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Regulation of the Cell Integrity Pathway by Rapamycin-sensitive TOR Function in Budding Yeast*

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The TOR (target of rapamycin) pathway controls cell growth in response to nutrient availability in eukaryotic cells. Inactivation of TOR function by rapamycin or nutrient exhaustion is accompanied by triggering various cellular mechanisms aimed at overcoming the nutrient stress. Here we report that in *Saccharomyces cerevisiae* the protein kinase C (PKC)-mediated mitogen-activated protein kinase pathway is regulated by TOR function because upon specific Tor1 and Tor2 inhibition by rapamycin, Mpk1 is activated rapidly in a process mediated by Sit4 and Tap42. Osmotic stabilization of the plasma membrane prevents both Mpk1 activation by rapamycin and the growth defect that occurs upon the simultaneous absence of Tor1 and Mpk1 function, suggesting that, at least partially, TOR inhibition is sensed by the PKC pathway at the cell envelope. This process involves activation of cell surface sensors, Rom2, and downstream elements of the mitogen-activated protein kinase cascade. Rapamycin also induces depolarization of the actin cytoskeleton through the TOR proteins, Sit4 and Tap42, in an osmotically suppressible manner. Finally, we show that entry into stationary phase, a physiological situation of nutrient depletion, also leads to the activation of the PKC pathway, and we provide further evidence demonstrating that Mpk1 is essential for viability once cells enter G_0.

Rapamycin is an antibiotic macrolide, with a strong antiproliferative action in eukaryotic cells. Its target is FK506-binding protein (FKBP)[1] 12, a small protein belonging to the FKBP family of peptidylprolyl isomerases (1, 2). An FKBP12-rapamycin complex is able to bind the TOR proteins (*t*arget *o*f *r*apamycin, also known as FRAP, RAFT, RAPT, or mTOR (3)) and to block TOR signaling to downstream effectors. The TOR proteins are members of the phosphatidylinositol kinase-related kinase family, and despite displaying significant homology to lipid kinases (4), they have been shown to be Ser/Thr protein kinases (5).

In *Saccharomyces cerevisiae* cells, the TOR proteins promote association between the Sit4 and Tap42 proteins under favorable nutrient conditions (6). Two other 2A protein phosphatases, Pph21p and Pph22p, also associate with Tap42 in a TOR-dependent rapamycin-sensitive manner (6). The *SIT4* gene codes for a Ser/Thr protein phosphatase closely related to the protein phosphatase 2A family (7, 8) and displays a high level of identity to human protein phosphatase 6. Tap42 shows sequence homology to the mammalian α4 protein, which in turn is able to associate with protein phosphatase 6 (9, 10). Tap42 can be phosphorylated directly by TOR, and this phosphorylation increases Tap42 affinity for the phosphatases (11). In yeast cells, inhibition of TOR function by rapamycin results in dissociation of the Sit4-Tap42 complex (6) and in cellular responses similar to those exhibited in nutrient-starved cells. These include down-regulation of translation initiation (6), repression of ribosome biogenesis (12), cell cycle arrest (13), induction of autophagy (14), and acquisition of thermotolerance (6). Both TOR and the Tap42-phosphatase complex are also involved in the repression of the starvation transcriptional program (15, 16), which is achieved by preventing the nuclear translocation of specific transcription factors (17), and in the Tap42-mediated stabilization of amino acid permeases (18). Recently, TOR signaling has been shown to control autophagy via an Apg1 protein kinase complex, although Tap42 has been proposed not to be involved in this specific signaling (19). Thus, most of TOR cellular functions imply the regulation of the Tap42-phosphatase complexes. Based on correlations established between the Sit4-Tap42 association state and the phosphorylation of downstream effectors, it has been proposed that Tap42 may inhibit Sit4 phosphatase activity (17, 18), although no direct target of the phosphatase has been described yet.

The *TOR* genes were originally identified in yeast by the fact that certain mutations in them conferred resistance to growth inhibition by rapamycin (3). The two yeast Tor proteins termed Tor1 and Tor2, can bind to the FKBP12 homolog (Fpr1)-rapamycin complex in budding yeast. *TOR1* and *TOR2* display a high degree of sequence homology. However, although both regulate the Sit4-Tap42 complex in response to nutrients, *TOR2* plays an additional essential function that is not shared by *TOR1* (20). The *TOR2* essential function has been related to the organization of the actin cytoskeleton (21). A temperature-sensitive $tor2^{ts}$ mutant displays lower activity levels of the GTPase exchange factor Rom2 (18), which in turn is needed to activate the essential Rho1 small GTPase (22). Growth and actin polarization defects of $tor2^{ts}$ alleles are rescued by high copy expression of Rho1 or any of the members of the protein kinase C (PKC)/cell integrity pathway (23), as well as by cell wall damage-mediated activation of the pathway (22). The PKC pathway has been proposed to maintain cell integrity by monitoring the cell wall state (for a recent review, see 24). It is accepted that the PKC pathway senses cell wall damage and

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[1] The abbreviations used are: FKBP, FK506-binding protein; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C.

TABLE I
Yeast strains used in this work

Strain	Relevant genotype	Refs.
CML128	MATα *leu2–3,112 ura3–52 trp1 his4 can1^r*	41
CML125	*MATα* As CML128	33
JA-110	CML128 *sit4*::*trp1*	75
MML382	CML128 *wsc1*::*caURA3*	33
MML384	CML128 *wsc2*::*natMX4*	33
MML387	CML128 *mid2*::*kanMX4*	33
MML392	CML128 *wsc1*::*caURA3 wsc2*::*natMX4*	33
MML393	CML128 *wsc1*::*caURA3 mid2*::*kanMX4*	33
MML391	CML125 *rom2*::*kanMX4*	This study
MML200	CML128 *bck1*::*kanMX4*	33
CML399	CML128 *mpk1*::*URA3*	33
HNY21	*MATa ura3 leu2 trp1 his3 ade2 rho1–104*	76
JK9–3da	*MATa leu2–3,112 ura3–52 rme1 trp1 his4 GAL+*	3
JH11–1c	JK9–3da *TOR1–1*	3
JH12–17b	JK9–3da *TOR2–1*	3
MML304	*MATα pkc1*::*LEU2(pBCK1–20)*	33
MML378	JK9–3da *tor1*::*kanMX4*	This study
MML380	JK9–3da *TOR2–1 tor1*::*kanMX4*	This study
MML447	CML128 *tor1*::*kanMX4 mpk1*::*URA*	This study
MML448	CML128 *tor1*::*kanMX4*	This study
CY4907	W303 *MATa ura3–1 leu2–3,112 his3–11,15 trp1–1 ade2–1 can1–100 tap42*::*TRP1 SSD1-v* {*TAP42* on *LEU2*/*CEN*}	6
CY4908	W303 *MATa ura3–1 leu2–3,112 his3–11,15 trp1–1 ade2–1 can1–100 tap42*::*TRP1 SSD1-v* {*tap42–11* on *LEU2*/*CEN*}	6

plasma membrane stress through Mid2 (25) and the Wsc family of cell surface sensors (26), which would directly transmit the signal to Rom2 and Rho1 (27). Among other targets, Rho1 can directly up-regulate the glucan synthase machinery (28) and is also needed to stimulate Pkc1 protein kinase activity allosterically (29). In turn, Pkc1 activates a module of MAPKs, constituted by MAPKKK (MAPK kinase kinase) Bck1, the redundant MAPKKs (MAPK kinases) Mkk1 and Mkk2, and MAPK Mpk1/Slt2. Mpk1 is phosphorylated on both Thr^{190} and Tyr^{192} residues, thus causing a conformational switch that results in its activation (30–32).

We have shown recently that deletion of the *SIT4* protein phosphatase gene leads to an increase in the activity of the PKC/MAPK cell integrity pathway (33). Sit4 is also involved in mediating rapamycin-sensitive TOR signaling by its association with Tap42 in response to nutrients (6). The involvement of *SIT4* in the regulation of both pathways prompted us to investigate the possible link between rapamycin signaling and the cell integrity pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains and Gene Disruptions—Yeast strains used in this study are listed in Table I. Yeast transformations were performed by the lithium acetate procedure (34). The *URA3* marker from *Candida albicans* (35) was used to disrupt the *WSC1* gene by the one-step disruption method (36). This method was also employed to disrupt the *MID2* and *TOR1* genes with the *kan*MX4 module (36), whereas the *WSC2* gene was disrupted with the *nat*MX4 module (37).

Media and Growth Conditions—Yeast strains were grown in YPD medium (2% yeast extract, 1% peptone, 2% glucose). To monitor entry into stationary phase, cells were grown in selective glucose minimal medium, SD (0.67% yeast nitrogen base, 2% glucose, and the required amino acids) (38). Osmotic stabilization was provided where indicated by adding sorbitol or KCl to a final concentration of 0.8 and 0.5 M, respectively. Except where stated, cells were grown at 25 °C. To inactivate the temperature-sensitive *rho1-104* allele, cells were shifted from 25 to 39 °C for 45 min (39). Rapamycin (from Sigma) was stored at −20 °C as a 1 mg/ml stock solution (90% ethanol and 10% Tween) and used at a final concentration of 200 ng/ml. Tunicamycin was stored at −20 °C as a 5 mg/ml stock solution (75% methanol) and used at a final concentration of 2.5 μg/ml.

Analysis of Microarray Data—Raw data (16, 40) corresponding to Mpk1-regulated genes were plotted as a function of time in a Microsoft Excel worksheet. To maximize inductions and to avoid punctual deviations, data were plotted as a stacked area profile.

Yeast Extracts and Immunoblot Analyses—For Western analysis, cultures were grown overnight, and cells were harvested by filtration through 0.22-μm Millipore membranes, washed with ice-cold medium, transferred to Eppendorf tubes, and centrifuged for 15 s at 14,000 rpm. Total yeast protein extracts were prepared as described by Gallego *et al.* (41). The protein concentration in the supernatants was determined by a Micro DC protein assay (Bio-Rad). Equivalent amounts of total protein extracts were run on 10% SDS-polyacrylamide gels. The anti-phospho-p44/p42 antibody (New England Biolabs) was used at a final dilution of 1:5,000 in TBST buffer, and the anti-GST-Mpk1 antibody (39, 42) at a 1:2,000 dilution in the presence of 5% fat milk. Horseradish peroxidase-linked anti-rabbit secondary antibody (NA931, Amersham Biosciences) was used at a 1:10,000 dilution and incubated in TBST buffer containing 1% fat milk for the anti-phospho-Mpk1 and 0.25% fat milk for the anti-GST-Mpk1 primary antibody. Chemoluminescent detection was performed using the Supersignal Substrate (Pierce) in a Lumi-Imager equipment (Roche Molecular Biochemicals).

Actin Staining—Cells were fixed in 4% formaldehyde for 10 min, centrifuged at 3,000 rpm 5 min, and fixed overnight in phosphate-buffered saline plus 4% formaldehyde. Cells were washed once with phosphate-buffered saline containing 10 mM ethanolamine, and once more with phosphate-buffered saline. For F-actin staining, rhodamine-phalloidin (from Sigma; stored as a 6.6 μM solution at −20 °C in methanol) was used at a final concentration of 0.6 μM. Cells were stained for at least 2 h in the dark and washed five times with phosphate-buffered saline before resuspending in mounting solution. All centrifugations were performed at 3,000 rpm.

RESULTS

The PKC Pathway Is Activated in Response to Rapamycin Treatment and upon Entry into Stationary Phase—To test whether rapamycin had any effect on the activity of the PKC pathway, we performed Western blot analysis of total cell extracts using anti-phospho-p44/42 MAPK antibodies. These antibodies specifically recognize the doubly phosphorylated form of Mpk1 and allow accurate monitoring of Mpk1 activity (26, 39). Rapamycin was added to wild type cultures growing exponentially at 25 °C in rich medium (YPD), and samples were taken at the indicated times (Fig. 1*A*). We observed a very rapid increase in the amount of the active Mpk1 form in response to rapamycin, already detectable after 15 min of treatment. Maximum levels of Mpk1 activity were reached after 45 min and remained high for the duration of the experiment (60 min). The induction of Mpk1 activity was not the result of an increase in the Mpk1 protein levels, which remained constant throughout the experiment (Fig. 1*A*), as observed after probing the same extracts with anti-Mpk1 polyclonal antibodies.

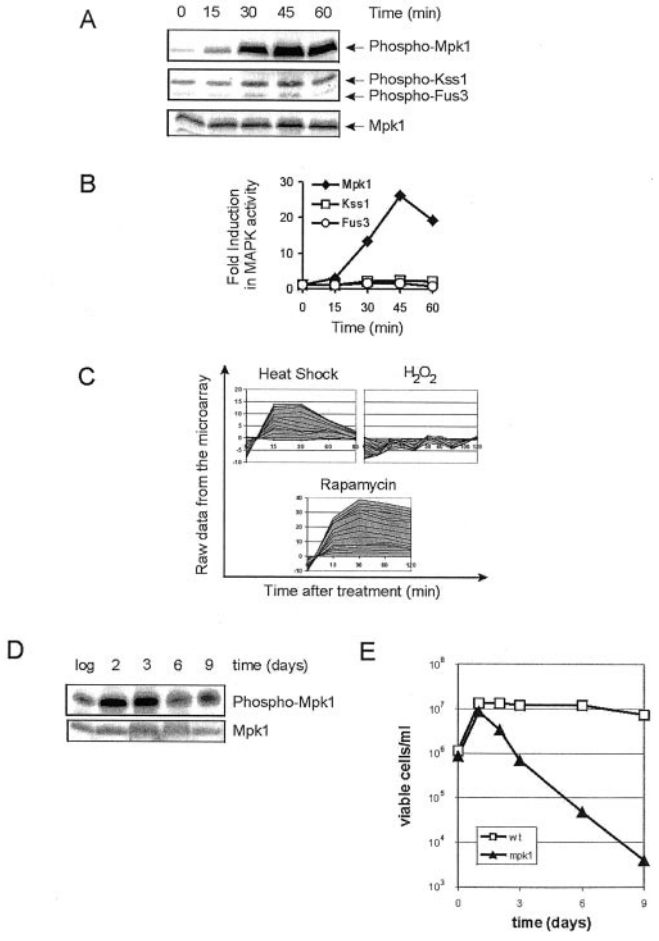

FIG. 1. **Mpk1 activity is induced by rapamycin treatment and upon entry into stationary phase.** *A*, time course of Mpk1 activation in response to rapamycin. Mid-log cultures of wild type (CML128) cells, growing exponentially in YPD medium at 25 °C, were treated with rapamycin to a final concentration of 200 ng/ml. Samples were taken at the indicated times. Western blots were done using anti-phospho-p44/p42 to detect Mpk1, Kss1, and Fus3 double phosphorylation, and anti-Mpk1 to quantify total amounts of Mpk1 protein. *B*, rapamycin-mediated MAPK activation is specific for Mpk1. MAPK phosphorylation levels from *A* were quantified, relativized to 1 at time 0 for each MAPK, and plotted as a function of time. *C*, induction of Mpk1-regulated genes by rapamycin treatment. Raw data corresponding to the expression of Mpk1-regulated genes upon heat shock from 25 to 37 °C (40), 0.3 mM hydrogen peroxide treatment (40), and 100 nM rapamycin treatment (16) were plotted as a function of time. Mpk1-regulated genes comprise *BGL2*, *CHS3*, *CIS3*, *CRH1*, *CWP1*, *DFG5*, *FKS1*, *FKS2*, *HSP150*, *MLP1*, *PIR1*, *PIR3*, *PST1*, *SEC28*, *SED1*, *SLT2* (*MPK1*), *SSR1*, *YIL117C*, *YLR194C*, *YMR295C*, and *YNL085C*. *D*, induction of Mpk1 activity upon entry into stationary phase. Wild type cells were exponentially grown in minimum medium at 25 °C. At an A_{600} of 0.3, a sample was collected to serve as the time 0 reference, and further samples were recovered at the indicated days. Immunoblots using anti-phospho-p44/p42 and anti-Mpk1 antibodies in the same protein extracts were performed as in *A*. *E*, loss of viability of *mpk1*Δ cells upon entry into stationary phase. Cell viability was determined for wild type and *mpk1*Δ cells in the same cultures as in *D*. The data shown are representative of three independent experiments.

Two related MAPKs, Fus3 and Kss1, are also recognized in their active, doubly phosphorylated state by the same antibody (43, 44). However, no significant change in their activity was detected after the addition of rapamycin (Fig. 1, *A* and *B*). The latter suggests that rapamycin-mediated MAPK activation is specific for Mpk1.

The transcripts up-regulated by Mpk1 activation have been described recently using whole-genome approaches and comprise mostly cell wall and stress-responsive genes (45). The induction of all of them is expected to occur under any stress that activates Mpk1, including rapamycin treatment. To check the validity of this hypothesis, we carried out an *in silico* study of the behavior of Mpk1-regulated genes under different environmental perturbations that either induce or do not affect Mpk1 activity. Data on Mpk1-regulated genes was extracted from previously published works (40) and plotted as a function of time (Fig. 1*C*). As expected, Mpk1-regulated genes are induced by heat shock and hypotonic shock, situations that give rise to Mpk1 activation, but not by hydrogen peroxide or menadione treatment (Fig. 1*C* and data not shown). In a further step, we carried out an analogous study on other published data (16) to check whether the same group of genes was also induced upon rapamycin treatment. As shown in Fig. 1*C*, expression of Mpk1-regulated genes is induced by rapamycin treatment in a fashion that correlates with the increase in Mpk1 activity (Fig. 1, *A* and *C*), although different concentrations of the inhibitor were used in each experiment. Overall, these observations evidence that the pattern of Mpk1-regulated gene expression serves as a good marker of Mpk1 activity. Moreover, because all of those genes are up-regulated by rapamycin, it shows that the signal from rapamycin to Mpk1 is transmitted to substrates downstream from this kinase, leading to the transcriptional induction of Mpk1-regulated genes.

The TOR proteins have been proposed to be central sensors of the quality of carbon and nitrogen sources (46), and rapamycin has been reported to cause effects similar to those exhibited by nutrient-starved cells. Besides, cells in stationary phase display phenotypes similar to those caused by rapamycin (6). Thus, we reasoned that cells entering stationary phase could also induce Mpk1 activity, as observed when TOR proteins are blocked by rapamycin (Fig. 1*A*). Wild type cells were inoculated in fresh minimal medium, and samples were collected at the times indicated. As shown in Fig. 1*D*, Mpk1 became strongly activated as cells progressively entered into stationary phase (2–4 days) and became less active at later time points. The increase in Mpk1 activity was not caused by changes in the levels of the Mpk1 protein, as checked with anti-Mpk1 polyclonal antibodies (Fig. 1*D*). Moreover, although entry into stationary phase does not affect viability in wild type cultures, *mpk1*Δ mutant cells rapidly lost viability upon exit from exponential growth (Fig. 1*E*). In a genomewide screen for genes whose deletion results in alterations on the growth response to rapamycin, it has been reported that both *MPK1* and *SWI6* (whose product is a Mpk1 target) confer rapamycin hypersensitivity when deleted (47). Other authors have shown recently that an intact PKC pathway is needed to maintain viability upon nutritional deprivation (48). These results, together with ours, suggest that Mpk1 activation, and the subsequent induction of its target genes, is essential for cells to remain viable once they enter stationary phase or are treated with rapamycin.

Both TOR1 and TOR2 Signaling Blockages Mediate Mpk1 Activation in Response to Rapamycin—To prove the direct involvement of members of the TOR pathway in mediating rapamycin signaling to Mpk1, we checked the behavior of rapamycin-resistant *TOR* mutants. *TOR1-1* and *TOR2-1* bear, respectively, chromosomal alleles of *TOR1* and *TOR2* which are insensitive to the immunosuppressant drug because their products cannot interact with the Fpr1-rapamycin complex. The JK9-3da wild type strain induced Mpk1 phosphorylation with kinetics identical to those of the CML128 wild type strain used above (Figs. 1*A* and 2*A*). However *TOR1-1* cells displayed no change in Mpk1 activity in response to rapamycin treatment, whereas in *TOR2-1* cells a mild activation was still detected (Fig. 2*A*). The results clearly indicate that Tor1 inhibition mediated by rapamycin signals to Mpk1 inducing its

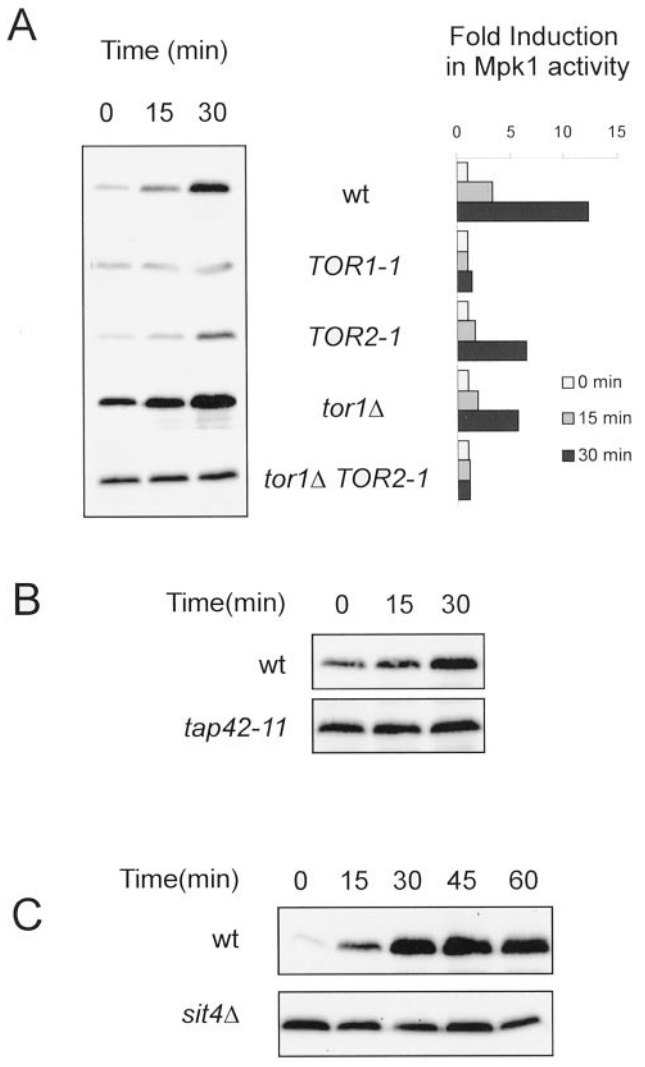

FIG. 2. **Tor1, Tor2, Sit4, and Tap42 mediate Mpk1 activation in response to rapamycin.** *A*, Mpk1 activation upon rapamycin treatment depends on both *TOR1* and *TOR2* genes. Exponential cultures of wild type (*wt*) (JK9-3da), *TOR1-1* (JH11-1c), *TOR2-1* (JH12-17b), *tor1*Δ (MML378), and *tor1*Δ*TOR2-1* (MML380) cells growing in YPD at 25 °C were treated with rapamycin to a final concentration of 200 ng/ml. Samples were taken at the indicated times and processed for analysis by Western blotting, using anti-phospho-p44/p42 to detect Mpk1 phosphorylation, as in Fig. 1*A*. Phosphorylation levels were quantified as in Fig. 1*B* and relativized to 1 at time 0 for each strain. *B*, *TAP42* mediates Mpk1 activation in response to rapamycin. Wild type (CY4907) and *tap42-11* (CY4908) cells were grown exponentially and treated with rapamycin as in *A*. *C*, Sit4 is needed for rapamycin-induced Mpk1 activation. Wild type (CML128) and *sit4*Δ (JA-110) mutant cells were grown exponentially and treated with rapamycin. Samples were taken at the indicated times and processed for immunoblotting as in *A*. In all cases in *A–C*, anti-Mpk1 immunoblot analysis of the same extracts was performed to verify that an equal amount of Mpk1 was present in each *lane* (not shown).

activity. Therefore, activation of the MAPK is not the result of a direct cell wall damage effect caused by rapamycin. We hypothesized that the slight Mpk1 activation still observed in *TOR2-1* cells could be the result of the rapamycin-mediated blockage of a fully inhibitable Tor1 protein. Thus, inhibition of Tor2 in wild type cells could still contribute to rapamycin signaling to Mpk1. Support for this hypothesis is the observation that Fpr1-rapamycin binding to Tor1 occurs at 10-fold lower rapamycin concentrations compared with those necessary to bind to Tor2 (49); hence, the inhibitory complex has more affinity for Tor1 than for Tor2.

To test whether Tor2 inhibition by rapamycin is also involved in the induction of the PKC pathway, we deleted the *TOR1* gene in both the wild type and the *TOR2-1* strains, and the levels of Mpk1 activation were checked in the single and double mutants. The *tor1*Δ mutant displayed activation of Mpk1 in response to rapamycin treatment (Fig. 2*A*), although the induction was less pronounced than in wild type cells. This result clearly indicates that in these conditions Tor2 inhibition by rapamycin mediates induction of Mpk1 activity. Furthermore, no increase in the activation of the MAPK was detectable in *tor1*Δ *TOR2-1* double mutant cells after addition of the drug (Fig. 2*A*). In light of these results, we propose that either Tor1 or Tor2 inhibition by rapamycin can mediate Mpk1 activation. Interestingly, *tor1*Δ mutant cells, which may be compromised for TOR function (because they rely on the single *TOR2* gene), displayed higher levels of both basal and induced Mpk1 activity than wild type cells, which suggests that partial elimination of TOR function leads to a constitutive increased activation of Mpk1 (as rapamycin does). Taken together, these data show that a block in TOR function may act by up-regulating the PKC pathway.

TAP42 and SIT4 Mediate Rapamycin Signaling to Mpk1—An active TOR pathway promotes the association of the Tap42 subunit with the protein phosphatase 2A and Sit4 (6). Therefore, we tested whether *TAP42* is also involved in mediating rapamycin signaling to Mpk1. The *tap42-11* allele is known to be both temperature-sensitive and rapamycin-resistant at the permissive temperature (6). In contrast to its isogenic wild type strain, cells carrying the *tap42-11* allele displayed no induction of Mpk1 activity after the addition of rapamycin at the permissive temperature, although the exponential levels of the MAPK activity were remarkably higher than those determined in wild type cells (Fig. 2*B*). Surprisingly, the isogenic wild type strain (W303 background) displayed higher levels of basal Mpk1 activity than the CML128 or JK9-3da strains, also used in this study, and although Mpk1 activity was induced by rapamycin, the up-regulation was not as pronounced as in the other two backgrounds. These results support the fact that Tap42 is also involved in the signaling to the PKC pathway which occurs as a consequence of TOR inhibition.

SIT4, one of the closest TOR downstream effectors, negatively modulates the activity of the Pkc1/MAPK pathway (33). We therefore checked the possible implication of *SIT4* in mediating rapamycin signaling to Mpk1. As described previously, *sit4*Δ mutant cells exhibited higher basal levels of Mpk1 activation than its isogenic wild type strain, but no change in Mpk1 activity levels was detected in the mutant strain after the addition of rapamycin (Fig. 2*C*). Mpk1 activity was monitored in a longer time course to check that its induction did not occur at later time points. This result suggests that *SIT4*, as shown above for *TAP42*, is involved in rapamycin-mediated induction of Mpk1. It is worth noting that mutation in either *TAP42* or *SIT4* (as well as in *tor1*Δ cells) resulted in a constitutive increase in the basal levels of Mpk1 activity relative to their isogenic wild type strains, which suggests that TOR and *TAP42*, as we have shown recently for *SIT4* (33), act negatively on Mpk1 activity under normal growth conditions.

The Signaling between the TOR and the PKC Pathways Takes Place at a Level Upstream from Rho1—In a further step, we attempted to elucidate the level at which the cross-talk between the TOR and PKC pathways occurs. Thus, we analyzed the response to rapamycin treatment in mutants of the cell integrity pathway. As expected, neither *bck1*Δ nor *pkc1*Δ mutant cells showed detectable basal levels of Mpk1 phosphorylation, and they were completely insensitive in their response to rapamycin treatment with respect to Mpk1 activation (Fig.

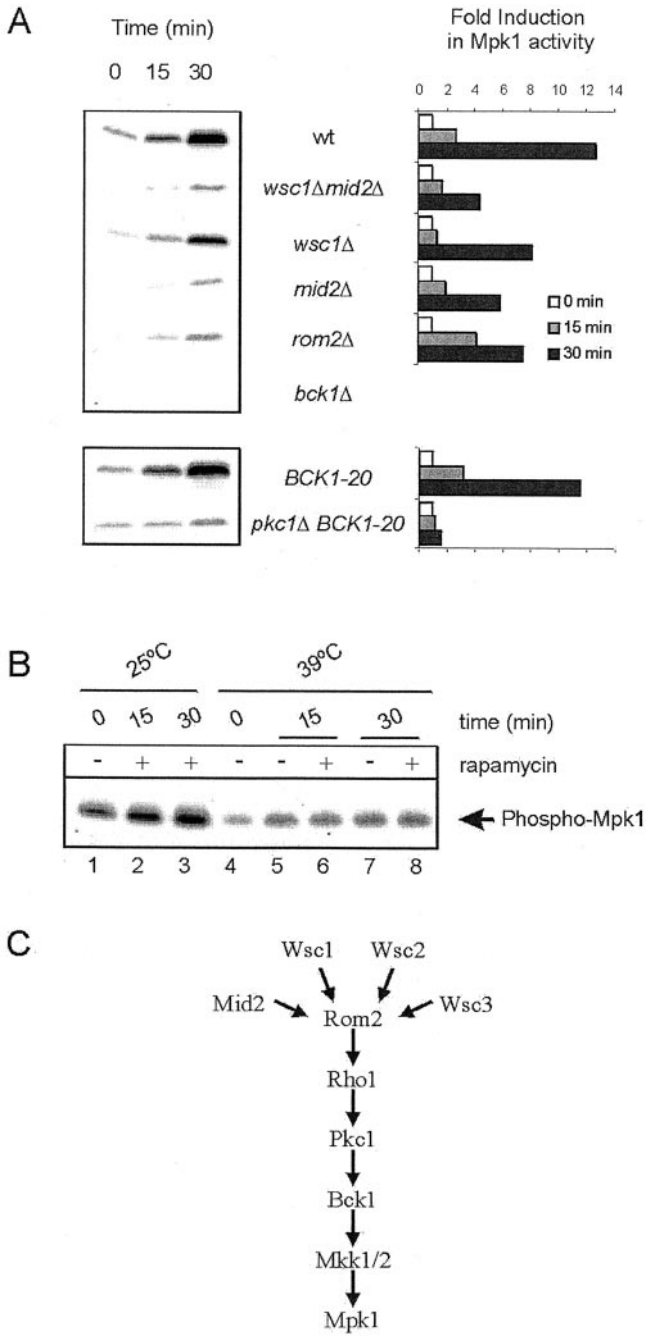

FIG. 3. **The PKC/MAPK module, Pkc1, and Rho1 mediate Mpk1 activation in response to rapamycin.** *A*, upstream components in the PKC pathway transmit the signal from rapamycin. Exponential cultures of wild type (*wt*) (CML128), *wsc1Δmid2Δ* (MML393), *wsc1Δ* (MML382), *mid2Δ* (MML387), *bck1Δ* (MML200), *pkc1Δ*/pBCK1-20 (MML304), and CML128/pBCK1-20 cells growing in YPD at 25 °C were treated with rapamycin to a final concentration of 200 ng/ml. Samples were taken at the indicated times and processed for analysis by Western blotting, using anti-phospho-p44/p42 to detect Mpk1 phosphorylation, as in Fig. 1*A*. Phosphorylation levels were quantified as in Fig. 1*B* and relativized to 1 at time 0 for each strain. *B*, a functional Rho1 is needed to transmit the signal from rapamycin to Mpk1. Exponential cultures of *rho1-104* cells (HNY21) growing in YPD at 25 °C (*lane 1*) were either treated with rapamycin to a final concentration of 200 ng/ml (*lanes 2* and *3*) or shifted to 39 °C to inactivate Rho1. After 45 min at the restrictive temperature (*lane 4*), the culture was spliced in two and kept at 39 °C. Half of the culture served as a control (*lanes 5* and *7*), and the rest was treated with rapamycin to a final concentration of 200 ng/ml (*lanes 6* and *8*). Samples were taken at the indicated times for Western blot analysis. *C*, scheme of the components of the PKC/cell integrity pathway. In *A* and *B*, anti-Mpk1 immunoblot analysis of the same extracts was performed to verify that an equal amount of Mpk1 was present in each *lane* (not shown).

3*A* and data not shown). These results suggest that the signaling from TOR proteins goes through the various elements of the PKC pathway to activate Mpk1, the last kinase of the cascade. To test this, we used a strain deleted for *PKC1* bearing the *BCK1-20* allele, which constitutively activates Mpk1 (33). In this strain, all of the activating signal coming from the upstream elements of the pathway become blocked by the absence of Pkc1. According to our hypothesis, rapamycin treatment would not provoke additional Mpk1 activation above the basal levels detected at 25 °C. Alternatively, if there existed a parallel activating pathway from the TOR proteins to Mpk1, independently on the cell integrity cascade, we would expect rapamycin to induce Mpk1 activity. As shown in Fig. 3*A*, inactivation of TOR function by rapamycin treatment induced Mpk1 activation in a wild type strain carrying the *BCK1-20* allele, whereas in the *pkc1Δ* background harboring the *BCK1-20* allele no induction of Mpk1 double phosphorylation was observed in such conditions. Taken together, from the above results we propose a model in which rapamycin-induced signaling to Mpk1 is conveyed by the Pkc1/MAPK module.

Rho1 is a small GTPase that acts as an essential element in the signaling cascade from the cell surface to Mpk1 (39). Because its deletion results in lethality, we made use of the temperature-sensitive *rho1-104* allele to analyze its implication in transducing the signal from rapamycin to Mpk1. Rho1-104 was inactivated by a temperature shift to 39 °C, which led to a decrease in the levels of Mpk1 activation (*lane 4* in Fig. 3*B*). After 45 min of inactivation, rapamycin was added to half of the culture, and the other half was used as an untreated control. Samples from both cultures were taken for Western analysis. Although rapamycin was able to activate Mpk1 at 25 °C, shifting the culture to 39 °C completely abolished its ability to activate the pathway (Fig. 3*B*). By contrast, wild type cells were able to activate Mpk1 by rapamycin treatment further after being shifted at 39 °C (data not shown). Therefore, Rho1 is also involved in rapamycin signaling from the TOR proteins to Mpk1.

The activity of small G proteins is tightly regulated, and several elements have been shown to modulate them (50). Among all of the regulatory elements controlling Rho1, the best characterized is Rom2 (51), which may transduce the signal from the cell surface sensors to Pkc1 via Rho1 (Fig. 3, *A* and *C*). In contrast to the role played by downstream elements in the PKC pathway, *ROM2* seemed to be partially involved in rapamycin signaling to Mpk1; a *rom2Δ* mutant strain displayed reduced basal levels of Mpk1 activity, but the MAPK was only partially induced in response to rapamycin (8-fold induction during the first 30 min of incubation in rapamycin in the mutant strain, in contrast to 13-fold induction in the wild type strain; Fig. 3*A*). However, a *rom2Δ* mutant strain has been shown to retain 60% of GTP binding to Rho1 (27), which suggests that the coordinated action of other GTPase exchange factors for Rho1 (such as Rom1, Bem4, or Tus1) may be responsible for Mpk1 activation after the addition of rapamycin in the *rom2Δ* mutant strain. Moreover, the inactivation of GTPase-activating proteins for Rho1 may also play a role in rapamycin signaling to Mpk1.

A Blockage of TOR Function Activates Cell Surface Sensors in an Osmotic Suppressible Manner—The *MID2* gene, coding for a transmembrane protein proposed to act as a cell surface sensor, has been described to block activation of the PKC pathway when deleted (25, 39, 52, 53). *WSC1* codes for another class of cell surface sensor and is thus another possible mediator of rapamycin signaling to Mpk1 (26). Deletion of either *WSC1* or *MID2* provoked a notable reduction in the basal signal of Mpk1 activity compared with wild type cells (Fig. 3*A*). In addition, the

induction of MAPK phosphorylation after rapamycin treatment was partially impaired in both individual mutants (induction reduced to about half of that in wild type cells after 30 min of rapamycin treatment in *wsc1*Δ cells and even lower in the *mid2*Δ strain). Moreover, the double *wsc1*Δ*mid2*Δ mutant strain led to a more marked effect on the basal and induced Mpk1 activity in the presence of the drug, and the induction at 30 min was reduced to one-third relative to wild type cells (Fig. 3*A*). These results indicate that there is a partial dependence on the Wsc1 and Mid2 sensors for rapamycin signaling to the PKC pathway. However, because Wsc proteins have been shown to act cooperatively and to overlap in function (26), we cannot discard the possibility that the whole family of Wsc sensors contributes to rapamycin-mediated activation of Mpk1.

Activation of Wsc sensors can occur in response to a block in the secretory pathway caused by tunicamycin or thermosensitive mutations in the *SEC* genes (54). However, the effects that problems in secretion have on Mpk1 activity are still contradictory (54–56). We checked whether a block in secretion caused by tunicamycin treatment paralleled Mpk1 activation in response to rapamycin. The kinetics of Mpk1 activation differed totally between both treatments. Whereas tunicamycin caused only a slight increase in Mpk1 activity at the time points tested, rapamycin provoked a much more intense and rapid activation of the MAPK (Fig. 4*A*). In view of these results, we suggest that rapamycin may not be acting on cell surface sensors by halting secretion, at least not at the same level as tunicamycin does.

Because Wsc and Mid2 sensors have also been proposed to sense changes in the yeast cell wall and plasma membrane, we wondered whether inhibition of TOR function by rapamycin induced some kind of cell envelope stress. Addition of 0.8 M sorbitol to the growing medium has been shown to prevent the activation of the PKC pathway by insults infringed on the cell envelope (53, 57). When rapamycin treatment was performed in cultures osmotically stabilized with sorbitol, the induction of the pathway was severely impaired (Fig. 4*B*). Similar results were obtained using 0.5 M KCl as an osmotic stabilizer (Fig. 4*B*). Thus, rapamycin-mediated inhibition of TOR function triggers activation of the PKC pathway by means that are suppressible by addition of an osmotic stabilizer to the growing culture. Because increasing the external osmolarity is predicted to relieve the osmotic gradient across the cell membrane, these results suggest that changes induced by rapamycin may be taking place on the yeast envelope, either on the cell wall or on the plasma membrane. However, we have detected no increase in zymoliase sensitivity in rapamycin-treated cells, but rather a slight increase in resistance to cell wall digestion (data not shown). Thus, rapamycin may not be inducing Mpk1 activity by weakening the cell wall.

To understand the above results better, we constructed a *tor1*Δ*mpk1*Δ double mutant. We reasoned that a lack of TOR function may either trigger damage in the cell envelope or alternatively affect its structure in such a way that the cell requires a functional PKC pathway to compensate for those changes. In Fig. 4*C* we can observe that the *tor1*Δ*mpk1*Δ double mutant, compared with both single ones, displayed a growth defect already at the permissive temperature 25 °C, which was more severe at 37 °C. Interestingly, the problems in *tor1*Δ*mpk1*Δ cell growth were totally rescued upon the addition of an osmotic stabilizer (Fig. 4*C*). These results support our hypothesis that TOR inhibition by rapamycin triggers a signal that, at least in part, is sensed at the cell envelope and then activates the cell integrity pathway.

The Actin Cytoskeleton Becomes Disorganized in Response to Rapamycin Treatment—As shown above, rapamycin inhibition

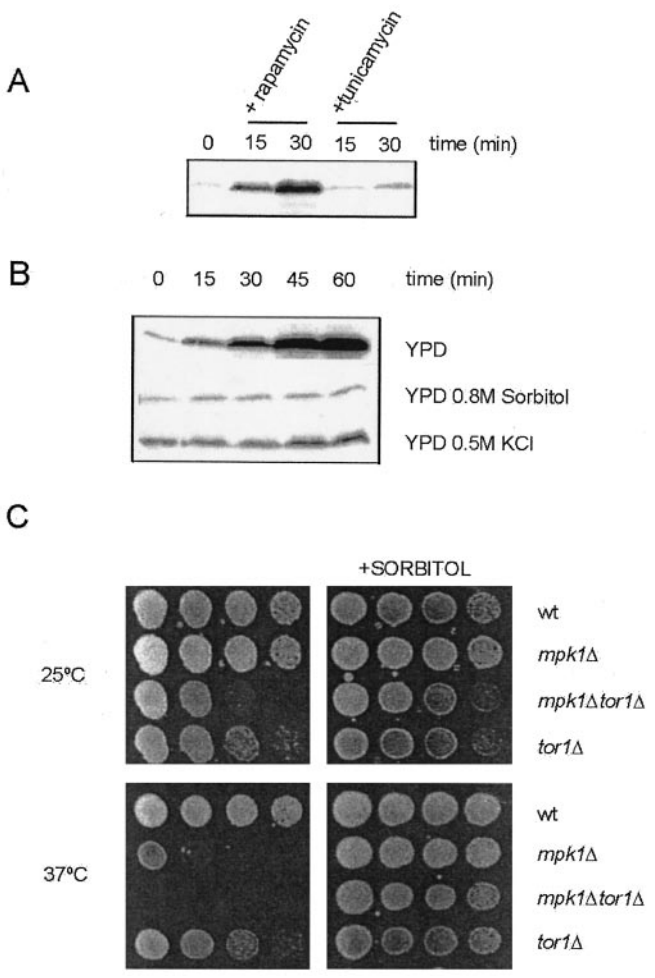

FIG. 4. **Cell signaling from TOR to Mpk1 is sensed at the cell envelope and suppressed upon osmotic stabilization.** *A*, a secretion block caused by tunicamycin treatment does not parallel rapamycin-mediated activation of Mpk1. Exponentially growing wild type cells (CML128) in YPD at 25 °C were treated with rapamycin or tunicamycin to a final concentration of 200 ng/ml or 2.5 μg/ml, respectively. Samples were taken at the indicated times. *B*, osmotic stabilization prevents activation of Mpk1 by rapamycin. Wild type cultures (CML128) were grown overnight at 25 °C in YPD alone or in YPD supplemented with either 0.8 M sorbitol or 0.5 M KCl to exponential phase. Rapamycin was added at time 0, and samples from each of the three cultures were taken at the indicated times for Western blot analysis. *C*, the cell growth defect provoked by the simultaneous absence of Mpk1 and Tor1 is rescued upon osmotic stabilization. Serial dilutions of CML128, *mpk1*Δ, *mpk1*Δ *tor1*Δ, and *tor1*Δ exponentially growing cultures were plated into YPD and YPD plus 1 M sorbitol and grown at 25 and 37 °C; *wt*, wild type. In *A* and *B*, anti-Mpk1 immunoblot analysis of the same extracts was performed to verify that an equal amount of Mpk1 was present in each lane (not shown).

of both Tor1 and Tor2 function leads to the activation of the PKC pathway probably by inducing cell wall or plasma membrane stress. Several stresses have been described to induce the depolarization of the actin cytoskeleton (58, 59), and it has been shown that this phenotype is specific and not caused by a halt in cell growth (60). Therefore, we sought to determine whether rapamycin inhibition of the TOR shared function also affected the organization of the actin cytoskeleton. The actin cytoskeleton became disorganized after 45 min of rapamycin treatment (data not shown), and complete depolarization was achieved within the 1st h of incubation in rapamycin in all of the strain backgrounds used in this study (Fig. 5 and data not shown). Both *TOR1-1* and *TOR2-1* efficiently suppressed the actin cytoskeleton depolarization phenotype (Fig. 5 and data not shown). The *SIT4* deletion, and to a lesser extent the

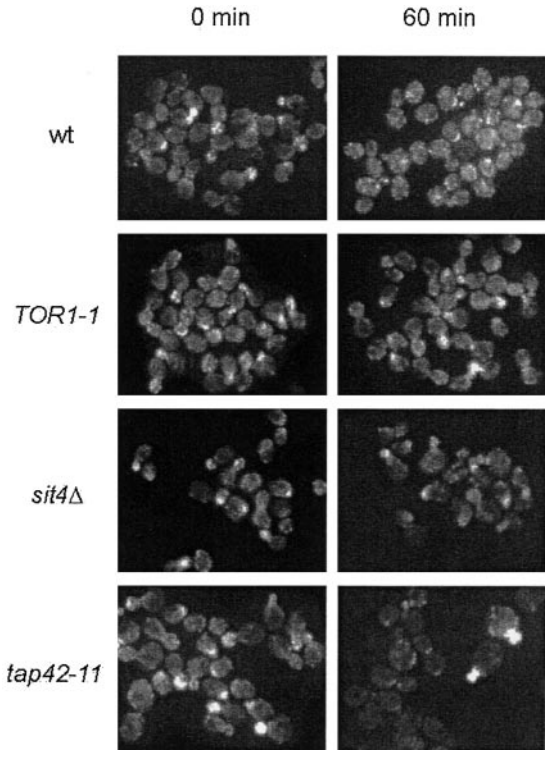

FIG. 5. ***TOR* genes and their downstream effectors *SIT4* and *TAP42* mediate actin cytoskeleton depolarization in response to rapamycin treatment.** Cultures of wild type (*wt*) (JK9-3da), *TOR1-1* (JH11-1C), *sit4*Δ (JA-110), and *tap42-11* (CY4908) cells were incubated up to logarithmic phase in YPD at 25 °C, and rapamycin was added to a final concentration of 200 ng/ml. At the times indicated, samples were removed, fixed, and processed for actin staining. The parental wild type strains of *sit4*Δ and *tap42-11* (CML128 and CY4907, respectively) underwent the same kinetics of actin depolarization as JK9-3da (not shown).

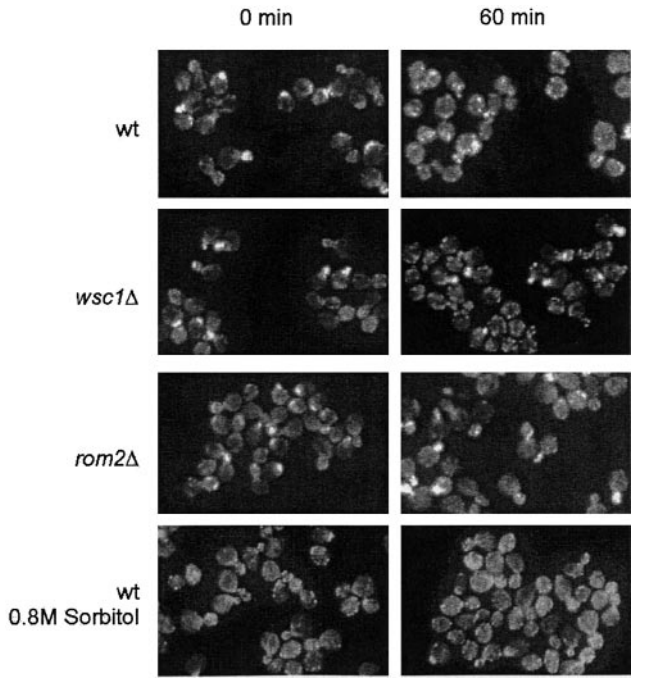

FIG. 6. **Rom2 and upstream components in the PKC pathway mediate actin cytoskeleton depolarization in response to rapamycin treatment in a sorbitol-suppressible manner.** Cultures of wild type (*wt*) (CML128), *rom2*Δ (MML391), and *wsc1*Δ (MML382) cells were incubated up to logarithmic phase in YPD (or YPD supplemented with 0.8 M sorbitol) at 25 °C, and rapamycin was added to a final concentration of 200 ng/ml. At the indicated times, samples were removed, fixed, and processed for actin staining.

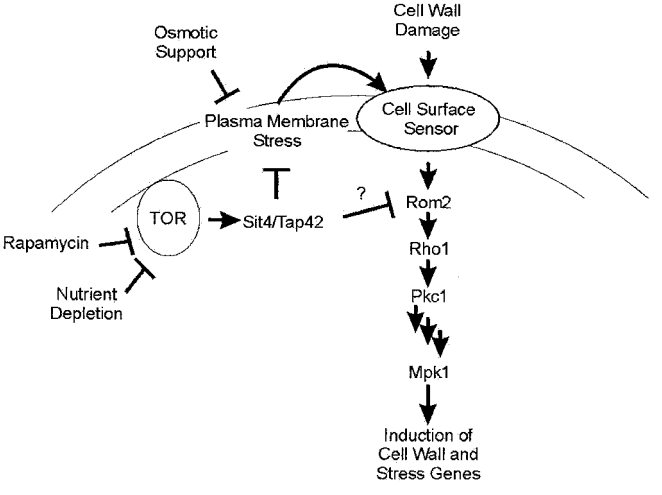

FIG. 7. **Model for TOR signaling to the PKC pathway.** TOR function promotes association of Sit4 and Tap42 (6), and this may lead to the down-regulation of the PKC pathway. Inactivation of TOR function by rapamycin treatment or upon entry into stationary phase causes the dissociation of the complex and the induction of the pathway in a process that may involve induction of plasma membrane stress and, in different degrees, all members of the PKC signaling cascade.

tap42-11 allele, also suppressed the actin cytoskeleton depolarization process, albeit more poorly than in the *TOR1-1* and *TOR2-1* rapamycin-resistant alleles (Fig. 5). These results indicate that the inhibition of the rapamycin-sensitive TOR shared function also affects the organization of the actin cytoskeleton. It has been described that mutations in *WSC1* and *ROM2* genes severely impair depolarization of the actin cytoskeleton in response to various cell wall stresses, otherwise known to induce depolarization in wild type cells (59).[2] We observed that both *WSC1*- and *ROM2*-deleted cells displayed a marked resistance to depolarize the actin cytoskeleton in response to rapamycin (Fig. 6), which suggests that both *WSC1* and *ROM2* also mediate the signal to the actin cytoskeleton. Moreover, osmotic stabilization by the addition of 0.8 M sorbitol to the growing medium also prevented depolarization of the actin cytoskeleton. These results, together with those shown in Figs. 3 and 4, suggest that inhibition of TOR function rapidly affects the activity of cell surface sensors by creating cell wall or plasma membrane stress.

DISCUSSION

We have shown that rapamycin-mediated blockage of TOR function leads to the up-regulation of the Mpk1 MAPK in all four of the strain backgrounds used in this study. Thus, TOR function negatively affects the activity of the PKC pathway, probably by preventing plasma membrane stress (Fig. 7). We have also presented evidence demonstrating that rapamycin-induced activation of Mpk1 is mediated by the TOR effectors Tap42 and Sit4 and takes place upstream in the cell integrity pathway, in a process that may involve activation of cell surface sensors.

TOR function has been reported to control cell growth in response to nutrient signals in eukaryotic cells (for review, see Refs. 61 and 62). In yeast, it has been suggested, based on genetic interactions between cell surface sensors and mutants in the protein kinase A pathway, that the cell integrity pathway would also be related to nutrient sensing via the Ras/cAMP pathway (26). *BCK1* kinase was also initially cloned as a

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

gene with a Ras/cAMP-independent role in nutrient sensing (63), and both mutants in *BCK1* and *WSC1* have been shown to display defects in cell cycle arrest upon nutrient deprivation (64, 65). The connection that rapamycin establishes between TOR and Mpk1 provides new evidence for a role of the cell integrity pathway in the nutrient stress response. Situations of limited nutrient availability, such as nitrogen starvation, sporulation, and entry into stationary phase, eventually affect remodeling of the cell wall, and regulatory mechanisms affecting PKC activity may play a central role in all of them. Cells that have entered into stationary phase or that have been treated with rapamycin acquire thicker cell walls and display thermotolerance (6, 66). Both processes are dependent, at least in heat-shocked cells, upon the previous induction of Mpk1 activity (53). In fact, here we show that Mpk1 is activated by both rapamycin and upon entry into stationary phase. This activation may be crucial for thermotolerance acquisition and for inducing changes in cell wall structure. Furthermore, cells deleted for *MPK1* lose viability when entering stationary phase. Also, as reported previously by other investigators, an *MPK1*-deleted strain is hypersensitive to rapamycin (47). Thus, Mpk1 seems to have a broader role beyond its known function in response to cell wall damage, and its activation may be essential to maintain cell viability once the nutrient supply is exhausted.

The observation that Mpk1 is activated when cells enter stationary phase raises the question about the nature of the signal monitored by TOR which eventually induces the PKC pathway. Although other types of signal being sensed by the cells cannot be discarded, we hypothesize that depletion of some nutrient(s) may lead to the up-regulation of Mpk1 activity. However, we have not detected significant changes in the activity of the PKC pathway (measured by Mpk1 activation) in cells undergoing nitrogen deprivation, amino acid starvation, or glucose depletion (data not shown). Thus, exhaustion of any other element(s) could be the event that triggers Mpk1 activation when cells enter G_0. We propose that TOR function maintains the signal(s) that prevent the cell integrity pathway from being activated when cells grow under favorable nutrient conditions.

Yeast cells have two *TOR* genes, *TOR1* and *TOR2*. It has been proposed that the TOR pathway has two essential functions (20), one of which is shared by *TOR1* and *TOR2*. The other function only depends on *TOR2*, is not inhibited by rapamycin, and is related to the polarization of the actin cytoskeleton through the cell integrity pathway (21–23). Up-regulation of the PKC pathway in response to TOR inhibition results from a block of the TOR shared function because it is sensitive to rapamycin, and it is mediated by both *TOR1* and *TOR2*. Although it remains to be determined whether the *TOR2* essential function also impinges on Mpk1 activity, it seems that both the TOR shared and the *TOR2* essential function may be directly or indirectly affecting the PKC pathway. The sharing of common regulatory elements of both TOR functions has been suggested previously (67) because overexpression of *PLC1* or *MSS4* (coding for phospholipase C and a phosphatidylinositol 4-phosphate 5-kinase, respectively) suppresses not only mutations in the *TOR2* essential function but also mutants defective in the TOR shared function, suggesting that TOR regulates two related signaling pathways.

Our results indicate that cell surface sensors contribute to Pkc1 signaling when TOR activity is affected. We have shown that *wsc1*Δ, *mid2*Δ, and *rom2*Δ cells partially suppress induction of the PKC pathway in response to rapamycin. We propose two interpretations for the above results. (i) The fact that all of those genes display redundancy with their functional homologs might explain why the single deletants do not totally abolish rapamycin-dependent activation of Mpk1 and why simultaneous deletion of *WSC1* and *MID2* has an additive effect in the blockage of rapamycin signaling to Mpk1. (ii) In addition to this mechanism, other entries to the upper elements upstream from Pkc1 (namely, Rom2, Rho1) could be functioning from the Sit4-Tap42 complex and thus also contribute to activate Mpk1. We have also shown that both *rom2*Δ and *wsc1*Δ cells partially block the actin depolarization process. Rapamycin signaling to cell surface sensors most probably diverges to actin organization and Mpk1 activation independently. As expected from previous reports (59), Rom2 may participate in both. However, the effects of the PKC pathway in polarity signaling are not clear: some *rho1* alleles and mutations in *MPK1* depolarize the actin cytoskeleton (23, 68), whereas hyperactivation of Rho1 and Pkc1 induces depolarization of the actin cytoskeleton (59), and *rom2*Δ cells display hyperpolarization phenotypes (69). Therefore, because the PKC pathway has also been proposed to be involved in polarization of the actin cytoskeleton, we do not discard the possibility that mutations in *WSC1* and *ROM2* preadapt cells in some way that they become less prone to depolarizing the actin cytoskeleton upon the appropriate environmental signal(s). Further experiments will be needed to clarify this point.

TOR function might not be actively inhibiting elements of the PKC pathway. Instead, TOR function could be involved in some aspects of cell wall integrity, and its failure would lead to the activation of the PKC pathway provided cells are not osmotically stabilized. However, the activation of cell surface sensors probably does not respond to cell wall damage because (i) Mpk1 activation by rapamycin treatment occurs in a very short lapse of time, and it is difficult to explain how cell wall architecture can be remodeled so rapidly; (ii) rapamycin-treated cells become thermotolerant (6); and (iii) rapamycin-treated cells are not more sensitive to zymoliase than non-treated cells within the time course when the PKC signaling is observed. Alternatively, TOR function may be involved in the maintenance of an adequate outward osmotic pressure at the plasma membrane. In this case, TOR inhibition would also lead to the up-regulation of Mpk1 activity in a sorbitol-suppressible manner. Support for this hypothesis is that (i) the TOR proteins have been mainly localized to the plasma membrane (70), where they could be involved in functions of the cell envelope; and (ii) the simultaneous lack of Tor1 and Mpk1 function affects cell viability in a way suppressible by osmotic stabilization. When cells are treated with rapamycin (as when they enter stationary phase), the TOR shared function would become blocked, giving rise to changes in the cell surface which in turn would trigger actin cytoskeleton depolarization, Pkc1 up-regulation, and the concomitant induction of Mpk1 activity and the expression of genes needed for cell wall construction. Therefore, the latter signaling from TOR to the PKC pathway is another cellular response to overcome or adapt to stressful nutritional conditions and to prevent cell death.

We have also shown that the TOR effectors Sit4 and Tap42 mediate rapamycin signaling to Mpk1. Because rapamycin induces the dissociation of the complex (6), this may lead to the up-regulation of Mpk1 activity. The latter is in accordance with the *sit4*Δ and *tap42-11* mutant phenotypes: both of them block rapamycin signaling to Mpk1 and up-regulate its basal activity levels. Although speculative, this hypothesis suggests that the *tor1*Δ strain may display higher basal levels of Mpk1 activity because of lower TOR activity consequently reduced levels of the Sit4-Tap42 complex. Therefore, we propose that the Sit4-Tap42 complex may be the active form of Sit4 regarding the rapamycin-sensitive regulation of the cell integrity pathway.

Although we cannot exclude the possibility that Sit4 already dissociated from Tap42 up-regulates the pathway, our previous observations strongly suggest that this may not be the case because *SIT4* acts as a negative modulator of the cell integrity pathway (33). The latter implies that in this signaling, Tap42 would act as a modulator of Sit4 rather than as an inhibitor. This is in agreement with the observation that simultaneous overexpression of *SIT4* and *TAP42* aggravates the growth defects of the single overexpression mutants, suggesting that *TAP42* may activate *SIT4* function(s) (6). These observations are reflected in a model in which TOR proteins positively signal to the Sit4-Tap42 complex, which in turn prevents (at least in part) activation of cell surface sensors by inhibiting changes in the yeast envelope (Fig. 7). Whether or not these changes mean damage or just structural changes that do not affect the cell wall resistance remains unclear. Another point we would like to make is that we do not discard the possibility that the Sit4-Tap42 complex might signal directly to other elements of the cell integrity pathway, upstream from Pkc1 but downstream from cell wall sensors. Although rapamycin-mediated activation of the PKC pathway is osmotically suppressible, we have shown previously that Sit4 negatively modulates the basal activity of the pathway in a sorbitol-independent manner and at a level downstream from cell surface sensors (33). Besides, further activation of the PKC pathway in a *sit4*Δ mutant can be induced by heat shock, whereas this mutation renders cells unable to induce Mpk1 by rapamycin (Ref. 33 and Fig. 2*C*). This suggests that Sit4 may impinge on cell integrity regulation via two different pathways. One involves Sit4 association with Tap42 and is exercised at the level of the cell envelope. The other one, which may not be necessarily related to rapamycin signaling, operates upstream from Pkc1 to modulate the activity of the MAPK module.

In mammalian cell lines, mTOR also controls several PKC isotypes by promoting the phosphorylation of the kinases in their hydrophobic C-terminal site (71, 72). This phosphorylation is rapamycin-sensitive, thus implying that mTOR positively regulates PKC phosphorylation in mammals. However, the affected residue is different from the site phosphorylated by phosphoinositide-dependent kinases in the activation loop of PKC (for a recent review, see Ref. 73). Moreover, mTOR-dependent phosphorylation has been proposed to be needed for optimum PKC function. All of those putative phosphorylation sites are conserved in yeast Pkc1, and phosphorylation by phosphoinositide-dependent kinases, along with activation by Rho1, plays an important role in regulating Pkc1 activity (74). However, as we have shown above, a crucial difference arises in yeast: contrary to what may occur in mammalian cells, the TOR shared function negatively affects Pkc1 activity. Thus, it seems that yeast and mammals have evolved different mechanisms for TOR control over PKC activity.

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