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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 G_1 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 downstream 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 pH (8). Sit4 also plays an important role in cell cycle regulation, as it is required for the proper G_1 to S phase transition (9, 10). Cells deleted for *SIT4* are either nonviable or display slow growth because of an expanded passage through G_1 (9, 11–13). This delay is partly because of the role of *SIT4* in the normal transcription control of the G_1 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 *sit4*Δ 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/Slg1, 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 Tyr^{192} and Thr^{190} 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 Msg5 (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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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; HA, hemagglutinin; FACS, fluorescence activated cell sorter; RP, ribosomal proteins.

TABLE I
Yeast strains used in this work

Strain	Relevant genotype	Ref.
CML125	*MATα leu2–3,112 ura3–52 trp1 his4 can1^r*	This work
CML128	*MAT***a** as CML125	Gallego *et al.* (39)
JA-110	*MAT***a** *sit4*::*TRP1*	Clotet *et al.* (13)
JA-117	*MAT***a** *sit4*::*kan*MX4	Clotet *et al.* (13)
CML399	*MAT***a** *mpk1*::*URA3*	This work
MML182	*MAT***a**/α *SIT4*/*sit4*::*TRP1 BCK1*/*bck1*::*kan*MX4	This work
MML200	*MATα bck1*::*kan*MX4	This work
MML203	*MAT***a** *sit4*::*TRP1 bck1*::*kan*MX4	This work
MML282	*MAT***a**/α *PKC1*/*pkc1*::*LEU2, SIT4*/*sit4*::*kan*MX4	This work
MML302	*MATα pkc1*::*LEU2 sit4*::*kan*MX4(pBCK1–20)	This work
MML304	*MATα pkc1*::*LEU2*(pBCK1–20)	This work
MML344	*MAT***a** *pkc1*::*LEU2*	This work
MML345	*MATα pkc1*::*LEU2 sit4*::*kan*MX4	This work
MML382	*MAT***a** *wsc1*::*CaURA3*	This work
MML384	*MAT***a** *wsc2*::*nat*MX4	This work
MML387	*MAT***a** *mid2*::*kan*MX4	This work
MML388	*MAT***a** *mid2*::*kan*MX4 *sit4*::*TRP1*	This work
MML392	*MAT***a** *wsc1*::*CaURA3 wsc2*::*nat*MX4	This work
MML393	*MAT***a** *wsc1*::*CaURA3 mid2*::*kan*MX4	This work
MML398	*MAT***a** *wsc2*::*nat*MX4 *sit4*::*TRP1*	This work
MML400	*MAT***a** *wsc1*::*CaURA3 wsc2*::*nat*MX4 *sit4*::*TRP1*	This work
MML400	*MAT***a** *wsc1*::*CaURA3 mid2*::*kan*MX4 *sit4*::*TRP1*	This work
MML402	*MAT***a** *wsc1*::*CaURA3 sit4*::*TRP1*	This work

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, MML216 from MML185, MML231 and MML233 from MML230, and MML344 and MML345 from MML282.

Yeast cells were usually grown in YPD medium (2% yeast extract, 1% peptone, 2% glucose) or in the selective glucose minimal medium, SD (0.67% yeast nitrogen base, 2% glucose, and the required amino acids) (39). Where indicated, D-sorbitol was added to a final concentration of 1 M. To repress expression of the *PKC1* gene under the *tetO*$_7$ 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 *kan*MX4 module and the *WSC2* gene with the *nat*MX4 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* (*TRP2*μ) plasmid harbors a genomic 2.65-kb *Sna*BI-*Nhe*I fragment containing the *SIT4* gene cloned in the *Sma*I-*Xba*I restriction site of YEplac195 (47). Plasmid pMM66 is a YEplac195 derivative (*URA3*/2μ) (48) containing *MSG5* under its own promoter cloned at the *Sma*I vector site. *MSG5* was amplified by PCR from yeast genomic DNA. Plasmid pMM69 is a YEplac195 derivative harboring (PCR-amplified) *PTP2* under its own promoter and cloned into the *Kpn*I and *Hin*dIII vector sites. Plasmid pMM126 is a pCM265 derivative (*URA3*/ CEN) (40) that contains *PCK1* under the *tetO*$_7$ 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 *Pme*I and *Pst*I vector sites (47). The pRS413-*BCK1–20* plasmid is described in Ref. 24. The pMpk1-HA plasmid is a YEP352 derivative, Mpk1 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 G_2 arrests were performed with hydroxyurea and nocodazole, respectively, at the concentrations indicated in the text. G_1 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 *sit4*Δ 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-μ Millipore 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 M KCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamido-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 nmol/μl). 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/p42 antibody (New England Biolabs) was used at a final dilution of 1:2000 in TBST buffer. The anti-Swi6 antibody was 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 1:10000 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. Chemiluminescent detection was performed using the Supersignal substrate (Pierce) in a Lumi-Imager (Roche Molecular Biochemicals).

Kinase Activity—Immunoprecipitation of Pkc1 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 Pkc1 activity, 4×10^{-9} 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 Slt2/Mpk1 MAP Kinase Activity—We had previously tested Mpk1 basal and heat shock-induced levels of activity in a *ppz1*Δ mutant and observed that they were severely impaired (data not shown).[2] To examine whether Mpk1 phosphorylation levels were higher in the absence in Sit4 than in wild type cells (antagonically to that observed in the *ppz1*Δ mutant), we used

[2] M. A. de la Torre-Ruiz, J. Torres, J. Ariño, and E. Herrero, unpublished observations.

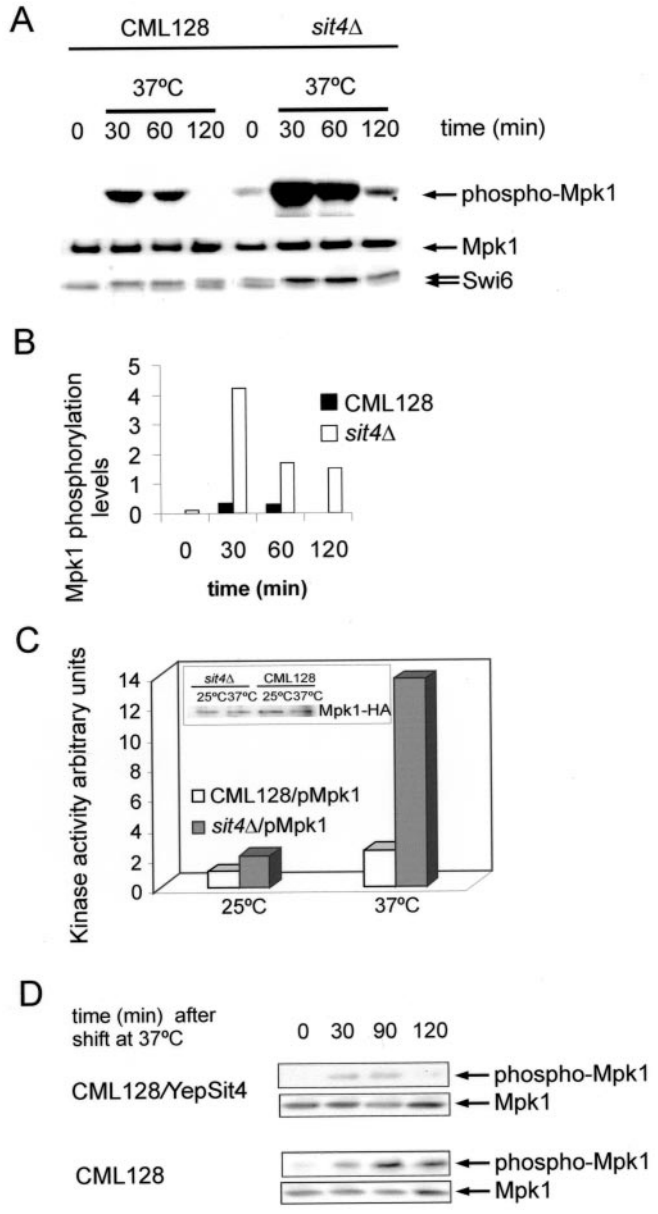

FIG. 1. **Mpk1 activity is up-regulated in the absence of *SIT4*.** *A*, log phase cultures of wild-type (CML128) and *sit4*Δ cells were grown in YPD at 25 °C (time 0) and shifted at 37 °C for the indicated periods. Anti-phospho-p44/p42 was used to detect Mpk1 phosphorylation, anti-Gst-Mpk1 to quantify total amounts of Mpk1 protein, and anti-Swi6 to detect the Swi6 phosphorylation state. *B*, histograms represent Mpk1 phosphorylation levels relative to total Mpk1 protein (arbitrary units) from a representative experiment, quantified using a CCD Lumi-Imager chamber (Roche Molecular Biochemicals). *C*, wild type and *sit4*Δ cells were transformed with a multicopy plasmid carrying Mpk1 HA-tagged in C-terminal. Samples were collected from exponentially growing cultures either at 25 °C or after 30 min of shifting cells at 37 °C. Histograms represent arbitrary units of Mpk1 kinase activity using myelin basic protein as a substrate. Mpk1-HA immunoprecipitates are shown in the *inset*. *D*, cultures from *sit4*Δ and CML128 cells, transformed with the Yep-Sit4 multicopy plasmid, were exponentially grown in SD media plus amino acids at 25 °C. Cultures were washed and transferred to fresh YPD media for 4 h at 25 °C (time 0), then shifted at 37 °C for the times indicated.

the anti-phospho-p44/42 MAPK antibodies raised against dually phosphorylated (Thr^{202}/Tyr^{204})-p44/42 MAPK. These antibodies allow accurate monitoring of Mpk1 activity (23, 37). Wild type and *sit4*Δ cells were grown at 25 °C and then shifted at 37 °C for various time periods (Fig. 1*A*). *sit4*Δ cells showed increased basal phosphorylation levels as compared with wild type cells (Fig. 1*A*). 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. 1*A*). Quantification and normalization of phosphorylation levels revealed that after 30 min of heat shock, Mpk1 phosphorylation levels in the *sit4*Δ mutant were 10 times higher than in wild type cells (Fig. 1*B*). 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 *sit4*Δ exponentially growing cells, both at 25 °C or after 30 min of shifting cells at 37 °C compared with the wild type (Fig. 1*C*). To prove that the increase in Mpk1 activity observed in a *sit4*Δ 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. 1*D*), 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, 53)).

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. 1*A*). In contrast, in *sit4*Δ cell extracts 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. 1*A*). Hyperphosphorylation of Swi6 in *sit4*Δ cells after heat shock was fully mediated by Mpk1, as no changes in mobility were observed in a *sit4*Δ-*mpk1*Δ 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 *sit4*Δ 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. 2*A*). However, the presence of the stabilizer did not prevent Mpk1 basal phosphorylation in a *sit4*Δ 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 *sit4*Δ mutant showed increased tolerance to treatment with zymolyase (a combination of 1,3-β-glucanase and protease en-

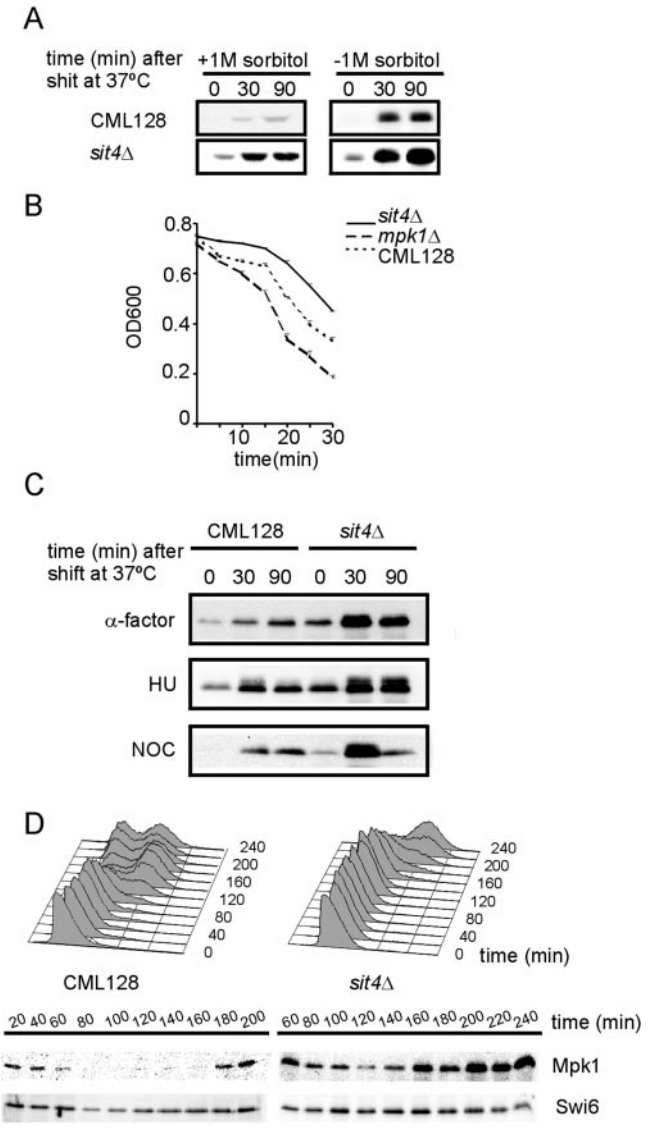

FIG. 2. **The high Mpk1 activity in *sit4*Δ mutants is not relieved by sorbitol and does not depend on the cell cycle stage.** *A*, CML128 and *sit4*Δ cells were exponentially grown at 25 °C (*time 0*) in YPD ± 1 M sorbitol and shifted to 37 °C. *B*, cells from wild type, *sit4*Δ, and *mpk1*Δ were cultured in YPD at 24 °C to mid-log phase and subsequently treated with 1 unit/ml zymolyase 100T for the indicated times, according to de Nobel *et al.* (34). Cell lysis was estimated by measuring A_{600}. Values represent the average of three independent experiments. *C*, cultures from wild type and *sit4*Δ cells were exponentially grown at 25 °C (*time 0*) in YPD medium and synchronized at different stages of the cell cycle: in G_1 with 10 μg/ml α-factor, in S phase with 200 μg/ml HU (hydroxyurea), and in G_2 with 10 μg/ml nocodazole (*NOC*). Following treatments, cultures were shifted from 25 to 37 °C. In all cases, arrest was maintained throughout the experiment. *D*, cells from wild type and *sit4*Δ log-phase cultures were elutriated in G_1 as described under "Materials and Methods." After collection, cells were transferred to fresh YPD medium at 25 °C and allowed to progress up to the second cell cycle. Immunoblot analyses using anti-Swi6, anti-phospho-p44/p42 (bands shown in the figure), and anti-GST-Mpk1 were performed as described in the legend to Fig 1. In all cases, we made a parallel Western blot using the same samples to quantify the total amount of Mpk1 with anti-GST-Mpk1 antibody and confirmed that equal levels of this protein were detected in all lines (data not shown).

zymes that degrade the yeast cell wall) with respect to wild type cells (Fig. 2*B*). In contrast, as previously described (34) the *mpk1*Δ mutant was hypersensitive to enzymatic digestion.

Exponentially growing cultures of *sit4*Δ are enriched in G_1 cells, and it has been reported that Mpk1 becomes phosphorylated at the G_1/S transition in a cell cycle-dependent manner (18). Increased Mpk1 dual-phosphorylation in a *sit4*Δ mutant could therefore result from the presence of a higher proportion of cells in G_1 in asynchronous cultures. To discard this possibility, we performed heat-shock experiments with cells synchronized with α-factor in G_1, in S with hydroxyurea, and in G_2 with nocodazole. In all three cases basal and heat-induced Mpk1 phosphorylation were higher in *sit4*Δ than in wild type cells (Fig. 2*C*). As these treatments are somewhat stressful for cells and might affect Mpk1 phosphorylation, G_1 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 *sit4*Δ cells was more intense than in wild type cells (Fig. 2*D*). 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 *sit4*Δ cells is not merely a circumstantial effect caused by the partial synchronization in G_1 in this mutant.

Sit4 Functions Upstream of Pkc1 and Negatively Regulates Its Activity—We constructed *pkc1*Δ-*sit4*Δ and *bck1*Δ-*sit4*Δ 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 *pkc1*Δ-*sit4*Δ (Fig. 3*A*, and data not shown) or *bck1*Δ,*sit4*Δ (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*/*pkc1*Δ*SIT4*/*sit4*Δ diploid strain using a plasmid bearing the *BCK1–20* allele. After sporulation, the *pkc1*Δ,*sit4*Δ, *pkc1*Δ, and *sit4*Δ strains (bearing the pBCK1–20 plasmid) were isolated and Mpk1 phosphorylation was subsequently analyzed. It is important to stress that after heat shock Mpk1 still became hyperactivated in the presence of a *BCK1–20* allele (not shown). Our reasoning was therefore as follows: if Sit4 acts downstream of Pkc1 as a negative regulator for the pathway, then both *sit4*Δ/pBCK1–20 and *pkc1*Δ-*sit4*Δ/pBCK1-20 cells would exhibit higher levels of Mpk1 activity than wild type and *pkc1*Δ cells transformed with pBCK1–20, respectively. Alternatively, if Sit4 acts upstream of Pkc1 we would first expect the constitutive phosphorylation level of Mpk1 in *sit4*Δ/pBCK1–20 cells to be higher than in any of the other strains tested, and second we would expect *pkc1*Δ-*sit4*Δ/pBCK1-20 and *pkc1*Δ/pBCK1–20 cells to exhibit the same constitutive levels of Mpk1 activity, revealing that in the absence of Pkc1 the lack of Sit4 would have no effect on Mpk1 phosphorylation. In fact, and as predicted by the second hypothesis, in *pkc1*Δ-*sit4*Δ/pBCK1-20 and *pkc1*Δ/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. 3*B*). However, this *sit4*Δ-mediated additive effect was suppressed when *PKC1* was deleted. This suggested that up-regulation of Mpk1 phosphorylation in *sit4*Δ/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 situates 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 *sit4*Δ cells were transformed with a centromeric plasmid bear-

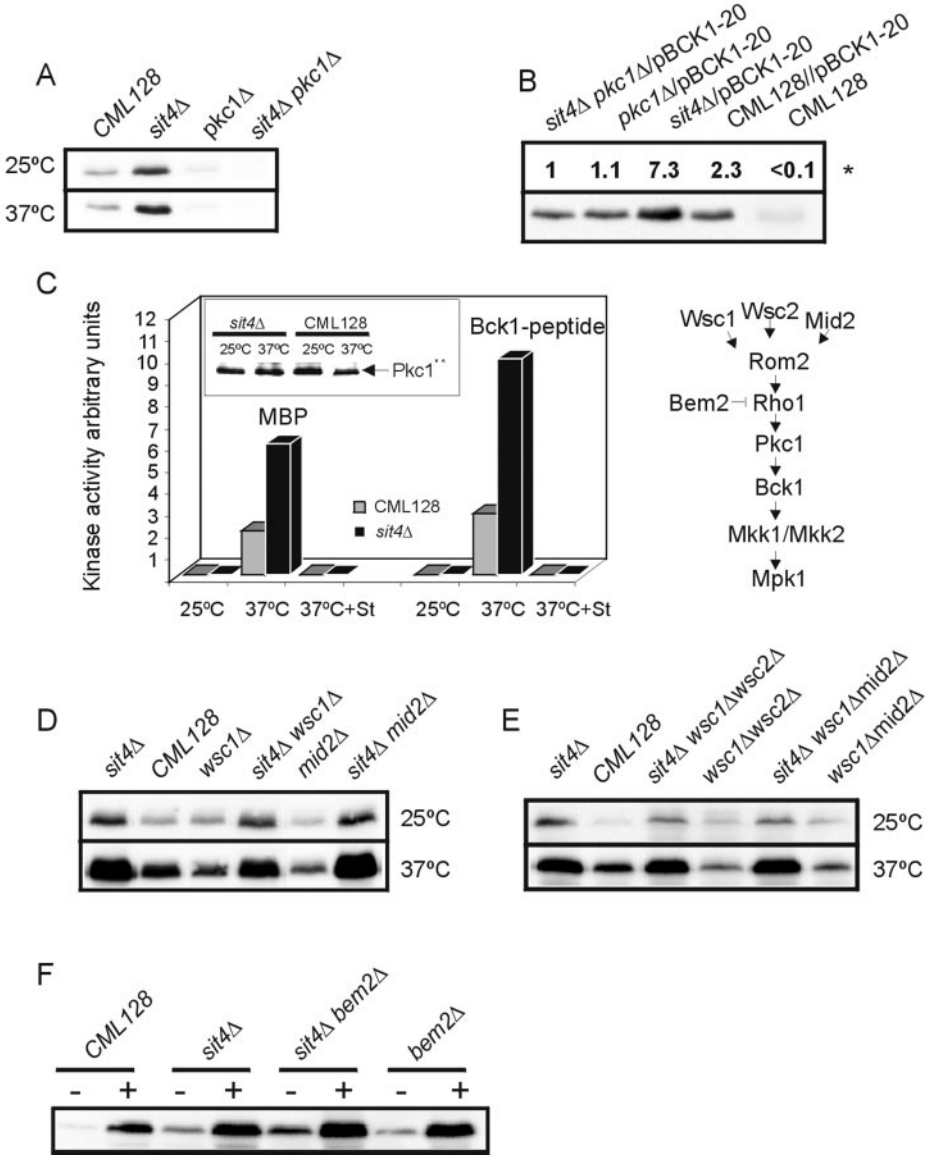

FIG. 3. **Sit4 is not epistatic to the *PKC1*-MAP pathway, and functions upstream of Pkc1 in the modulation of the pathway activity.** *A*, mid-log cultures from wild type, *sit4*Δ-*pkc1*Δ, and *sit4*Δ-*pkc1*Δ strains growing at 25 °C were shifted to 37 °C for 30 min. *B*, exponential cultures of the following strains at 25 °C: *sit4*Δ/pBCK1–20, *sit4*Δ-*pkc1*Δ/pBCK1–20, *pkc1*Δ/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 *sit4*Δ cells were transformed with a plasmid carrying the Pkc1-HA-tagged protein under the control of the $tetO_7$ 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 *sit4*Δ, wild type, *wsc1*Δ, *sit4*Δ-*wsc1*Δ, *mid2*Δ, and *sit4*Δ-*mid2*Δ strains growing at 25 °C were shifted at 37 °C for 30 min. *E*, mid-log cultures from *sit4*Δ, wild type, *sit4*Δ-*wsc1*Δ-*wsc2*Δ, *wsc1*Δ-*wsc2*Δ, *sit4*Δ-*wsc1*Δ-*mid2*Δ, and *wcs1*Δ-*mid2*Δ strains growing at 25 °C were shifted at 37 °C for 30 min. *F*, exponentially growing wild type, *bem2*Δ-*sit4*Δ, and *bem2*Δ 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).

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 *sit4*Δ than in wild type cells, and was suppressed after the addition of the Pkc1 inhibitor staurosporine (Fig. 3*C*). 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 *sit4*Δ-*wsc1*Δ, and the *sit4*Δ-*mid2*Δ double mutants, Mpk1 phosphorylation was markedly more induced than in *wsc1*Δ-*mid2*Δ, and wild type cells at the respective temperatures (Fig. 3*D*). To simplify the figure we did not include the results obtained with the *wsc2*Δ mutant as they were similar to those obtained with *wsc1*Δ. 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 *wsc1*Δ-*wsc2*Δ and *wsc1*Δ-*mid2*Δ double mutants and corresponding triple mutants in combination with *sit4*Δ. Again, in both *wsc1*Δ-*wsc2*Δ-*sit4*Δ and *wsc1*Δ-*mid2*Δ-*sit4*Δ triple mutants, both basal and induced Mpk1 activity was higher in wild type cells and double mutants, and was equivalent to the Mpk1 phosphorylation levels observed in the *sit4*Δ strain (Fig. 3*E*). 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 *bem2*Δ compared with wild type cells (37), we wondered whether Sit4 might be regulating Bem2 activity. We observed that in the *sit4*Δ-*bem2*Δ 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. 3*F*). 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 from Pkc1.

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 Pkc1 hyperactivation also induces depolarization of the actin cytoskeleton in the absence of heat stress (22). We examined actin polarization in both wild type and *sit4*Δ cells to determine whether Sit4 was also involved in the regulation of this process. Both wild type

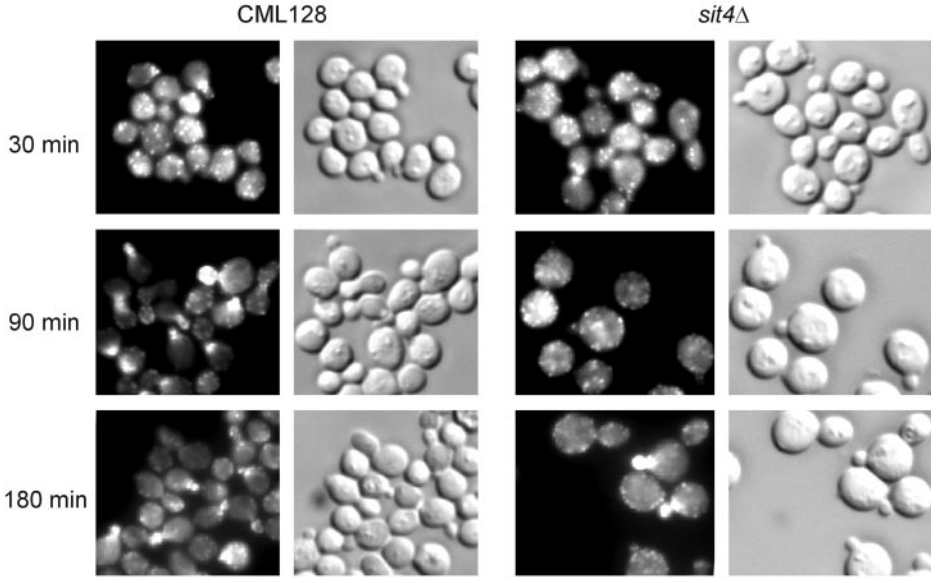

FIG. 4. **Sit4 was required for Pkc1-dependent normal depolarization of the actin cytoskeleton.** Wild type (CML128) and *sit4*Δ cells were grown in rich medium at 25 °C, shifted at 37 °C for the indicated times to be fixed, and then processed for actin cytoskeleton visualization.

and *sit4*Δ cells showed normal actin distribution at 25 °C, whereas random actin disorganization occurred after 30 min at 37 °C. However, whereas normal actin distribution was restored in wild type cells after 90 min, in the case of the *sit4*Δ mutant this was only achieved 3 h after heat shock (Fig. 4). This effect could be explained by the fact that Pkc1 activity after heat shock is greater in a *sit4*Δ mutant and remains greater for longer than in wild type cells (data not shown). The actin defect observed in *sit4*Δ cells was not because of the slow growth rate described for this mutant, because in the double mutant *sit4*Δ-*ppz1*Δ, in which the cell cycle defect caused by the absence of Sit4 was largely relieved (13), the actin cytoskeleton remained depolarized long after heat shock as in *sit4*Δ cells (data not shown). We conclude from these results that Sit4 also affects another known Pkc1 function, namely the transient depolarization of the actin cytoskeleton that occurs after environmental stress.

Sit4 Also Regulates the PCK1 Function Required for Transcriptional 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 *sit4*Δ and wild type cells to test whether this process was also affected by Sit4 function. We shifted *sit4*Δ and wild type cultures from 25 at 37 °C for half an hour to maximize the differences in Pkc1 activity between *sit4*Δ 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 *sit4*Δ and wild type cells after 30 min at 37 °C (Fig. 5), despite the large differences in Pkc1 activity reflected in Mpk1 phosphorylation (Fig. 1*A*). However, upon addition of tunicamycin, RP relative mRNA levels decreased significantly faster in *sit4*Δ 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 *sit4*Δ 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 *sit4*Δ mutant. The lack of RP mRNA repression observed in a *pkc1*Δ mutant, under conditions in which the secretory pathway was altered by tunicamycin, was also observed in the *sit4*Δ *pkc1*Δ 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 *sit4*Δ 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.

Pkc1 Is Involved in the G_1 to S Delay Observed in the Absence of Sit4—As previously reported, cells lacking Sit4 display a marked defect in the G_1 to S progression through the cell cycle (Ref. 9 and Fig. 6*B*), which results in slower growth compared with wild type cells (Fig. 6*C*). Interestingly, when we overexpressed Pkc1 under the *tet* promoter we induced an extended G_1 phase in wild type (Fig. 6*A*) and *sit4*Δ (not shown) cultures that provoked an increase in generation time in both strains. This was reflected in the accumulation of cells at G_1 in exponentially growing cultures (Fig. 6*A*), which resembled *sit4*Δ FACS profiles. This delay in G_1 was also observed when we overexpressed the constitutively active allele *PKC1* (R398A,R405A,K406A) under the Gal promoter (not shown). Pkc1 overexpression also provoked an increase in Mpk1 activity (Fig. 6*A*). Given the correlation observed between Mpk1 basal activity and G_1 delay between the *sit4*Δ mutant and cells overexpressing Pkc1, we speculated that high levels of PKC1-MAPK activity would induce a G_1 delay. This could explain why, in the absence of Sit4, the cell cycle was extended in G_1, in comparison with wild type cells. Following this line of reasoning, we would expect *sit4*Δ cells not to display a G_1 defect in the absence of Pkc1. As shown in Fig. 7*B*, *pkc1*Δ deletion efficiently suppressed the accumulation of cells in *sit4*Δ cultures at G_1. 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 Pkc1. In support to this, *sit4*Δ-*pkc1*Δ and also *pkc1*Δ 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. 6*C*). Even so, the *sit4*Δ cells doubling time was twice as long as that observed in the above mentioned cultures. This phenotype must be specific to

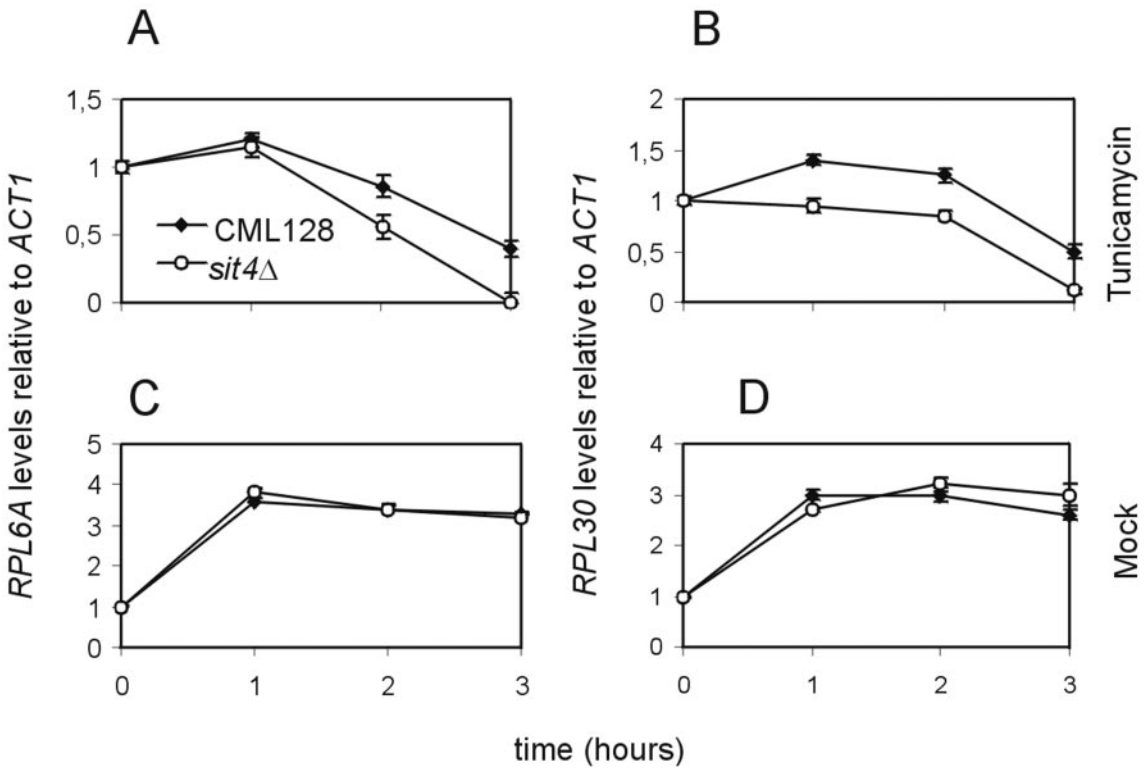

FIG. 5. **Ribosomal gene expression upon alteration of the secretory pathway is also affected by the absence of Sit4.** CML128 and *sit4*Δ cells were grown exponentially in YPD at 25 °C, then shifted to 37 °C for 30 min (time 0). After that, cultures were either treated with 2.5 μg/ml tunicamycin (*A* and *B*) or mock treated (*C* and *D*). Relative mRNA levels obtained at time 0 were assigned the value 1, the rest of the mRNA relative levels obtained in the subsequent 3-h experiment were normalized with respect to this value. *Error bars* represent standard deviations calculated from three independent experiments.

Pkc1 because the absence of Mpk1 did not rescue the G_1 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 G_1 to S phase progression, and that the G_1 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 exercises in the cellular processes after heat shock, although it is known that Swi6 is required for Pkc1-dependent transcriptional induction of some cell wall genes after heat shock (31). In addition, it has been reported that heat shock and osmotic stress cause cells to transitorily accumulate at G_1, which correlates with a descent in the transcription of G_1 cyclin genes *CLN1* and *CLN2* (61, 62). However, no clear relationship was observed between Swi6 phosphorylation after heat shock and cyclin expression, either in *sit4*Δ 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 G_1 phase in exponentially growing cultures. This function might help to explain the G_1 to S defect observed in *sit4*Δ mutants (14, 13), because such a defect is rescued to wild type levels in a *sit4*Δ-*pkc1*Δ double mutant. Furthermore, the fact that the absence of Mpk1 does not compensate for the *sit4*Δ growth defect suggests that the functional interaction between Sit4 and Pkc1 in G_1 cell cycle regulation is specific to Pkc1 activity. Therefore, the G_1 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 G_1 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 *sit4*Δ 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 observations[2] show that Sit4 is functionally independent from the Ptp2 and Msg5 protein phosphatases, and that these directly down-regulate Mpk1 activity (36, 37).

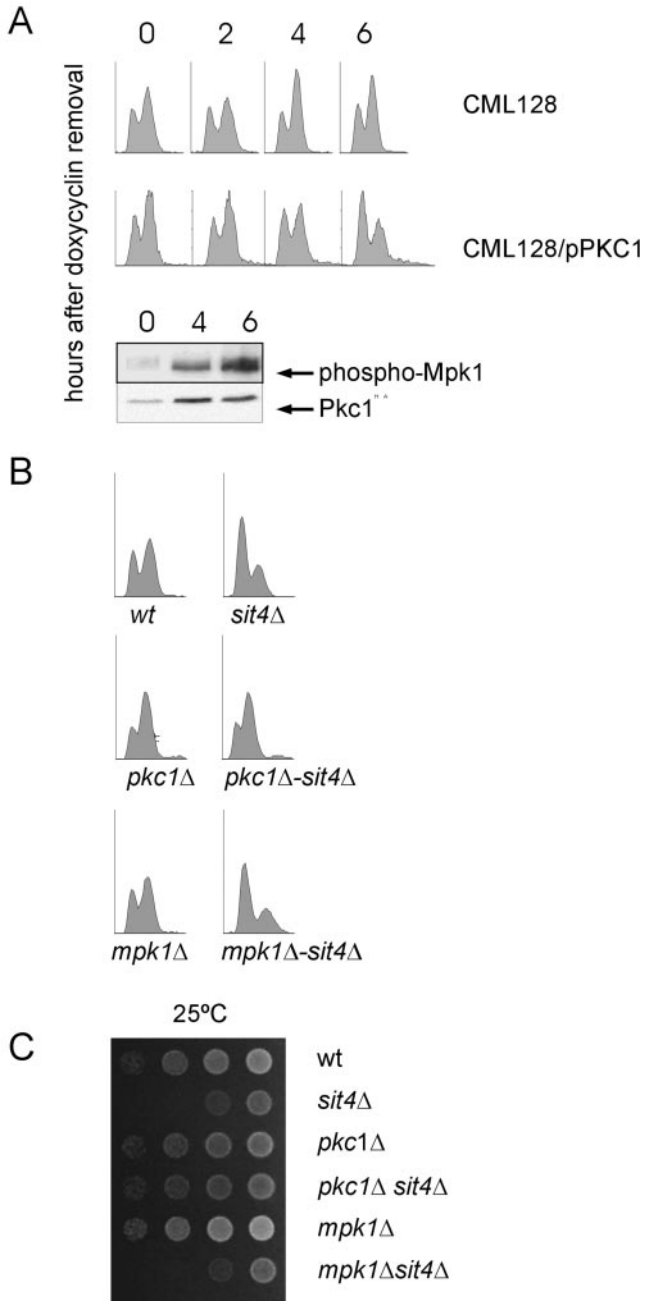

FIG. 6. **Pkc1 overexpression induces a G_1 arrest.** *A*, cultures of CML128 and CML128 harboring a plasmid with Pkc1 under the *tet*O_7 promoter were grown in minimum medium to log phase, then washed several times to eliminate doxycycline to induce Pkc1 expression (see "Materials and Methods"). Samples were taken at the indicated periods for FACS analyses and Western blot. *B*, FACS profiles of the indicated strains grown exponentially at 25 °C in YPD + 1 M sorbitol. *C*, serial dilutions of CML128, *sit4*Δ, *pkc1*Δ, *sit4*Δ-*pkc1*Δ, *mpk1*Δ, and *sit4*Δ-*mpk1*Δ exponentially growing cultures in YPD plus 1 M sorbitol.

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 *bck2*Δ cells are very similar to those of wild type cells.[2]

The location of Sit4 upstream of Pkc1 in the cell integrity pathway led us to hypothesize that it could contribute to the modulation of other Pkc1-dependent biological processes that

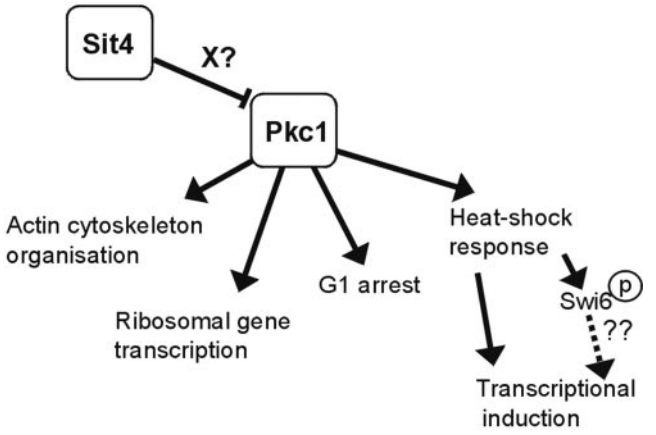

FIG. 7. **Schematic model of the down-regulatory effect that Sit4 exercises on Pkc1 biological functions.** These are: actin cytoskeleton organization, ribosomal gene transcription, cell cycle regulation at G_1, and Mpk1 basal and induced activity that results in the subsequent activation of a number of cellular responses to cell wall stress, including transcriptional induction of genes involved in cell-wall repair. Whether Swi6 phosphorylation signals the latter process remains unclear. The function we attribute to Sit4 can probably be exerted through regulation of various elements termed *X*, which in turn directly interacts with Pkc1. Candidates for this are the known and/or unknown upstream elements of the PKC1-MAPK pathway (cell wall receptors, GTP-exchange factors, GTPase activating proteins, or G-proteins).

are not directly regulated by Mpk1. Pkc1 plays a role in the organization of the actin 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 Pkc1, 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 Pkc1 and Sit4 and in turn correlates to higher levels of Pkc1 activity in the absence of Sit4.

Transcriptional repression of ribosomal genes upon impairment of the secretory machinery is also dependent on Pkc1, but independent of downstream elements of the pathway (58). This process is also affected in *sit4*Δ cells, which again supports the model in which the absence of Sit4 would affect a number of biological processes acting through Pkc1 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 Pkc1 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 Pkc1.

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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REFERENCES

1. Arndt, K. T., Styles, C. A., and Fink, G. R. (1989) *Cell* **56,** 527–5371
2. Doseff, A. I., and Arndt, K. T. (1995) *Genetics* **141,** 857–871
3. Shimanuki, M., Kinoshita, N., Ohkura, H., Yoshida, T., Toda, T., and Yanagida, M. (1993) *Mol. Biol. Cell.* **4,** 303–313
4. Bastians, H., and Ponsting, H. (1996) *J. Cell Sci.* **109,** 2865–2874
5. Di Como, C. J., and Arndt, K. T. (1996) *Genes Dev.* **10,** 1904–1916
6. Beck, T., and Hall, M. N. (1999) *Nature* **402,** 689–692
7. Jiang, Y., and Broach, J. R. (1999) *EMBO J.* **18,** 2782–2792
8. Masuda, C. A., Ramírez, J., Peña, A., and Montero-Lomelí, M. (2000) *J. Biol. Chem.* **40,** 30957–30961
9. Sutton, A., Inmanuel, D., and Arndt, K. T. (1991) *Mol. Cell. Biol.* **11,** 2133–2148
10. Mann, D. J., Dombradi, V., and Cohen, P. T. (1993) *EMBO J.* **12,** 4833–4842
11. Di Como, C. J., Bose, R., and Arndt, K. T. (1995) *Genetics* **139,** 95–107
12. Sutton, A., and Freiman, R. (1997) *Genetics* **147,** 57–71
13. Clotet, J., Garí, E., Aldea, M., and Ariño, J. (1999) *Mol. Cell. Biol.* **19,** 2408–2415
14. Fernandez-Sarabia, M. J., Sutton, A., Zhong, T., and Arndt, K. T. (1992) *Genes Dev.* **6,** 2417–2428
15. Di Como, C. J., Chang, H., and Arndt, K. T. (1995) *Mol. Cell. Biol.* **15,** 1835–1846
16. Posas, F., Casamayor, A., Morral, N., and Ariño, J. (1992) *J. Biol. Chem.* **267,** 11734–11740
17. Lee, K. S., Hines, L. K., and Levin, D. E. (1993) *Mol. Cell. Biol.* **13,** 5843–5853
18. Zarzov, P., Mazzoni, C., and Mann, C. (1996) *EMBO J.* **15,** 83–91
19. Davenport, K. R., Sohaskey, M., Kamada, Y., Levin, D. E., and Gustin, M. C. (1995) *J. Biol. Chem.* **270,** 30157–30161
20. Kamada, Y., Jung, U. S., Piotrowski, J., and Levin, D. E. (1995) *Genes Dev.* **9,** 1559–1571
21. Ketela, T., Green, R., and Bussey, H. (1999) *J. Bacteriol.* **181,** 3330–3340
22. Delley, P. A., and Hall, M. N. (1999) *J. Cell Biol.* **147,** 163–174
23. Verna, J., Lodder, A., Lee, K., Vagts, A., and Ballester, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13804–13809
24. Lee, K. S., and Levin, D. E. (1992) *Mol. Cell. Biol.* **12,** 172–182
25. Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K., and Oshima, Y. (1993) *Mol. Cell. Biol.* **13,** 3076–3083
26. Torres, L., Martin, H., Garcia-Saez, M. I., Arroyo, J., Molina, M., Sánchez, M., and Nombela, C. (1991) *Mol. Microbiol.* **5,** 2845–2854
27. Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin, D. E. (1993) *Mol. Cell. Biol.* **13,** 3067–3075
28. Lim, Y. M., Tsuda, L., Inoue, Y. H., Irie, K., Adachi-Yamada, T., Hata, M., Nishi, Y., Matsumoto, K., and Nishida, Y. (1997) *Genetics* **146,** 263–273
29. Sidorova, J. M., Mikesell, G. E., and Breeden, L. L. (1995) *Mol. Biol. Cell* **6,** 1641–1658
30. Madden, K., Sheu, Y. J., Baetz, K., Andrews, B., and Snyder, M. (1997) *Science* **275,** 1781–1784
31. Igual, J. C., Johnson, A. L., and Johnston, L. H. (1996) *EMBO J.* **15,** 5001–5013
32. Zhao, C., Jung, U. S., Garret-Engele, P., Roe, T., Cyert, M. S., and Levin, D. E. (1998) *Mol. Cell. Biol.* **18,** 1013–1022
33. Jung, U. S., and Levin, D. E. (1999) *Mol. Microbiol.* **34,** 1049–1057
34. de Nobel, H., Ruiz, C., Martin, H., Morris, W., Bru, S., Molina, M., and Klis, F. M. (2000) *Microbiology* **146,** 2121–2132
35. Helliwell, S. B., Schmidt, A., Ohya, Y., and Hall, M. N. (1998) *Curr. Biol.* **8,** 1211–1214
36. Mattison, C. P., Spencer, S. S., Kresge, K. A., Lee, J., and Ota, I. M. (1999) *Mol. Cell. Biol.* **19,** 7651–7660
37. Martín, H., Rodriguez-Pachón, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000) *J. Biol. Chem.* **14,** 1511–1519
38. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
39. Gallego, C., Garí, E., Colomina, N., Herrero, E., and Aldea, M. (1997) *EMBO J.* **16,** 7196–7206
40. Garí, E., Piedrafita, L., Aldea, M., and Herrero, E. (1997) *Yeast* **13,** 837–848
41. Watanabe, M., Chen, C. Y., and Levin, D. E. (1994) *J. Biol. Chem.* **269,** 16829–16836
42. Gietz, R. D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **220,** 1425
43. Goldstein, A. L., Pan, X., and McCusker, J. H. (1999) *Yeast* **15,** 507–511
44. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* **10,** 1793–1808
45. Goldstein, A. L., and McCusker, J. H. (1999) *Yeast* **15,** 1541–1553
46. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) *Current Protocols in Molecular Biology,* Wiley-Interscience, New York
47. Gietz, R. D., and Sugino, A. (1988) *Gene* (*Amst.*) **74,** 527–534
48. Johnston, L. H., and Hohnson, A. L. (1997) *Methods Enzymol.* **283,** 342–350
49. Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, A. B. (1988) *EMBO J.* **7,** 4335–4346
50. Pringle, J. R., Adams, A. E. M., Drubin, D. G., and Haarer, B. K. (1991) *Methods Enzymol.* **194,** 565–602
51. Martín, H., Arroyo, J., Sánchez, M., Molina, M., and Nombela, C. (1993) *Mol. Gen. Genet.* **241,** 177–184
52. Antonsson, B., Montessuit, S., Friedli, L., Payton, M. A., and Paravicini, G. (1994) *J. Cell Biol.* **269,** 16821–16828
53. Sheu, Y. J., Santos, B., Fortin, N., Costigan, C., and Snyder, M. (1998) *Mol. Cell. Biol.* **18,** 4053–4059
54. Wang, T., and Bretscher, A. (2000) *Mol. Biol. Cell* **6,** 1011–1024
55. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) *Nature* **415,** 123–124
56. Chowdhury, S., Smith, K. W., and Gustin, M. C. (1992) *J. Cell Biol.* **118,** 561–571
57. Lillie, S. H., and Brown, S. S. (1994) *J. Cell Biol.* **125,** 825–842
58. Li, Y., Moir, R. D., Sethy-Coraci, I. K., Warner, J. R., and Willis, I. M. (2000) *Mol. Cell. Biol.* **20**, 3843–3851
59. Li, Y., Moir, R. D., Sethy-Coraci, I. K., Warner, J. R., and Willis, I. M. (2000) *Mol. Cell. Biol*. **20,** 3843–3851
60. Philip, B., and Levin, D. E. (2001) *Mol. Cell. Biol.* **21,** 271–280
61. Rowley, A., Johnston, G. C., Butler, B., Werner-Washburne, M., and Singer, R. A. (1993) *Mol. Cell. Biol.* **13,** 1034–1041
62. Bellí, G., Garí, E., Aldea, M., and Herrero, E. (2001) *Mol. Microbiol.* **39,** 1022–1035
63. Evans, D. R. H., and Stark, M. J. R. (1997) *Genetics* **145,** 227–241
64. Andrews, P. D., and Stark, M. J. R. (2000) *J. Cell Sci.* **113,** 507–520

MECHANISMS OF SIGNAL TRANSDUCTION:

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