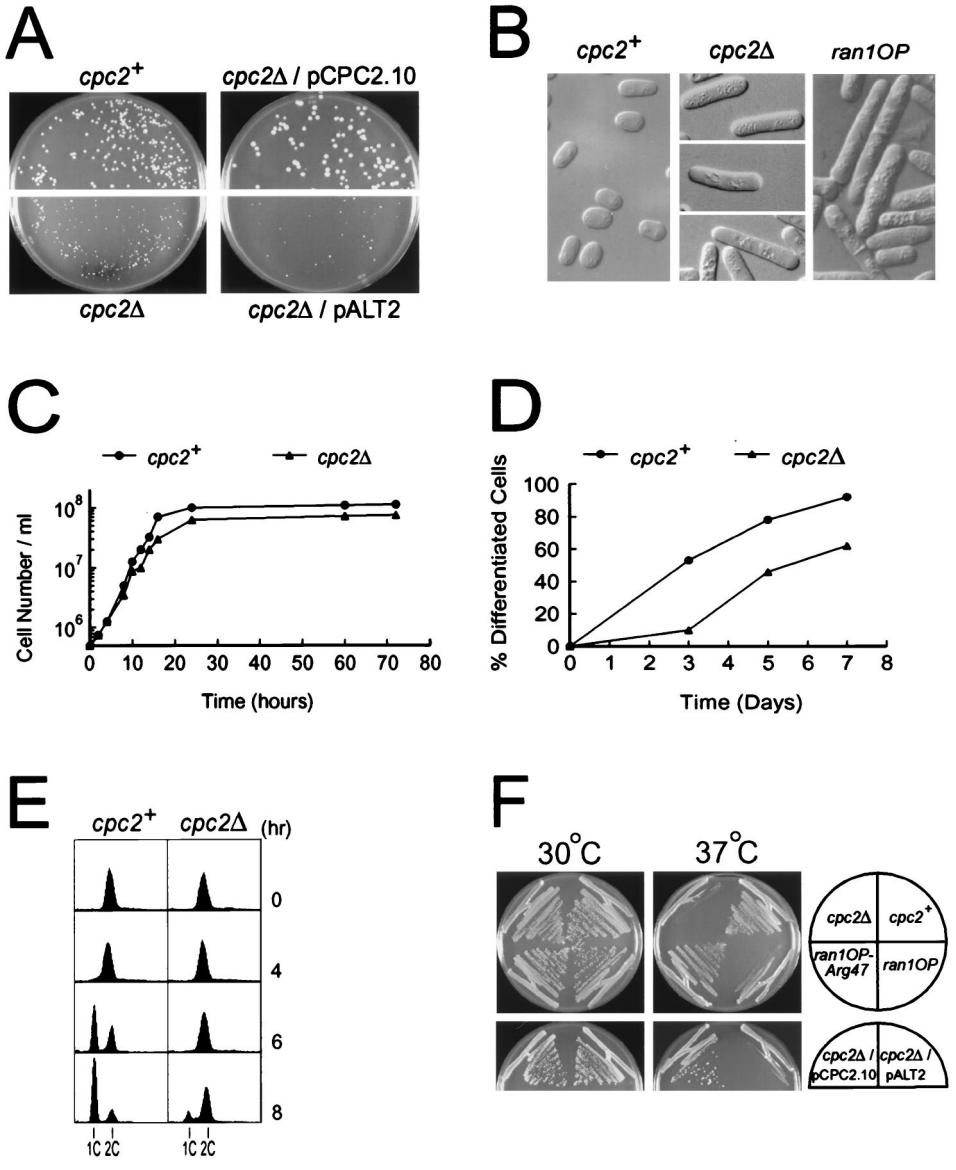

FIG. 3. Loss of *cpc2* causes pleiotropic defects. (A) *cpc2*$^+$ (SPB173), *cpc2*Δ (SPB182) (left side), or *cpc2*Δ (SPB188) (right side) cells transformed with the indicated plasmids, spread on minimal medium plates, and incubated for 60 h at 30°C. (B) Photomicrographs of *cpc2*$^+$ (SPB173), *cpc2*Δ (SPB182), and *ran1OP* (SP712) cells. (C) Growth curve constructed by growing cells (*cpc2*$^+$ [SPB173] or *cpc2*Δ [SPB182]) to stationary phase and inoculating them at a density of 10^5/ml into fresh YEA medium. A portion of each culture was removed at the indicated times, and cells were counted using a hemocytometer. (D) *cpc2*$^+$ (SPB173) or *cpc2*Δ (SPB182) cells patched onto minimal-medium plates and incubated at 30°C. At the indicated times, a portion of the cells was resuspended in water and the cells were counted and scored as either a vegetative cell, a zygote, or an ascus-containing spores. Differentiated cells include diploid zygotes plus asci. (E) Flow cytometry of *cpc2*$^+$ (SPB173) or *cpc2*Δ (SPB182) cells shifted to nitrogen-free medium for the indicated times prior to fluorescence-activated cell sorter analysis. (F) *cpc2*$^+$ (SPB173), *cpc2*Δ (SPB182), *ran1OP* (SP712), and *ran*^{K47R}*OP* (SP713) cells were streaked on minimal-medium plates and incubated at either 30 or 37°C.

cpc2. We observed that neither wild-type cells nor *cpc2*Δ cells sporulated to a significant level after 20 h on malt extract medium at 30°C. At that time, 80% of the *ran1-114* cells contained spores. Importantly, *ran1-114* sporulation was only partially independent of *cpc2*. Inactivation of *ran1* reversed the differentiation defect caused by loss of *cpc2*, but sporulation was accelerated if cells contained a functional *cpc2* allele (Fig. 5A). We infer from this result that loss of *cpc2* enhances *ran1* residual activity. Since inactivation of *ran1* allows *cpc2*Δ cells to regain a function, then *cpc2* is most likely upstream of *ran1* if, in fact, they are on the same pathway.

Inactivation of Ran1 causes cells to accumulate in G_1 (3), but activated Ran1 (*ran1OP*) leads to a G_2 delay (23). Considering that loss of *cpc2* also causes a G_2 delay (Fig. 2B and E), the effect of producing high levels of *cpc2* was examined. Expression of *cpc2* using the *adh* promoter had no observable effect in either wild-type or *ran1OP* cells (data not shown). However, the inverse experiment, expression of Ran1 in *cpc2*Δ cells, caused an additive phenotype (Fig. 5B). Specifically, growth of *ranOP cpc2*Δ cells was retarded compared to that of *cpc2* cells transformed with an empty vector (Fig. 5B). Microscopic examination of the cells revealed that they were highly elongated (data not shown). In comparison, expression of Ran1 in wild-type cells caused far less severe defects in colony formation and cell length. Similar results were obtained if *ran1* was expressed using either the *adh* or the *nmt1* promoter. One

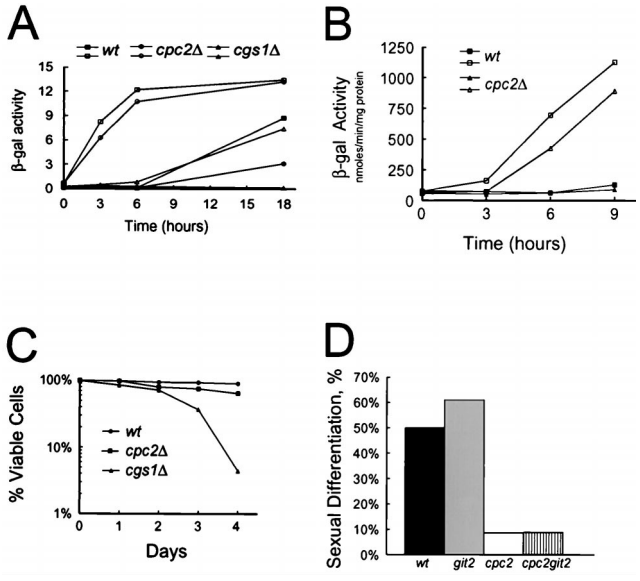

FIG. 4. Interactions between *cpc2* and the cAMP pathway. (A) Induction of the *fbp1-lacZ* fusion gene. Cells with the indicated relevant genotype and the *ura4::fbp-lacZ* reporter gene were shifted from glucose (filled symbols) to glycerol (empty symbols) medium. β-Galactosidase activity was measured at the indicated times using a permeabilized-cell assay. Each point represents three samples (standard error of the mean, <2.0%). Wild-type (wt; SPB90), *cgs1* mutant (SPB93), and *cpc2* mutant (SPB320) cells were used. (B) Induction of the *mei2-lacZ* fusion gene. Cells were grown to 5×10^6/ml and washed in medium devoid of a nitrogen source. The culture was divided between two flasks. NH_4Cl was added to one sample (closed symbols); the other represents nitrogen-depleted medium (open symbols). β-Galactosidase activity was measured at the indicated times using 20 μg of soluble cell protein. Wild-type (SPB68) and *cpc2* mutant (SPB307) cells were used. (C) Survival in stationary phase. Cells were grown to saturation (2×10^8/ml; day 0) and incubated for an additional 4 days (days 1 to 4). A portion of the culture was removed each day and stained with FUN-1. Wild-type (SPB173) *cgs1* mutant (SPB76), and *cpc2* mutant (SBP182) cells were used. (D) Sexual differentiation of cells grown on phorbol myristate acetate for 3 days at 30°C. Asci and zygotes in a portion of each sample were measured using a hemocytometer. Wild-type (SPB273), *git2* mutant (SPB318), *cpc2* mutant (SPB317), and *git2 cpc2* mutant (SPB319) cells were used.

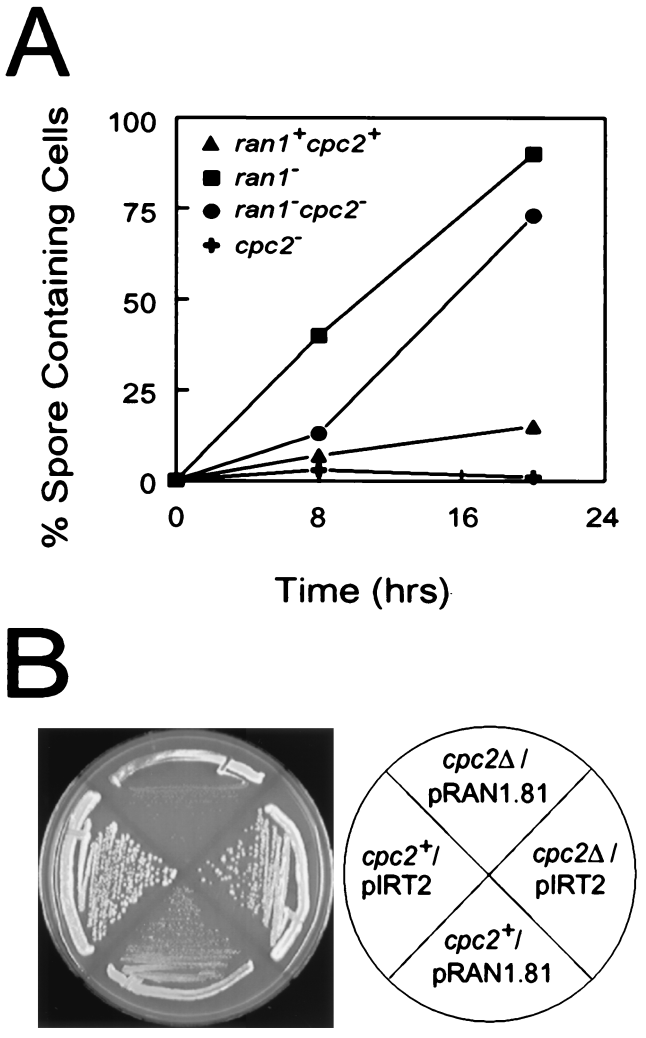

FIG. 5. Genetic interactions between *ran1* and *cpc2*. (A) Wild-type (SP66), *ran1* mutant (SPB217) *cpc2* mutant (SPB188), or *ran1* and *cpc2* mutant (SPB214) cells were patched onto malt extract plates and incubated at 32°C for the indicated periods of time. A portion of the patches was resuspended in water, and the cells were counted and scored as a vegetative cell, a zygote, or an ascus containing spores. A differentiated cell includes zygotes and cells with spores. (B) Either wild-type (SP66) or *cpc2* mutant (SPB182) cells were transformed with a control plasmid (pALT2) or a plasmid containing *adh-ran1* (pRAN1-81).

interpretation of these data is that Ran1 and Cpc2 act in opposition on independent pathways. However, since the epistasis test indicates a role for *cpc2* as a regulator of *ran1*, we favor the hypothesis that the exaggerated Ran1 phenotypes observed when Ran1 is expressed in *cpc2*Δ cells result from increased Ran1 activity that would be constrained by Cpc2 if it were present. With regard to the experiments showing that high-level production of *cpc2* causes no observable phenotypes, we suggest the presence of an as yet unidentified activator for Cpc2 or perhaps a structural role for the protein.

Cpc2 allows nuclear accumulation of Ran1. Mammalian RACK1 is believed to regulate PKC function by serving as an anchoring protein for the activated kinase (37). Thus, the cellular localization of Ran1 in wild-type and *cpc2*Δ cells was examined. For these experiments, Ran1 was fused to GFP and expressed under control of the *nmt1* promoter. Induction of the fusion protein was accomplished by removal of thiamine from the medium. A detectable GFP signal was first noted 7 h following induction, and the signal was concentrated in the nucleus (Fig. 6A and reference 47). Early nuclear concentration of Ran1 was independent of *cpc2*. At 12 h following induction, a striking difference in the cellular distribution of Ran1-GFP became evident. Ran1-GFP displayed a prominent, punctate cytoplasmic staining in *cpc2*Δ cells that was not observed in wild-type cells. In addition, the fusion protein did not accumulate to high levels in the nucleus in the absence of *cpc2* (Fig. 6A and data not shown). The pattern is specific for Ran1-GFP because it was not observed for the other nuclear proteins tested, such as Ste11-GFP and Mei3-GFP, or for GFP alone (data not shown). The level of Ran1-GFP in the cells was examined in an immunoblot. This experiment showed that the fusion protein had the predicted molecular weight and that the steady-state level of Ran1-GFP in *cpc2*Δ cells was comparable to that in wild-type cells (Fig. 6B).

Cpc2 does not alter Ran1 activity in vitro. One hypothesis consistent with the results obtained to this point is that *cpc2* functions upstream of *ran1* and negatively regulates its activity. The mechanism employed by Cpc2 to inactivate Ran1 was further investigated. An in vitro kinase assay was used to measure Ran1 activity in partially purified yeast lysates to determine if Cpc2 inhibits Ran1 substrate phosphorylation. Ran1 was expressed as a GST fusion protein in wild-type cells, *cpc2*Δ cells, or cells expressing high levels of *cpc2*. Affinity-purified

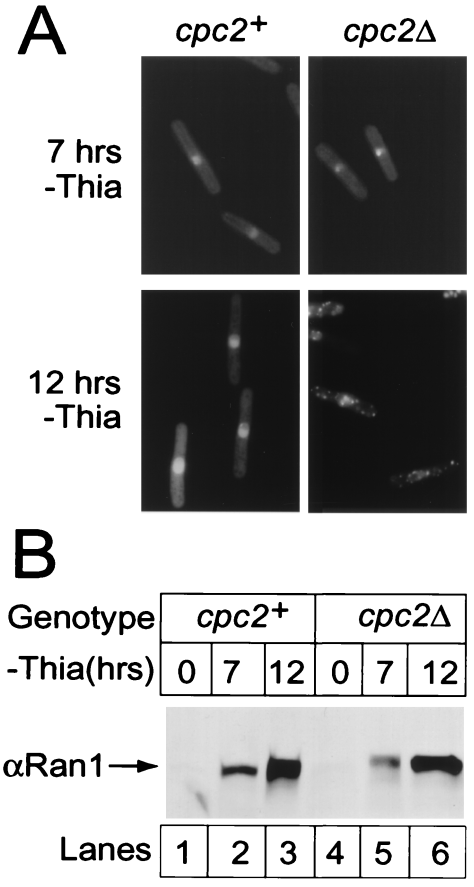

FIG. 6. Localization of the Ran1-GFP fusion protein. (A) *cpc2*$^+$ (SPB5) or *cpc2*Δ (SPB188) cells were transformed with a plasmid (pRAN1-GFP) expressing Ran1-GFP under control of the *nmt* promoter. Transformants were grown to 5×10^6 cells/ml in minimal medium containing thiamine (Thia). The cells were washed thoroughly in thiamine-free medium, and incubation was continued in the absence of thiamine for 7 or 12 h. The cells were then examined using a microscope. (B) The above cells were used to prepare whole-cell extract. A portion of the extract was separated by SDS-PAGE and used in a Western blot assay. The proteins were detected using anti-Ran1 antibody.

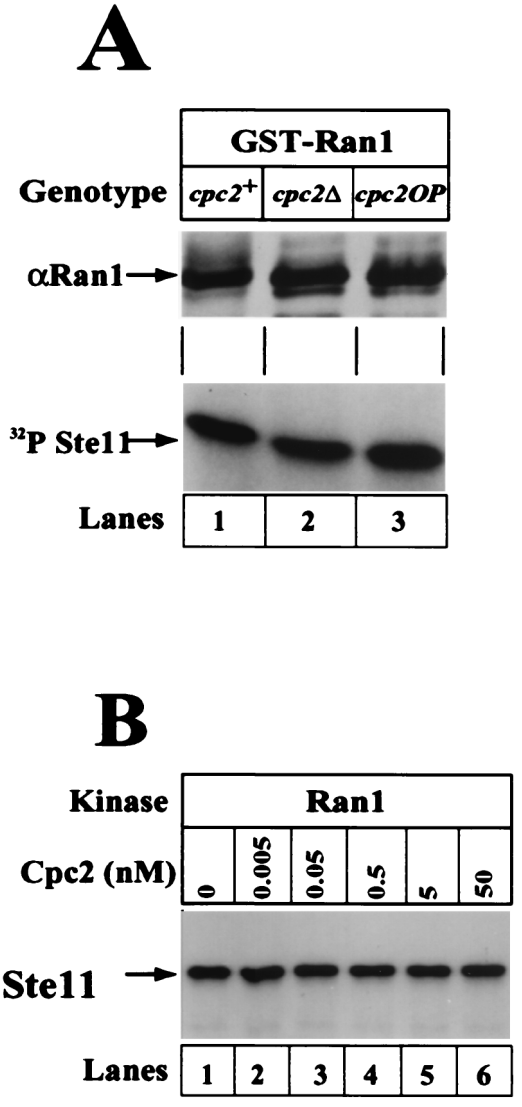

FIG. 7. Cpc2 does not alter Ran1 substrate phosphorylation in vitro. (A) Western blot (top) and in vitro kinase (bottom) assays of extracts obtained from *cpc2*$^+$ (SP66), *cpc2*Δ (SPB188), or *cpc2OP* (SPB870::CPC2-24) cells transformed with a plasmid (pRAN1-95) producing Ran1 under control of the *nmt* promoter. Ran1 is tagged at the amino terminus with GST-HA1 sequences. Twenty hours following induction of Ran1, cell lysate was prepared. Ran1 was affinity purified from the lysates and incubated in the in vitro kinase reaction. p39^{ste11} was used as the substrate (20). (B) Recombinant p52^{ran1} and recombinant p39^{ste11} were incubated with [^{32}P]ATP in the absence (lane 1) or the presence (lanes 2 to 6) of increasing concentrations of recombinant Cpc2. Phosphorylation of p39^{ste11} was monitored using SDS-PAGE and autoradiography.

Ran1 was incubated with p39^{ste11} as the substrate and radioactive ATP (Fig. 7A). This experiment revealed that Ran1 substrate phosphorylation was unaltered by Cpc2. To more accurately quantitate the effect of Cpc2 on Ran1, both proteins were expressed in and purified from bacteria. $(HIS)_6$-tagged Cpc2 was purified by Ni^{2+} affinity chromatography, and increasing amounts of Cpc2 were added to $(HIS)_6$-Ran1 in an in vitro kinase assay (Fig. 7B). This analysis showed that addition of Cpc2 had no effect on Ran1 substrate phosphorylation. Thus, Cpc2 does not inhibit Ran1 activity under these conditions. As shown in Fig. 1B, Cpc2 contains serine and threonine residues (Ser-39 and Thr-128) at positions corresponding to the phosphoacceptor serine and threonine residues in Ste11 (20). However, the in vitro kinase assays also demonstrate that Cpc2 is not likely to function as a substrate for Ran1 (data not shown).

Mammalian RACK1 is a functional homologue of the fission yeast gene. We wished to determine if Cpc2 and mammalian RACK1 are functional, as well as structural, homologues. Recently, it was shown that *N. crassa cpc-2* is a functional homologue of the *S. cerevisiae CPC2* gene (15). To investigate this, rat RACK1 (which is identical to the human protein) was expressed in yeast under control of the *adh* promoter. This plasmid was introduced into a *cpc2*Δ strain. As controls, the cells were transformed with the vector alone or with a plasmid expressing fission yeast *cpc2*. Individual transformants were examined in several assays (Fig. 8). As previously observed, loss of *cpc2* caused temperature-sensitive growth. Additionally, *cpc2*Δ cells formed small colonies compared to wild-type cells. Expression of mammalian RACK1 reversed both defects as efficiently as yeast *cpc2*. The transformants were also examined to determine if the mammalian gene was able to reverse the *cpc2*Δ sexual differentiation defect. For this assay, cells were

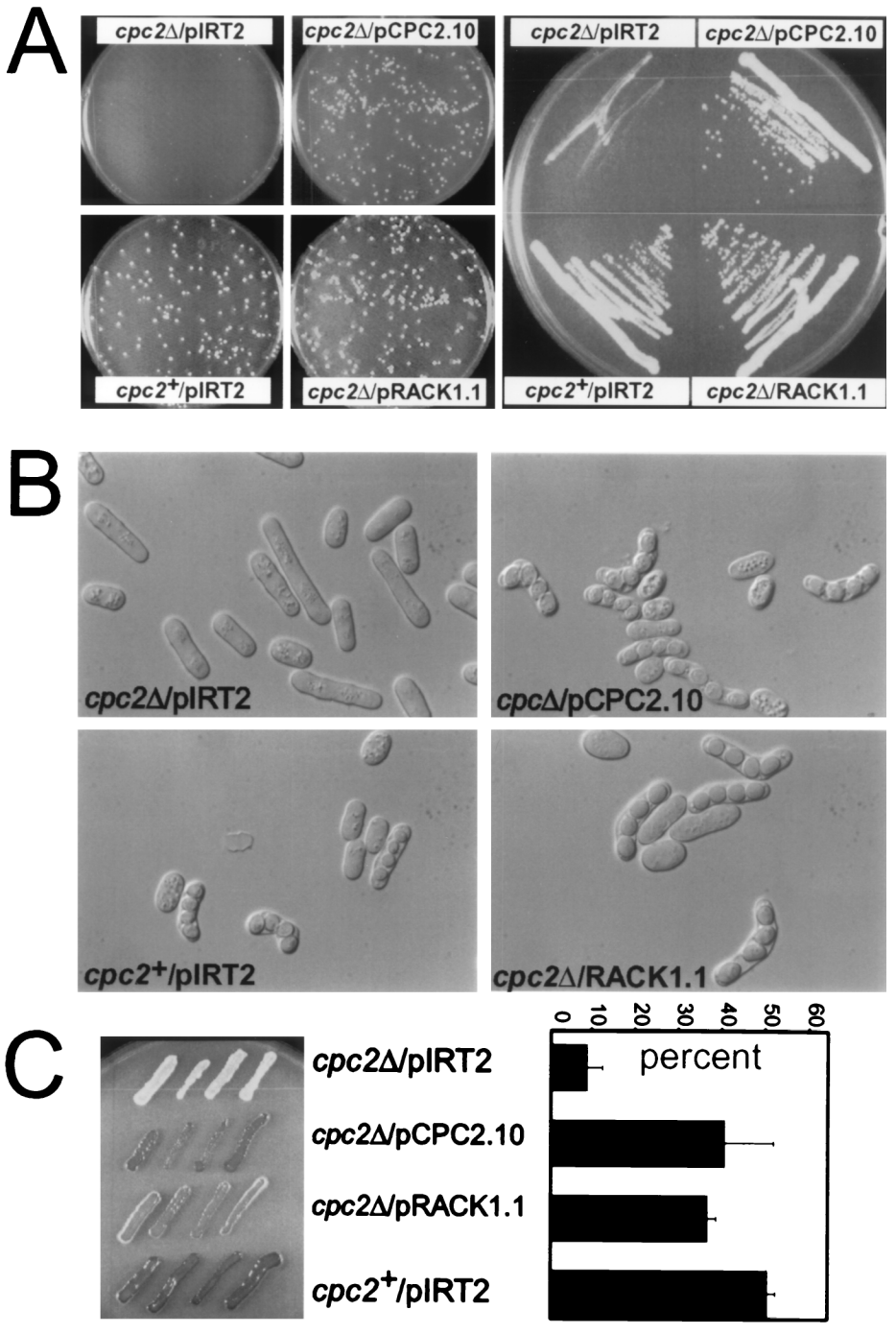

FIG. 8. Functional complementation of *cpc2*Δ by expression of the mammalian gene. Either *cpc2*$^+$ (SP66) or *cpc2*Δ (SPB190) cells were transformed with a control plasmid (pIRT2), the fission yeast *cpc2* gene (pCPC2.10), or the gene for rat RACK1 (pRACK1.1). (A) The transformants were spread on minimal-medium plates and incubated at 30°C to observe colony morphology (left side of panel). In addition, a representative colony was streaked for single colonies and incubated at 37°C to examine viability (right side of panel). (B) The morphology of the cells was examined using Nomarski optics. (C) Patches of cells were incubated at 30°C for 2 days and stained using iodine vapor to observe spore-containing cells. The patched cells were examined under a microscope to count vegetative cells, zygotes, and spore-containing cells. The bar graph represents the percentages of cells that have conjugated or formed spores. Three independent samples were measured.

plated on minimal medium for 2 days and stained with iodine vapor to observe spore-containing cells. Both yeast *cpc2* and mammalian RACK1 restored the ability of *cpc2*Δ cells to sporulate. This experiment provides evidence that mammalian RACK1 is a structural and functional homologue of fission yeast *cpc2*.

DISCUSSION

Ran1 is a key regulator of the developmental switch between mitotic cell growth and meiosis. Activation of Ran1 inhibits sexual differentiation (23), while inactivation of the kinase initiates G_1 arrest, conjugation, and meiosis (3, 16, 17, 32). We

screened a fission yeast library using a two-hybrid interaction assay to identify protein partners for Ran1. Here, we describe one of these, the product of the *cpc2* gene. Fission yeast Cpc2 is a structural and functional homologue of mammalian RACK1 proteins. RACK1 was initially isolated as a βPKC-binding protein. It is hypothesized to function as an anchoring protein for activated βPKC to enable access of the kinase to physiologically important substrates. Evidence is presented that Cpc2 interacts with Ran1. Analysis of cells containing the *cpc2* null allele indicates that it is important for cell cycle progression, as well as efficient conjugation and meiosis. Cpc2 may regulate Ran1 activity by controlling cellular localization of the kinase.

The interaction between Ran1 and Cpc2 is specific and may be direct. Three lines of evidence obtained using different experimental approaches indicate that Ran1 and Cpc2 interact. Firstly, Cpc2 was identified as a Ran1-interacting protein in a two-hybrid library screen. In support of this finding, we found Ran1 and Cpc2 physically associated in cell extracts. Cpc2 contains two regions that resemble a motif (known as RKD) previously identified in both the Ste11 substrate and the Mei3 inhibitor of Ran1. Thus, the interaction between Ran1 and Cpc2 may be mediated by the RKD-like sequences of *Cpc2*. Mutagenesis of specific residues in both Cpc2 RKD regions diminishes the interaction observed in the two-hybrid assay. One interpretation of this observation is that the RKD regions mediate Cpc2 interaction with Ran1. However, it can be argued that the mutations, which are in conserved regions of the protein, are required for functional folding of the protein. In that case, failure of the polypeptide to interact with Ran1 could be a general consequence of misfolding. We favor the former interpretation. Expression of Cpc2 RKD on a plasmid in *cpc2*Δ cells causes partial suppression of *cpc2*-associated defects (data not shown). This suggests that *cpc2* RKD is a weak but functional, allele, perhaps producing a product with reduced affinity for Ran1. High-level expression of the altered protein may compensate for an affinity defect but is less likely to restore a function lost because of incorrect folding.

Genetic assays indicate that the association between Ran1 and Cpc2 is specific and provide a functional link between the two proteins. Ran1 controls the transition between mitosis and meiosis in response to nutritional (and perhaps other) signals. In cells unable to inactivate Ran1, conjugation and sporulation are blocked and the cells are highly elongated. Loss of *cpc2* leads to similar phenotypes. The differentiation defect of *cpc2*Δ is reversed by inactivation of *ran1*. These observations are consistent with the hypothesis that Cpc2 negatively regulates Ran1 activity.

What, then, is the function of Cpc2? Ran1 associated with Cpc2 does not exhibit altered kinase activity in vitro, even though a wide range of Cpc2 concentrations was tested. If, as suggested by the two-hybrid results presented in Fig. 1, the Cpc2 RKD regions contribute to its association with the kinase, then Cpc2 is expected to be a competitive inhibitor of Ran1 substrate phosphorylation. Further biochemical experiments are required to examine the role of Cpc2 as an inhibitor. However, in vivo data suggest that Cpc2 does not function simply as a competitive inhibitor of Ran1. Cells producing high levels of Ran1 are sterile, and this defect can be reversed by expression of Ste11 (20) but not by expression of Cpc2. Moreover, the differentiation defect of *cpc2*Δ cells is not reversed by expression of Ste11 (B. Shor and M. McLeod, unpublished data). These results suggest either that Cpc2 has a more extensive role in meiosis than as a competitive inhibitor of Ran1 or that another component is required for Cpc2 to function.

The interaction between RACK1 and PKC regulates the cellular localization of activated PKC. Our data suggest that Cpc2 may regulate Ran1, not by altering the catalytic activity of the kinase but by influencing its subcellular localization. As shown in Fig. 6, *cpc2*Δ cells accumulate Ran1-GFP as bright dots in the cytoplasm with little nuclear concentration of the fusion protein. Both phenomena are absent in cells containing functional Cpc2. This result is specific to Ran1-GFP and is unlikely to be an insignificant artifact of the expression system. No other genetic backgrounds tested alter Ran1 localization. The mechanism used to localize Ran1 is not clear. Cpc2 could function as an anchoring protein to dock the kinase to a site proximal to substrates or regulators. In favor of this hypothesis, Cpc2 structurally resembles the β subunit of heterotrimeric G proteins. The βγ subunits of heterotrimeric G proteins have recently been shown to function as anchoring molecules for protein kinases (4, 34). Alternatively, it is possible that Cpc2 participates in a posttranslational processing event required to control the regional concentration of Ran1. There is evidence that, in addition to a RACK1-binding site, PKC contains a pseudoanchoring site that resembles sequences in RACK1. The binding site and the pseudoanchoring site associate in an intramolecular reaction. In this conformation, βPKC must be activated by phosphatidylserine, diacylglycerol, and calcium to function as a kinase. Upon activation, the RACK1-binding region is liberated from the pseudoanchoring site and becomes available to bind RACK1. In this conformation, the kinase becomes independent of its normal activators and translocates to membranes (38). We have not identified a pseudo-Cpc2 region in the Ran1 polypeptide, although one may exist. However, the phenotype of cells devoid of Cpc2 is intriguing in light of the RACK1 model. *cpc2* is not absolutely required for development but determines both the timing and extent of meiotic differentiation. If Cpc2, like RACK1, can stabilize Ran1 in an intermediate state of activation, the kinase may no longer be responsive to minor signal fluctuations. Loss of a stabilizer ought not to prevent the execution of an event but may influence its progression.

Structural and functional conservation of Cpc2. One of the most significant findings presented here is the identification of a structurally and functionally conserved RACK1 homologue as a partner for a kinase. It is somewhat surprising to identify the highly conserved RACK1 protein interacting with a protein that does not appear to have a structural counterpart in other organisms. The closest relative to Ran1 in budding yeast is Sks1p. The two proteins share 52% homology, and this is limited to their kinase domains. Conversely, the PKA pathway is conserved from yeast to mammals. As reported here, the phenotypes of cells containing *cpc*Δ resemble those caused by high cAMP levels. These observations, and the finding that RACK1 interacts with a mammalian phosphodiesterase, provide a rationale for the hypothesis that Cpc2 regulates cAMP levels in yeast. However, our studies failed to identify a connection between Cpc2 and the PKA pathway. We also investigated phenotypes associated with loss of the two identified fission yeast PKC genes to search for PKC interaction with Cpc2 (Shor and McLeod, unpublished data). To date, no interaction has been demonstrated in yeast. The interaction between RACK1 proteins and specific signal transduction pathways may vary depending on the particular cell type examined or the signal to which the cell is responding. However, one common theme emerging from studies of Cpc2 (RACK1) in diverse systems is that Cpc2 (RACK1) alters cell cycle progression and, in particular, may augment exit from the mitotic cell cycle into either G_0 or a differentiation pathway. *S. pombe cpc2*Δ cells are cell cycle delayed and slow to respond to signals that induce G_1 arrest. In NIH 3T3 cells, expression of RACK1

causes a G_1/G_0 delay, as well as a reduced growth rate (5). Budding yeast cells devoid of *CPC2* enter G_0 prematurely (15). Both *S. pombe* and *N. crassa* devoid of *cpc-2* exhibit fertility defects (28). On the other hand, RACK1 specifically associates with the integrin β subunit in lymphoblastoid cells (21), suggesting that some integrin signaling pathways function through a RACK1-PKC pathway. Notably, yeast cells appear to lack integrins. Recent evidence indicates that RACK1 binds pleckstrin homology domains from some proteins. The authors speculate that RACK1 scaffolding proteins are multivalent organizers that coordinate complex signaling systems (35). With this intriguing observation in mind, further genetic analysis is under way to identify other potential partners for Cpc2.

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REFERENCES

1. **Alfa, C., P. Fantes, J. Hyams, M. McLeod, and E. Warbrick.** 1993. Experiments with fission yeast. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
2. **Basi, G., E. Schmid, and K. Maundrell.** 1993. TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene **123:**131–136.
3. **Beach, D., L. Rodgers, and J. Gould.** 1985. *ran1+* controls the transition from mitotic division to meiosis in fission yeast. Curr. Genet. **10:**297–311.
4. **Bell, B., H. Xing, K. Yan, N. Gautam, and A. J. Muslin.** 1999. KSR-1 binds to G-protein βγ subunits and inhibits βγ-induced mitogen-activated protein kinase activation. J. Biol. Chem. **274:**7982–7986.
5. **Chang, B. Y., K. B. Conroy, E. M. Machleder, and C. A. Cartwright.** 1998. RACK1, a receptor for activated C kinase and a homolog of the β subunit of G proteins, inhibits activity of Src tyrosine kinases and growth of NIH 3T3 cells. Mol. Cell. Biol. **18:**3245–3256.
6. **Costello, G., L. Rodgers, and D. Beach.** 1986. Fission yeast enters the stationary phase G_0 state from either mitotic G_1 or G_2. Curr. Genet. **11:**119–125.
7. **Davey, J.** 1998. Fusion of a fission yeast. Yeast **14:**1529–1566.
8. **Davey, J., and O. Nielsen.** 1994. Mutations in *cyr1* and *pat1* reveal pheromone-induced G_1 arrest in the fission yeast *Schizosaccharomyces pombe*. Curr. Genet. **26:**105–112.
9. **DeVoti, J., G. Seydoux, D. Beach, and M. McLeod.** 1991. Interaction between *ran1+* protein kinase and cAMP dependent protein kinase as negative regulators of fission yeast meiosis. EMBO J. **10:**3759–3768.
10. **Egel, R.** 1973. Commitment to meiosis in fission yeast. Mol. Gen. Genet. **121:**277–284.
11. **Egel, R., and M. Egel-Mitani.** 1974. Premeiotic DNA synthesis in fission yeast. Exp. Cell Res. **88:**127–134.
12. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell **75:**805–816.
13. **Hoffman, C. S., and F. Winston.** 1991. Glucose repression of transcription of the *Schizosaccharomyces pombe fbp1* gene occurs by a cAMP signaling pathway. Genes Dev. **5:**561–571.
14. **Hoffman, C. S., and F. Winston.** 1989. A transcriptionally regulated expression vector for the fission yeast *Schizosaccharomyces pombe*. Gene **84:**473–479.
15. **Hoffmann, B., H. U. Mosch, E. Sattlegger, I. B. Barthelmess, A. Hinnebusch, and G. H. Braus.** 1999. The WD protein Cpc2p is required for repression of Gcn4 protein activity in yeast in the absence of amino-acid starvation. Mol. Microbiol. **31:**807–822.
16. **Iino, Y., and M. Yamamoto.** 1985. Mutants of *Schizosaccharomyces pombe* which sporulate in the haploid state. Mol. Gen. Genet. **198:**416–421.
17. **Iino, Y., and M. Yamamoto.** 1985. Negative control for the initiation of meisois in *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **82:**2447–2451.
18. **Imai, Y., and M. Yamamoto.** 1994. The fission yeast mating pheromone P-factor: its molecular structure, gene structure, and ability to induce gene expression and G1 arrest in the mating partner. Genes Dev. **8:**328–338.
19. **Kelly, M., J. Burke, M. Smith, A. Klar, and D. Beach.** 1988. Four mating-type genes control sexual differentiation in the fission yeast. EMBO J. **7:**1537–1547.
20. **Li, P., and M. McLeod.** 1996. Molecular mimicry in development: identification of *ste11+* as a substrate and *mei3+* as a pseudosubstrate inhibitor of *ran1+* kinase. Cell **87:**869–880.
21. **Liliental, J., and D. D. Chang.** 1998. Rack1, a receptor for activated protein kinase C, interacts with integrin β subunit. J. Biol. Chem. **273:**2379–2383.
22. **Maeda, T., N. Mochizuki, and M. Yamamoto.** 1990. Adenylyl cyclase is dispensable for vegetative cell growth in the fission yeast *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **87:**7814–7818.
23. **McLeod, M., and D. Beach.** 1988. A specific inhibitor of the *ran1+* protein kinase regulates entry into meiosis in *Schizosaccharomyces pombe*. Nature **332:**509–514.
24. **McLeod, M., M. Stein, and D. Beach.** 1987. The product of the *mei3+* gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast. EMBO J. **6:**729–736.
25. **Millard, P. J., B. L. Roth, H. P. Thi, S. T. Yue, and R. P. Haugland.** 1997. Development of the FUN-1 family of fluorescent probes for vacuole labeling and viability testing of yeasts. Appl. Environ. Microbiol. **63:**2897–2905.
26. **Mochizuki, N., and M. Yamamoto.** 1992. Reduction in the intracellular cAMP level triggers initiation of sexual development in fission yeast. Mol. Gen. Genet. **233:**17–24.
27. **Mochly-Rosen, D., H. Khaner, J. Lopez, and B. L. Smith.** 1991. Intracellular receptors for activated protein kinase C. Identification of a binding site for the enzyme. J. Biol. Chem. **266:**14866–14868.
28. **Muller, F., D. Kruger, E. Sattlegger, B. Hoffmann, P. Ballario, M. Kanaan, and I. B. Barthelmess.** 1995. The *cpc-2* gene of *Neurospora crassa* encodes a protein entirely composed of WD-repeat segments that is involved in general amino acid control and female fertility. Mol. Gen. Genet. **248:**162–173.
29. **Neer, E. J., C. J. Schmidt, R. Nambudripad, and T. F. Smith.** 1994. The ancient regulatory-protein family of WD-repeat proteins. Nature **371:**297–300.
30. **Neer, E. J., T. F. Smith, E. J. Neer, and T. F. Smith.** 1996. G protein heterodimers: new structures propel new questions. Cell **84:**175–178.
31. **Nielsen, O., J. Davey, and R. Egel.** 1992. The *ras1* function of *Schizosaccharomyces pombe* mediates pheromone-induced transcription. EMBO J. **11:**1391–1395.
32. **Nurse, P.** 1985. Mutants of the fission yeast *Schizosaccharomyces pombe* which alter the shift between cell proliferation and sporulation. Mol. Gen. Genet. **198:**497–502.
33. **Nurse, P., and Y. Bissett.** 1981. Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. Nature **292:**558–560.
34. **Pitcher, J. A., J. Inglese, J. B. Higgins, J. L. Arriza, P. J. Casey, C. Kim, J. L. Benovic, M. M. Kwatra, M. G. Caron, and R. J. Lefkowitz.** 1992. Role of βγ subunits of G proteins in targeting the β-adrenergic receptor kinase to membrane-bound receptors. Science **257:**1264–1267.
35. **Rodriguez, M. M., C. H. Chen, B. L. Smith, and D. Mochly-Rosen.** 1999. Characterization of the binding and phosphorylation of cardiac calsequestrin by epsilon protein kinase C. FEBS Lett. **454:**240–246.
36. **Ron, D., C. H. Chen, J. Caldwell, L. Jamieson, E. Orr, and D. Mochly-Rosen.** 1994. Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. Proc. Natl. Acad. Sci. USA **91:**839–843.
37. **Ron, D., and D. Mochly-Rosen.** 1994. Agonists and antagonists of protein kinase C function, derived from its binding proteins. J. Biol. Chem. **269:**21395–21398.
38. **Ron, D., and D. Mochly-Rosen.** 1995. An autoregulatory region in protein kinase C: the pseudoanchoring site. Proc. Natl. Acad. Sci. USA **92:**492–496.
39. **Russell, P. R., and B. D. Hall.** 1983. The primary structure of the alcohol dehydrogenase gene from the fission yeast *Schizosaccharomyces pombe*. J. Biol. Chem. **258:**143–149.
40. **Shan, X., H. Luo, B. Houle, and J. Wu.** 1994. Expression of a G-protein beta subunit-related gene during lymphocyte activation. Int. Immunol. **6:**739–749.
41. **Shiozaki, K., and P. Russell.** 1996. Conjugation, meiosis, and the osmotic stress response are regulated by *Spc1* kinase through *Atf1* transcription factor in fission yeast. Genes Dev. **10:**2276–2288.
42. **Stettler, S., E. Warbrick, S. Prochnik, S. Mackie, and P. Fantes.** 1996. The *wis1* signal transduction pathway is required for expression of cAMP-repressed genes in fission yeast. J. Cell Sci. **109:**1927–1935.
43. **Sugimoto, A., Y. Iino, T. Maeda, Y. Watanabe, and M. Yamamoto.** 1991. *Schizosaccharomyces pombe ste11+* encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. Genes Dev. **5:**1990–1999.
44. **Toone, W. M., S. Kuge, M. Samuels, B. A. Morgan, T. Toda, and N. Jones.** 1998. Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (exportin) and the stress-activated MAP kinase Sty1/Spc1. Genes Dev. **12:**1453–1463.
45. **Van Heeckeren, W. J., D. R. Dorris, and K. Struhl.** 1998. The mating-type

proteins of fission yeast induce meiosis by directly activating *mei3* transcription. Mol. Cell. Biol. **18:**7317–7326.
46. **Vassarotti, A., and J. D. Friesen.** 1985. Isolation of the fructose-1,6-bisphosphatase gene of the yeast *Schizosaccharomyces pombe*. Evidence for transcriptional regulation. J. Biol. Chem. **260:**6348–6353.
47. **Wang, W., P. Li, A. Schettino, Z. Peng, and M. McLeod.** 1998. Characterization of functional regions in the *Schizosaccharomyces pombe mei3* developmental activator. Genetics **150:**1007–1018.
48. **Watanabe, Y., S. Shinozaki-Yabana, Y. Chikashige, Y. Hiraoka, and M. Yamamoto.** 1997. Phosphorylation of RNA-binding protein controls cell cycle switch from mitotic to meiotic in fission yeast. Nature **386:**187–190.
49. **Willer, M., L. Hoffmann, U. Styrkársdóttir, R. Egel, J. Davey, and O. Nielsen.** 1995. Two-step activation of meiosis by the *mat1* locus in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **15:**4964–4970.
50. **Wu, S.-Y., and M. McLeod.** 1995. The *sak1*$^+$ gene of *Schizosaccharomyces pombe* encodes an RFX family DNA-binding protein that positively regulates cyclic AMP-dependent protein kinase-mediated exit from the mitotic cell cycle. Mol. Cell. Biol. **15:**1479–1488.
51. **Yamamoto, M., Y. Imai, and Y. Watanabe (ed.).** 1997. Mating and sporulation in *Schizosaccharomyces pombe*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
52. **Yarwood, S. J., M. R. Steele, G. Scotland, M. D. Houslay, and G. B. Bolger.** 1999. The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. J. Biol. Chem. **274:** 14909–14917.
53. **Zhao, Y., M. Yu, M. Chen, R. T. Elder, A. Yamamoto, and J. Cao.** 1998. Pleiotropic effects of HIV-1 protein R (Vpr) on morphogenesis and cell survival in fission yeast and antagonism by pentoxifylline. Virology **246:**266–276.