Sit4 Is Required for Proper Modulation of the Biological Functions Mediated by Pkc1 and the Cell Integrity Pathway in Saccharomyces cerevisiae*

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Maintenance of cellular integrity in Saccharomyces cerevisiae is carried out by the activation of the protein kinase C-mediated mitogen-activated protein kinase (PKC1-MAPK) pathway. Here we report that correct down-regulation of both basal and induced activity of the PKC1-MAPK pathway requires the SIT4 function. Sit4 is a protein phosphatase also required for a proper cell cycle progression. We present evidence demonstrating that the G1 to S delay in the cell cycle, which occurs as a consequence of the absence of Sit4, is mediated by up-regulation of Pkc1 activity. Sit4 operates down-stream of the plasma membrane sensors Mid2, Wsc1, and Wsc2 and upstream of Pkc1. Sit4 affects all known biological functions involving Pkc1, namely Mpk1 activity and cell wall integrity, actin cytoskeleton organization, and ribosomal gene transcription.

The Saccharomyces cerevisiae gene SIT4 codes for a Ser/Thr protein phosphatase member of the PPP phosphatase family that is closely related to the PP2A family (1, 2). Sit4 displays a high level of identity to both the fission yeast phosphatase ppe1 and the human protein phosphatase 6, which are involved in cell cycle regulation (3, 4). Sit4 participates in a number of cellular processes such as the TOR pathway-mediated response to nutrients (5–7) and the regulation of monovalent ion homeostasis and intracellular pHi (8). Sit4 also plays an important role in cell cycle regulation, as it is required for the proper G1 to S phase transition (9, 10). Cells deleted for SIT4 are either nonviable or display slow growth because of an expanded passage through G1 (9, 11–13). This delay is partly because of the role of SIT4 in the normal transcription control of the G1 cyclin genes CLN1 and CLN2, and also in the control of SWI4, coding for a DNA-binding protein required for transcriptional modulation of CLN1/CLN2 (14, 11). In addition, SIT4 is believed to function in a pathway parallel to CLN3 for the activation of CLN1 and CLN2 expression through BCK2 (15).

Ppz1 and Ppz2 (16, 17) represent another subset of Ser/Thr protein phosphatases, which play an opposite role to Sit4 in cell cycle regulation (13). The absence of PPZ1 compensates for the delay in cyclin accumulation and also alleviates the budding defect observed in a sit4Δ mutant (13). PPZ1 has been reportedly involved in the maintenance of cell integrity in cooperation with the PKC1-mitogen activated protein kinase (MAPK)1 pathway (17). Overproduction of Ppz1 suppresses the lysis phenotype of null mutants in PKC1 and MPK1 (17). The PKC1-MAPK pathway is a phosphorylation cascade that responds to signals related to yeast cell integrity, such as: mating pheromone (18), low osmolarity (19), and high temperatures (20). Mpk1/Slt2 is the last kinase member of the pathway. Simultaneous deletion of MPK1 and PPZ1 is lethal for the cell (17).

Cell wall stress is detected by the plasma membrane sensors Mid2 (21), Wsc1/Hsc77/Sig1, Wsc2, and Wsc3 (22, 23), and the signal is transmitted downstream via the GTP-binding protein Rho1 that activates the PKC1-MAPK module (21, 22). Pkc1 phosphorylates the MAPK kinase kinase Bck1 (24), which in turn, transmits the signal to the redundant MAPK kinases: Mkk1 and Mkk2 (25). These finally phosphorylate the Slt2/Mpk1 MAPK (26) on both Tyr192 and Thr190 residues (19, 27, 28) causing the activation of the kinase. Phosphorylation and activation of Mpk1 leads to a number of cellular responses. Thus, activation of Mpk1 results in phosphorylation of the transcriptional factor Swi6 through which the pathway is linked to the cell cycle regulatory machinery (29, 30). The PKC1 pathway is also involved in budding control (18) and cell wall synthesis (21), by regulating (i) the expression (often in a cell cycle-dependent fashion) of several genes coding for proteins related to cell wall synthesis and structure (31–34), and (ii) the organization of the actin cytoskeleton (35).

Genetic evidence indicates that Ppz1/Ppz2 phosphatases act independently of the PKC1-MAPK pathway (17). Their role therefore seems to be different from that of other phosphatases, such as Ptp2/Ptp3 (36) or Mag5 (37), which are known to dephosphorylate and negatively regulate certain components of the pathway. The observation that Ppz1 and Sit4 exhibit a functional antagonism in cell cycle regulation (13) prompted us to investigate whether this antagonism could also be extended to the functional connection with the PKC1 pathway.

Here we demonstrate that Sit4 is required for down-regulation of Pkc1 activity, and is consequently needed for a number of functions that depend on this kinase, such as Mpk1 activity, cytoskeleton organization, ribosomal gene expression, and cell cycle progression.

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The abbreviations used are: MAPK, mitogen-activated protein kinase; HA, hemagglutinin; FACS, fluorescence activated cell sorter; RP, ribosomal proteins.
Sit4 Phosphatase and Cell Integrity

MATERIALS AND METHODS

 Yeast Strains, Culture Medium, and Genetic Methods—The S. cerevisiae strains used in this work are listed in Table I. Unless otherwise stated, they are derived from either CML125 or CML128 wild type strains (38). The following strains were obtained from their corresponding diploids by tetrad analysis: MML200 and MML203 from MML182, MML344 and MML345 from MML282, and MML400 and MML402 from MML282.

 Yeast cells were usually grown in YPD medium (2% yeast extract, 1% peptone, 2% glucose) or in the selective glucose minimal medium, S5 (0.67% yeast nitrogen base, 2% glucose, and the required amino acids) (39). Where indicated, b-sorbitol was added to a final concentration of 1 M. To repress expression of the PKK1 gene under the teto promoter (40), cells were, respectively, grown in SD plus 10 μg/ml doxycycline until early log phase, then filtered and washed in the same medium without doxycycline. Cells were then resuspended in YPD and incubated for 6 h at 25 °C. After that, cultures were split in two. One-half was kept at the same temperature and the other was shifted to 37 °C for 30 min. Cells were subsequently collected by filtration and treated for total protein extraction as described in Ref. 41.

 Yeast transformations were performed as described in Ref. 42. The MPK1 gene was disrupted using a URA3 cassette (26). The URA3 marker from Candida albicans (43) was used to disrupt the WSC1 gene according to the one-step disruption method (44). This method was also employed to disrupt the MID2, BCK1, and MSG5 genes with the kanMX4 module and the WSC2 gene with the natMX4 module (45).

 DNA Manipulation and Plasmids—DNA manipulation, plasmid recovery, and bacterial transformation were performed according to standard methods (46). Escherichia coli DH5α (Invitrogen) was used for plasmid amplification.

 Yeplac195-SIT4 (TRP2α) plasmid harbors a genomic 2.65-kb Smal-NheI fragment containing the SIT4 gene cloned in the Smal-XhoI restriction site of Yeplac195 (47). Plasmid pMM66 is a Yeplac195 derivative (URA3/2μ) (48) containing MSG5 under its own promoter cloned at the Smal vector site. MSG5 was amplified by PCR from yeast genomic DNA. Plasmid pMM69 is a Yeplac195 derivative harboring the MSG5 gene under its own promoter and cloned into the KpnI and HindIII vector sites. Plasmid pMM126 is a pCM265 derivative (URA3/CEN) (40) that contains PCK1 under the teto promoter, and is tagged with three copies of the HA epitope at the N terminus of the protein. The PCK1-coding sequence was obtained from genomic DNA by PCR and directionally cloned into PmeI and PstI vector sites (47). The pHRS15-BCK1-20 plasmid is described in Ref. 24. The pMM1-1/HA plasmid is a YEp352 derivative. Mpkl ORF is cloned under its own promoter and HA-tagged in C-terminal (a gift from Maria Molina, University Complutense, Madrid, Spain).

 Cell Synchronization—For synchronization experiments, cells were exponentially grown to 10^7 cells/ml. S and G2 arrestes were performed with hydroxyurea and nocodazole, respectively, at the concentrations indicated in the text. G2 arrests were performed either by α-factor treatment (10 μg/ml) or by elutriation. All cell cycle arrests were performed at 25 °C for 2 h in the case of the wild type strain and for 4 h in the case of the sit4Δ mutant. Cells were elutriated according to the protocol described in Ref. 48. Fluorescence-activated cell sorting (FACS) sample analysis (49) was used to confirm correct synchronization.

 Actin Staining—Cells were stained with rhodamine-phalloidin as described in Ref. 50.

 Yeast Extracts and Immunoblot Analyses—For Western analysis, cells were collected on ice, filtered through 0.22-μm pore membranes, washed with ice-cold medium, transferred to Eppendorf tubes with 1 ml of ice-cold medium, and then centrifuged for 30 s at 13,000 rpm. Total yeast protein extracts were prepared in ice-cold lysis buffer (75 mM Tris-HCl, pH 7.5, 0.45 mM KCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM t-1-lysylamido-2-phenylethyl chloromethyl ketone, 1 mM pepstatin, 1 mM β-glycerophosphate, 1 mM EGTA, 5 mM sodium pyrophosphate, and the protease inhibitors chymostatin, leupeptin, and antipain each at 5 mmol/l). After the addition of SDS to a final concentration of 1%, lysates were then boiled at 95 °C for 5 min. Equivalent amounts of total protein extracts were run on SDS-PAGE gels with 10% acrylamide. The anti-phospho-p44/24 antibody (New England Biolabs) was used at a final dilution of 1:2000 in TBST buffer. The anti-Slt2/2 antibodies were used at a dilution of 1:10,000 in the same buffer, and the anti-GST-Mpk1 antibody (37, 51) at a dilution of 1:1000 in the presence of 5% fat milk (51). Horseradish peroxidase-linked secondary antibodies, anti-rabbit, or anti-mouse (NA931 and NA934, Amersham Biosciences), were used at a 1:10,000 dilution and incubated in TBST buffer containing 2% fat milk for the anti-phospho-Mpk1 and 0.25% fat milk for the other two primary antibodies. Chemiluminescence detection was performed using the Supersignal substrate (Pierce) in a Lumi-Imager (Roche Molecular Biochemicals).

 Kinase Activity—Immunoprecipitation of Pk1 and in vitro protein kinase assays were performed using either myelin basic protein (fragment 4-14, Sigma) or a Bck1-Ser939 synthetic peptide according to Ref. 41. To inhibit Pk1 activity, 4 × 10^-6 M staurosporine was added to the kinase reaction (52). Mpk1 immunoprecipitation and protein kinase assay were conducted following the protocol described in Ref. 20.

 RESULTS

 The Absence of Sit4 Leads to a Specific Up-regulation of Sit2/Mpk1 MAP Kinase Activity—We had previously tested Mpk1 basal and heat shock-induced levels of activity in a ppp2C1 mutant and observed that they were severely impaired (data not shown). To examine whether Mpk1 phosphorylation levels were higher in the absence of Sit4 than in wild type cells (antagonically to that observed in the ppp2C1 mutant), we used

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TABLE I

| Yeast strains used in this work | Relevant genotype | Ref. |
|-------------------------------|------------------|------|
| CML125                        | MATα leu2-3,112 ura3-52 trp1 his4 can1Δ | This work |
| CML128                        | MATα as CML125 | Gallego et al. (39) |
| JA-110                        | MATα sit4Δ TRP1 | Clotet et al. (13) |
| JA-117                        | MATα sit4Δ kanMX4 | Clotet et al. (13) |
| CML399                        | MATα mpk1Δ URA3 | This work |
| MML182                        | MATαΔ Sit4/Δ sit4Δ TRP1 BCK1/bck1Δ kanMX4 | This work |
| MML200                        | MATα bck1Δ kanMX4 | This work |
| MML203                        | MATα sit4Δ TRP1 bck1Δ kanMX4 | This work |
| MML282                        | MATαΔ PKC1/pkc1Δ LEU2Δ SIT4/sit4Δ kanMX4 | This work |
| MML302                        | MATα pkc1Δ LEU2Δ sit4Δ kanMX4 (pBCK1-20) | This work |
| MML304                        | MATα pkc1Δ LEU2Δ sit4Δ kanMX4 | This work |
| MML344                        | MATα pkc1Δ LEU2Δ sit4Δ kanMX4 | This work |
| MML345                        | MATα pkc1Δ LEU2Δ sit4Δ kanMX4 | This work |
| MML382                        | MATα wsc1Δ CaURA3 | This work |
| MML384                        | MATα wsc2Δ natMX4 | This work |
| MML387                        | MATα mid2Δ kanMX4 | This work |
| MML388                        | MATα mid2Δ kanMX4 sit4Δ TRP1 | This work |
| MML392                        | MATα wsc1Δ CaURA3 wsc2Δ natMX4 | This work |
| MML393                        | MATα wsc1Δ CaURA3 mid2Δ kanMX4 | This work |
| MML398                        | MATα wsc2Δ natMX4 sit4Δ TRP1 | This work |
| MML400                        | MATα wsc1Δ CaURA3 wsc2Δ natMX4 sit4Δ TRP1 | This work |
| MML401                        | MATα wsc1Δ CaURA3 mid2Δ kanMX4 sit4Δ TRP1 | This work |
| MML402                        | MATα wsc1Δ CaURA3 sit4Δ TRP1 | This work |

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* M. A. de la Torre-Ruiz, J. Torres, J. Arinño, and E. Herrero, unpublished observations.
the anti-phospho-p44/42 MAPK antibodies raised against dually phosphorylated (Thr202/Tyr204)-p44/42 MAPK. These antibodies allow accurate monitoring of Mpk1 activity (23, 37). Wild type and sit4Δ cells were grown at 25 °C and then shifted at 37 °C for various time periods (Fig. 1A). sit4Δ cells showed increased basal phosphorylation levels as compared with wild type cells (Fig. 1A). Upon heat shock, Mpk1 phosphorylation was much more intense in mutant than wild type cells, and remained higher for longer periods. These changes could not be ascribed to variations in the total amount of Mpk1 protein, as their levels remained constant in all cases, a fact deduced by probing the same protein samples with an anti-GST-Mpk1 antibody (Fig. 1A). Quantification and normalization of phosphorylation levels revealed that after 30 min of heat shock, Mpk1 phosphorylation levels in the sit4Δ mutant were 10 times higher than in wild type cells (Fig. 1B). We performed an in vitro Mpk1 kinase assay in order confirm that the dual Mpk1 phosphorylation detected with the p44/42 antibody correlated to Mpk1 activity. As expected, we were able to detect greater Mpk1 kinase activity levels in sit4Δ exponentially growing cells, both at 25 °C or after 30 min of shifting cells at 37 °C compared with the wild type (Fig. 1C). To prove that the increase in Mpk1 activity observed in a sit4Δ strain was not because of an indirect effect caused by the lack of this gene, we performed overexpression analyses by using a multicopy plasmid carrying Sit4 under its own promoter. Sit4 overexpression provoked a dramatic decrease in both the basal and induced phosphorylation state of Mpk1 in wild type cells (Fig. 1D), which demonstrates that Sit4 exercises specific regulatory control over Mpk1 activity.

We reasoned that if Mpk1 was hyperphosphorylated in cells lacking Sit4, this could result in biological changes derived from kinase activation. To test this we examined the phosphorylation of Swi6 (a transcription factor whose phosphorylation after heat shock depends on Pkc1-mediated Mpk1 activation (29, 30, 33)).

We monitored Swi6 phosphorylation with polyclonal antibodies that detect two forms of the protein in wild type cells: a faster migrating band corresponding to the hypophosphorylated Swi6 state and a slower mobility hyperphosphorylated Swi6 band. In nonstressed wild type cells only the hypophosphorylated form was detected, and shifting the cells to 37 °C resulted in the appearance of hyperphosphorylated Swi6 (Fig. 1A). In contrast, in sit4Δ cells extracellular hyperphosphorylated Swi6 was readily observed at 25 °C. Moreover, after heat shock the proportion of this form dramatically increased with respect to wild type cells, and remained higher throughout the experiment (Fig. 1A). Hyperphosphorylation of Swi6 in sit4Δ cells after heat shock was fully mediated by Mpk1, as no changes in mobility were observed in a sit4Δ-mpk1Δ double mutant (data not shown). In conclusion, the above results point to Sit4 being necessary for negative modulation of Mpk1 activity and, consequently also for downstream processes dependent on this activity.

The Absence of Sit4 Affects Mpk1 Phosphorylation at All Phases of the Cell Cycle and Is Not the Result of Intrinsic Cell Wall Defects—It could be hypothesized that the increased activation of Mpk1 found in sit4Δ cells derives from intrinsic cell wall defects in the mutant that would lead to constitutive hyperactivation of the PKC1-MAPK pathway. To test this possibility, we monitored Mpk1 phosphorylation in cells growing in the presence of 1 M sorbitol used as an osmotic stabilizer. This condition prevented Mpk1 phosphorylation when the cell wall was severely stressed (34). Growth in the presence of sorbitol resulted in reduced Mpk1 phosphorylation after the shift to 37 °C (Fig. 2A). However, the presence of the stabilizer did not prevent Mpk1 basal phosphorylation in a sit4Δ mutant with respect to other wild type cells. Therefore, hyperactivation of Mpk1 in the absence of Sit4 is not a consequence of hypothetical cell wall defects derived from the lack of Sit4. In fact, a sit4Δ mutant showed increased tolerance to treatment with zymolyase (a combination of 1,3-β-glucanase and protease en-
enzymes that degrade the yeast cell wall) with respect to wild type cells (Fig. 2B). In contrast, as previously described (34) the mpk1Δ mutant was hypersensitive to enzymatic digestion.

Exponentially growing cultures of sit4Δ are enriched in G1 cells, and it has been reported that Mpk1 becomes phosphorylated at the G1/S transition in a cell cycle-dependent manner (18). Increased Mpk1 dual phosphorylation in a sit4Δ mutant could therefore result from the presence of a higher proportion of cells in G1 in asynchronous cultures. To discard this possibility, we performed heat-shock experiments with cells synchronized with α-factor in G1, in S with hydroxyurea, and in G2 with nocodazole. In all three cases basal and heat-induced Mpk1 phosphorylation were higher in sit4Δ than in wild type cells (Fig. 2C). As these treatments are somewhat stressful for cells and might affect Mpk1 phosphorylation, G1 small daughter cells were recovered by elutriation, and allowed to progress through the cell cycle, to monitor Mpk1 phosphorylation. At all tested time points, Mpk1 phosphorylation in sit4Δ cells was more intense than in wild type cells (Fig. 2D). It can therefore be concluded that the absence of Sit4 affects Mpk1 phosphorylation regardless of its position in the cycle. This also demonstrates that the higher activity detected in sit4Δ cells is not merely a circumstantial effect caused by the partial synchronization in G1 in this mutant.

Sit4 Functions Upstream of Pkc1 and Negatively Regulates Its Activity—We constructed pck1Δ–sit4Δ and bck1Δ–sit4Δ double mutants to investigate whether the induction of Mpk1 activity that occurs in the absence of Sit4 was dependent on Pkc1. However, neither Mpk1 phosphorylation nor Swi6 hyperphosphorylation were observed in pck1Δ–sit4Δ (Fig. 3A), and data not shown) or bck1Δ–sit4Δ (not shown) double mutants at 25 or 37°C. We conclude that an intact PKC1-MAPK module is required for the Mpk1 activation caused by the absence of Sit4 function. This points against Sit4 defining a Mpk1-inactivating pathway independent from Pkc1.

We followed two different approaches to functionally situate Sit4 with respect to Pkc1. First, we used a BCK1–20 allele that constitutively activates Mpk1 (24). We transformed the PKC1/pkc1ΔSIT4/sit4Δ diploid strain using a plasmid bearing the BCK1–20 allele. After sporulation, the pck1Δ/sit4Δ, pck1Δ/pBCK1 strains were isolated (Fig. 3A, and data not shown) or bck1Δ/sit4Δ (not shown) double mutants at 25 or 37°C. We conclude that an intact PKC1-MAPK module is required for the Mpk1 activation caused by the absence of Sit4 function. This points against Sit4 defining a Mpk1-inactivating pathway independent from Pkc1.

We constructed pck1Δ20/sit4Δ, bck1Δ20/sit4Δ mutant strains (bearing the pBCK1–20 plasmid) were isolated (Fig. 3A, and data not shown). Our reasoning was therefore as follows: if Sit4 acts downstream of Pkc1 as a negative regulator for the pathway, then both pck1Δ20/pBCK1–20 and pck1Δ20/sit4Δ/pBCK1–20 cells would exhibit higher levels of Mpk1 activity than wild type and pck1Δ20 cells transformed with pBCK1–20, respectively. Alternatively, if Sit4 acts upstream of Pkc1 we would first expect the constitutive phosphorylation level of Mpk1 in sit4Δ/pBCK1–20 cells to be higher than in any of the other strains tested, and second we would expect pck1Δ20/sit4Δ/pBCK1–20 and pck1Δ20/sit4Δ/pBCK1–20 cells to exhibit the same constitutive levels of Mpk1 activity. This was indeed the case (Fig. 3B), and predicted by the second hypothesis, in pck1Δ20/sit4Δ/pBCK1–20 and pck1Δ20/pBCK1–20 strains, the constitutive activity of Mpk1 was lower than that observed in wild type/pBCK1–20 cells. We also observed that the absence of Sit4 together with the presence of the BCK1–20 allele had an additive effect on basal levels of Mpk1 activity (Fig. 3B). However, this sit4Δ-mediated additive effect was suppressed when PKC1 was deleted. This suggested that up-regulation of Mpk1 phosphorylation in sit4Δ/pBCK1–20 cells was the result of two independent processes, one because of constitutive Mpk1 activation caused by the BCK1–20 allele and the other because of up-regulation of Pkc1 activity in the absence of Sit4. These results support the hypothesis that sitiutes Sit4 upstream of Pkc1. To confirm this, in a second approach we sought to determine Pkc1 kinase activity using two different substrates. In both, wild type and sit4Δ cells were transformed with a centromeric plasmid bear-
Fig. 3. Sit4 is not epistatic to the PKC1-MAP pathway, and functions upstream of Pkc1 in the modulation of the pathway activity. A, mid-log cultures from wild type, sit4Δ-pkc1Δ, and sit4Δ-pkc1Δ strains growing at 25°C were shifted to 37°C for 30 min. B, exponential cultures of the following strains at 25°C: sit4Δ/pBCK1-20, sit4Δ-pkc1Δ/pBCK1-20, pck1Δ/pBCK1-20, pck1Δ/pBCK1-20, and CML128/pBCK1-20 were grown as described in the legend to Fig. 2 and samples were taken at 25°C for Western blot. Asterisk represent values corresponding to the amount of phosphorylated Mpk1 relative to total GST-Mpk1 protein for each strain. C, wild type and sit4Δ cells were transformed with a plasmid carrying the Pkc1-HA-tagged protein under the control of the tetO promoter. Growth conditions and protein preparation are described under “Materials and Methods.” Histograms represent arbitrary units of Pkc1 kinase activity using two different Pkc1 substrates. Pkc1-HA immunoprecipitates are shown in the inset. St, staurosporine. Schematic representation of some of the elements of the cell integrity pathway used in this study. D, mid-log cultures from sit4Δ, wild type, wsc1Δ, sit4Δ-wsc1Δ, mid2Δ, and sit4Δ-mid2Δ strains growing at 25°C were shifted at 37°C for 30 min. E, mid-log cultures from sit4Δ, wild type, sit4Δ-wsc1Δ, wsc1Δ-wsc2Δ, sit4Δ-wsc1Δ-mid2Δ, and wsc1Δ-mid2Δ strains growing at 25°C were shifted at 37°C for 30 min. F, exponentially growing wild type, bem2Δ-sit4Δ, and bem2Δ cells at 25°C were shifted to 37°C for 30 min (+). Equal amounts of total protein extracts were loaded onto SDS-polyacrylamide gels and subsequently immunoblotted with the anti-phospho-p44/p42 antibody. Except in part C, the bands shown in the figure correspond to phospho-Mpk1. The levels of total Mpk1 detected with the anti-GST-Mpk1 antibody were similar in each sample (data not shown).

The Absence of Sit4 Stimulates Transient Heat Shock-induced PKC1-dependent Actin Cytoskeleton Depolarization—Heat shock induces transient depolarization of the actin cytoskeleton (56, 57) mediated by upper cell integrity pathway components WSC1 and ROM2 (22). Rho1 and Pck1 hyperactivation also induces depolarization of the actin cytoskeleton in the absence of heat stress (22). We examined actin polarization in both wild type and sit4Δ cells to determine whether Sit4 was also involved in the regulation of this process. Both wild type phosphorylation levels observed in the sit4Δ strain (Fig. 3E). Bem2 is a GTPase activating protein that down-regulates Rho1 (54). Recent proteomic studies have shown that Bem2 and Sit4 along with other proteins form part of the same protein complex (55). Because both basal and induced Mpk1 activity are increased in bem2Δ compared with wild type cells (37), we wondered whether Sit4 might be regulating Bem2 activity. We observed that in the sit4Δ-bem2Δ double mutant there was an additive effect in the increase of basal Mpk1 (2.5-fold) and heat shock-induced phosphorylation (2-fold) with respect to both single mutants (Fig. 3F). This result discards the possibility of Sit4 being a regulator for Bem2 GTPase. Taken together, these results suggest that Sit4 operates downstream of Mid2, Wsc1, and Wsc2 membrane receptors, independently of Bem2 and upstream of Pkc1.

ing the Pkc1-HA-tagged protein under control of a regulatable tet promoter (40). We obtained the same results with both substrates: basal activity was barely detectable. However, Pkc1 activity induced after heat shock was much higher in sit4Δ than in wild type cells, and was suppressed after the addition of the Pkc1 inhibitor staurosporine (Fig. 3C). These results confirmed that Sit4 is required to negatively modulate Pkc1 activity in the cell integrity pathway.

We next studied Mpk1 phosphorylation in mutants lacking SIT4 and WSC1, WSC2, or MID2 plasma membrane receptor genes. In the case of both sit4Δ-wsc1Δ, and the sit4Δ-mid2Δ double mutants, Mpk1 phosphorylation was markedly more induced than in wsc1Δ-mid2Δ, and wild type cells at the respective temperatures (Fig. 3D). To simplify the figure we did not include the results obtained with the wsc2Δ mutant as they were similar to those obtained with wsc1Δ. To ascertain whether Sit4 is a regulator of more than one cell wall receptor (this could explain why we were unable to detect such a regulation when using single mutants), we constructed the wsc1Δ-wsc2Δ and wsc1Δ-mid2Δ double mutants and corresponding triple mutants in combination with sit4Δ. Again, in both wsc1Δ-wsc2Δ-sit4Δ and wsc1Δ-mid2Δ-sit4Δ triple mutants, both basal and induced Mpk1 activity was higher in wild type cells and double mutants, and was equivalent to the Mpk1
greater for longer than in wild type cells (data not shown). The mutant growth rate described for this mutant, because in the double process was also affected by Sit4 function. We shifted after heat shock is greater in a mutant this was only achieved 3 h after heat shock (Fig. 4). 37° and sit4 relative levels of with this theory, no significant differences were observed in than that responding to a secretory defect (59). In accordance shock, and that this repression occurs via a different pathway not required for repression of ribosomal mRNA after a heat ples at various time intervals. It has been reported that Pkc1 is tunicamycin to block their secretory machinery and took sam-

**Sit4 Also Regulates the PCK1 Function Required for Tran-
scriptional Repression of Ribosomal Genes—Transcriptional repression of ribosomal proteins (RP) after alteration of the yeast secretory pathway requires Pkc1 but not Mpk1 activity (58). We studied the transcriptional levels of the RPL30 and RPL6A ribosomal genes in sit4Δ and wild type cells to test whether this process was also affected by Sit4 function. We shifted sit4Δ and wild type cultures from 25 at 37 °C for half an hour to maximize the differences in Pkc1 activity between sit4Δ and wild type cells (see Fig. 1). We then treated or mock treated cells with tunicamycin to block their secretory machinery and took samples at various time intervals. It has been reported that Pkc1 is not required for repression of ribosomal mRNA after a heat shock, and that this repression occurs via a different pathway than that responding to a secretory defect (59). In accordance with this theory, no significant differences were observed in relative levels of RPL30 and RPL6A mRNAs or between sit4Δ and wild type cells after 90 min at 37 °C (Fig. 5), despite the large differences in Pkc1 activity reflected in Mpk1 phosphorylation (Fig. 1A). However, upon addition of tunicamycin, RP relative mRNA levels decreased significantly faster in sit4Δ than in wild type cells (Fig. 5, A and B), although with no apparent changes in ACT1 and U1 mRNA levels (data not shown). Nevertheless in mock treated cells mRNA levels showed a significant increase after heat shock that was maintained throughout the experiment (Fig. 5, C and D), in accordance with Ref. 59. Using anti-invertase polyclonal antibodies we could not detect differences in the invertase accumulation between wild type and sit4Δ cultures (data not shown) by Western blot, which indicates that the results shown above were not a reflection of intrinsic problems in secretion in the sit4Δ mutant. The lack of RP mRNA repression observed in a pck1Δ mutant, under conditions in which the secretory pathway was altered by tunicamycin, was also observed in the sit4Δ pck1Δ double mutant (data not shown), which means that in the absence of Sit4, Pkc1 activity was still required for this response. The greater RP mRNA repression observed in sit4Δ cells is therefore a Pkc1-dependent response to secretory problems. Our data indicate that Sit4 is also needed in a signaling process leading to ribosomal gene expression and that this is dependent on Pkc1 activity but not on downstream elements of the MAP kinase pathway.

Pck1 Is Involved in the G1 to S Delay Observed in the Absence of Sit4—As previously reported, cells lacking Sit4 display a marked defect in the G1 to S progression through the cell cycle (Ref. 9 and Fig. 6B), which results in slower growth compared with wild type cells (Fig. 6C). Interestingly, when we overexpressed Pck1 under the tet promoter we induced an extended G1 phase in wild type (Fig. 6A) and sit4Δ (not shown) cultures that provoked an increase in generation time in both strains. This was reflected in the accumulation of cells at G1 in exponentially growing cultures (Fig. 6A), which resembled sit4Δ FACS profiles. This delay in G1 was also observed when we overexpressed the constitutively active allele PCK1 (R398A,R405A,K406A) under the Gal promoter (not shown). Pck1 overexpression also provoked an increase in Mpk1 activity (Fig. 6A). Given the correlation observed between Mpk1 basal activity and G1 delay between the sit4Δ mutant and cells overexpressing Pck1, we speculated that high levels of PCK1-MAPK activity would induce a G1 delay. This could explain why, in the absence of Sit4, the cell cycle was extended in G1, in comparison with wild type cells. Following this line of reasoning, we would expect sit4Δ cells not to display a G1 defect in the absence of Pck1. As shown in Fig. 7A, pck1Δ deletion efficiently suppressed the accumulation of cells in sit4Δ cultures at G1. In all cases, cells were grown in rich medium plus the osmotic stabilizer sorbitol (1 M) to prevent cell lysis because of the absence of Pck1. In support to this, sit4Δ pck1Δ and also pck1Δ populations displayed similar generation times of about 180 min at 25 °C growing in rich medium plus sorbitol, very similar to that of wild type cells (Fig. 6C). Even so, the sit4Δ cells doubling time was twice as long as that observed in the above mentioned cultures. This phenotype must be specific to...
Pkc1 because the absence of Mpk1 did not rescue the G1 defect because of the absence of Sit4 function (Fig. 6, B and C). This is not strange since not all Pkc1 functions are mediated by Mpk1. We conclude from these results that high PKC1-MAPK activity induces a delay in the G1 to S phase progression, and that the G1 delay that occurs in the absence of Sit4 would be mediated by Pkc1.

**DISCUSSION**

One major finding of this study is that Sit4, a phosphatase whose function has been related to cell cycle control and nutritional state, is also involved in the functional regulation of the Pkc1 pathway. Sit4 functions upstream of Pkc1 and components of the PKC1-MAPK cascade, but downstream of the Wsc1, Wsc2, and Mid2 plasma membrane receptors, and the latter signal changes in cell wall integrity to Pkc1 via Rom2 and Rho1 (60). We also present evidence demonstrating that for this function Sit4 also acts independently of Bem2 (a GTPase-activating protein which regulates Rho1). The role of Sit4 in the modulation of the pathway is reflected in: (i) maintenance of a normal/basal level of Mpk1 activity throughout the cell cycle, and (ii) down-regulation the PKC1-MAPK module once this has been activated by external signals that affect cell membrane integrity, such as heat shock. Therefore, two biological processes that depend on Pkc1 activity via Mpk1 are affected by Sit4: phosphorylation of Swi6, and basal expression of FKS1 (data not shown) involved in cell wall assembly (31, 33). Little is known about the role that Pkc1-dependent Swi6 phosphorylation exerts in the cellular processes after heat shock (31). In addition, it has been reported that heat shock and osmotic stress cause cells to transitorily accumulate at G1, which correlates with a descent in the transcription of G1 cyclin genes CLN1 and CLN2 (61, 62). However, no clear relationship was observed between Swi6 phosphorylation after heat shock and cyclin expression, either in sit4Δ or in wild type cells (data not shown).

Another major novel finding addressed in this study is the observation that Pkc1 overexpression and activation of the pathway induce a prolonged G1 phase in exponentially growing cultures. This function might help to explain the G1 to S defect observed in sit4Δ mutants (14, 13), because such a defect is rescued to wild type levels in a sit4Δ-pkc1Δ double mutant. Furthermore, the fact that the absence of Mpk1 does not compensate for the sit4Δ growth defect suggests that the functional interaction between Sit4 and Pkc1 in G1 cell cycle regulation is specific to Pkc1 activity. Therefore, the G1 delay as a consequence of the absence of Sit4 function is mediated by the up-regulation of Pkc1 activity. Pkc1-mediated cell cycle regulation is not a trivial matter, because overexpression of CLN2, which shortens the G1 phase, does not compensate for the greater Mpk1 activity observed in the mutant.2 Future studies will contribute to clarify this function.

We have also addressed the question of whether the high levels of Pkc1 and Mpk1 activity observed in sit4Δ cells were merely a direct consequence of intrinsic cell wall problems. This is apparently not the case, as osmotic stabilization of the cell wall does not suppress up-regulation of Mpk1 activity. On the contrary, the observation that this mutant displayed a cell wall more resistant to zymolyase digestion could reflect two processes: (i) an up-regulation of Rho1 in the absence of Sit4 that could lead to greater Fks1 activity, and (ii) an increase in cell wall gene expression mediated by greater Mpk1 activity. Our unpublished observations2 show that Sit4 is functionally independent from the Ptp2 and Msg5 protein phosphatases, and that these directly down-regulate Mpk1 activity (36, 37).
**BCK2** is a suppressor of the lethality caused by mutations in the cell integrity pathway (27), and is also involved in the **SIT4** pathway for CLN activation (15). This raised the possibility of a functional relationship between **BCK2** and **SIT4** in controlling the activity in the PKC1-MAPK pathway. However, **BCK2** does not act in the same pathway as **SIT4** for this function, because the kinetics of Mpk1 phosphorylation in **bck2Δ** cells are very similar to those of wild type cells.  

The location of **SIT4** upstream of Pck1 in the cell integrity pathway led us to hypothesize that it could contribute to the modulation of other Pck1-dependent biological processes that are not directly regulated by Mpk1. Pck1 plays a role in the organization of the cell cytoskeleton but apparently does not depend on Mpk1 (22). Heat shock stress induces a transient depolarization of actin patch distribution in the cytoplasm. This process, and the subsequent repolarization of actin, both depend on Pck1, although only the latter could also be mediated by Mpk1 (22). The observation that in the absence of functional **SIT4** the actin cytoskeleton remains depolarized for longer periods than in wild type cells is another indication of the functional connection between Pck1 and Sit4 and in turn correlates to higher levels of Pck1 activity in the absence of Sit4.

Transcriptional repression of ribosomal genes upon impairment of the secretory machinery is also dependent on Pck1, but independent of downstream elements of the pathway (58). This process is also affected in **sit4Δ** cells, which again supports the model in which the absence of Sit4 would affect a number of biological processes acting through Pck1 both in a Mpk1-dependent and independent way. All these results are summarized in Fig. 7.

**Pph22**, a type 2A protein phosphatase, has been reported as having a positive role in cell wall integrity and cytoskeleton organization (63). **Glc7**, a catalytic subunit of type 1 protein serine/threonine phosphatases, functions positively to Pck1 in promoting cell integrity and polarization of the actin cytoskeleton (64). However, Sit4 is the only phosphatase, described to date, whose role in PKC1-MAPK modulation would produce a negative modulation upstream of Pck1.

**Sit4** is a phosphatase by sequence, and it is generally accepted that it influences the phosphorylation of a number of substrates. However, there is no evidence of active dephosphorylation of such substrates by Sit4, because no specific biochemical assay for this protein has yet been published.

Cell integrity pathway activity is necessary for survival. However, if this pathway were not shut down when not required, a number of processes such as cell cycle, cytoskeleton organization, and gene transcription, among others, would be deregulated and this would affect cell growth and viability. In this regard, Sit4 could contribute to maintaining correct phys-
iological levels of PKC1-MAPK activity in cells. Further studies are needed to characterize the direct substrate(s) on which Sit4 operates.

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