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Pkc1 and the Upstream Elements of the Cell Integrity Pathway in *Saccharomyces cerevisiae*, Rom2 and Mtl1, Are Required for Cellular Responses to Oxidative Stress*

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In this study we analyze the participation of the PKC1-MAPK cell integrity pathway in cellular responses to oxidative stress in *Saccharomyces cerevisiae*. Evidence is presented demonstrating that only Pkc1 and the upstream elements of the cell integrity pathway are essential for cell survival upon treatment with two oxidizing agents, diamide and hydrogen peroxide. Mtl1 is characterized for the first time as a cell-wall sensor of oxidative stress. We also show that the actin cytoskeleton is a cellular target for oxidative stress. Both diamide and hydrogen peroxide provoke a marked depolarization of the actin cytoskeleton, being Mtl1, Rom2 and Pkc1 functions all required to restore the correct actin organization. Diamide induces the formation of disulfide bonds in newly secreted cell-wall proteins. This mainly provokes structural changes in the cell outer layer, which activate the PKC1-MAPK pathway and hence the protein kinase Slt2. Our results led us to the conclusion that Pkc1 activity is required to overcome the effects of oxidative stress by: (i) enhancing the machinery required to repair the altered cell wall and (ii) restoring actin cytoskeleton polarity by promoting actin cable formation.

Cells are constantly exposed to a series of environmental stresses, which are sensed by complex signal transduction pathways that are responsible for cellular damage repair and adaptation responses. These responses are mediated by (mitogen-activated protein kinase (MAPK)[1] cascades in eukaryotic cells.

In the *Saccharomyces cerevisiae*, the PKC1-MAPK cell integrity pathway is involved in responses to a wide variety of stresses, including heat-shock (1), hypoosmotic shock (2), nutritional stress (3), and those associated with any other injuries that alter the integrity of the outer layer of cells. These environmental signals are generally sensed by Mid2 (4) and Wsc family (5) cell surface proteins. Mtl1 is a putative cell membrane sensor with 50% homology to Mid2 (6, 4). Mtl1 has been characterized as a multicopy suppressor of the absence of Rgd1, a protein that functions as a GTPase-activating protein for Rho3 and Rho4 proteins (7) and as a multicopy suppressor of Rho1 functions (8).

Cell surface sensors transmit signals to Rom2, a guanine exchange factor protein of the GTP-binding protein Rho1. Rho1 then activates the Pkc1 protein kinase, which in turn activates an MAPK module; Pkc1 phosphorylates Bck1, a MAPK kinase kinase, which transmits the signal to both MAPK kinases, Mkk1 and Mkk2. Those finally activate the last member of the cascade, Slt2/Mpk1, by phosphorylating both the Thr^{190} and Tyr^{192} residues of this MAPK (the PKC1-MAPK pathway reviewed in Refs 9 and 10). Rlm1, a transcription factor involved in the activation of cell wall genes, and Swi6 (a transcription factor involved in cell cycle regulation) are both targets for Slt2. Activation of Slt2 correlates to activation of both downstream events (11) (Fig. 1).

The upper elements of the PKC1-MAPK pathway are involved in the organization of the actin cytoskeleton upon cell wall stress. It has been reported that hyperactivation of either Pkc1 or Rho1 alone causes depolarization of actin cables in patches dispersed throughout the cytoplasm (12). In addition, the function of both Rho1 and Pkc1 is necessary for actin to become depolarized following heat stress (12).

Actin cable assembly relies on the activity of the two redundant formins, Bni1 and Bnr1 (13, 14). Rho1 is able to localize to actin patches (15) and is required for the activation of formins at high temperatures through Pkc1 (16). This emphasizes the role of these proteins in polarized growth and morphogenesis.

Oxygen is a vital molecule, but at the same time, it is responsible for provoking oxidative stress in cells, and causing cellular damage to various macromolecules (17). Budding yeast constitutes an optimal eukaryotic model for studying oxidative stress responses (18). However, although in the case of environmental stresses, such as osmotic, nutritional, and heat-shock stresses, there are specific signal transduction pathways that sense and transmit the different signals to specific components in *S. cerevisiae* (19), there are no well characterized MAPK pathways for sensing and signaling oxidative stresses to the cytoplasm and nucleus. Nevertheless, there is considerable information in the literature detailing the nuclear transcriptional factors responsible for the induction of specific genes in response to oxidative stress in yeast. These are Yap1, Skn7, and Msn2/4 (for a review, see Ref. 18).

In this study, we decided to investigate whether the cell integrity pathway in *S. cerevisiae* played a role in oxidative stress responses based on the following observations: (i) Skn7 has been shown to interact with the GTPase Rho1 (20); (ii) it has been reported that Msn2/Msn4 participate with the PKC1-MAPK pathway in the compensatory mechanism that is triggered by cell wall mutations (21); (iii) genome-wide analysis (22) indicates that the agent diamide induces the activation of

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; PKC, protein kinase C; GFP, green fluorescent protein; HA, hemagglutinin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol.

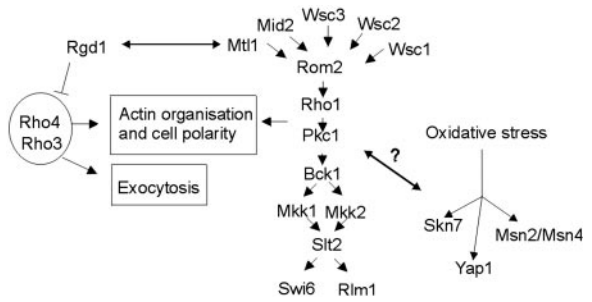

FIG. 1. **A schematic diagram showing some elements directly or indirectly related with some of the components of the cell integrity pathway, which play a role in the cellular responses against oxidative stress in *S. cerevisiae*.**

genes regulated by Rlm1; and (iv) in human cells, isoforms of PKC are known to be either activated or inactivated by oxidative stress, and this is causally associated with tumorigenesis (for a review see Ref. 23).

We demonstrate that Pkc1, and the upper elements of the cell integrity pathway, are required for survival and adaptation to the oxidative stress provoked by two different oxidizing agents: hydrogen peroxide and diamide. We also report a new function for Mtl1 as a cell membrane receptor for oxidative stress. We present a detailed study demonstrating that the major effect caused by diamide is the oxidation of cell wall proteins, which activates the cell integrity pathway. Hydrogen peroxide exercises its action at the intracellular level, mainly by affecting a cellular function that is closely related to the polarization of the actin cytoskeleton. Finally, we demonstrate that restoration of cell polarity and recovery from oxidative stress occurs in a Pkc1-dependent manner.

MATERIALS AND METHODS

Yeast Strains and Gene Disruptions—The yeast strains used in this study are listed in Table I. *MTL1* was disrupted by the one-step disruption method using the *kan*MX4 module (24), whereas the *WSC4* gene was disrupted using the *nat*MX4 module (25).

GFP-Pkc1 was constructed as follows: a 1-kb XhoI-KpnI fragment from pRS416-sGFP (26) was cloned at the SmaI site of the pFA6a plasmid containing the *kan*MX4 cassette. The resulting plasmid, pCYC86, was provided by Dr. Marti Aldea's laboratory. We then used PCR to amplify the sGFP-*kan*MX4 module from pCYC86. We did this by using oligonucleotides designed to insert the product in-frame, just before the stop codon of the genomic *PKC1* sequence to obtain the fusion protein Pkc1-GFP. These were: MMO218, 5′-gcaagaagagtttagaggatttt-CCTTTATGCCAGATGATTTGGATTTACCAGCTGAAGCTTCGTA-CGC; MMO219, 5′-CCGCTTAGATGTTTTATATAAAATTAAATAAAT-CATGGCATGACCTTTTCTgcataggccactagtggatc, where the use of lowercase letters represents the sequence used to amplify the *kan*MX4 cassette, and the use of capital letters represents the sequence homologous to the C-terminal domain of *PKC1* designed for recombinational integration.

Plasmids—Plasmid pMM126 contains *PKC1* under the *tet*O_7 promoter and is tagged with the HA epitope, as described before (27). To detect the total amount of Slt2 protein in protein extracts, we used yeast strains transformed with a YEP352 plasmid derivative containing the Slt2 open reading frame under its own promoter and tagged with the HA epitope in C-terminal (a gift from Dr. Maria Molina). All the strains used in this report were transformed with this plasmid, and the total amount of Slt2 was determined by using the anti-hemagglutinin monoclonal antibody (*right panel*), which was established as a loading control throughout this study.

Media and Growth Conditions—Yeasts were grown in SC (0.67% yeast nitrogen base, 2% glucose, and auxotrophic requirements) or SD (SC plus drop-out mixture) media (28) plus the required amino acids. Where needed, sorbitol was added to a final concentration of 0.8 M final concentration. Diamide (Sigma) was prepared in dimethyl sulfoxide, whereas hydrogen peroxide (Sigma) was diluted with sterile Milli Q water.

Yeast Extracts and Immunoblot Analyses and Conditions for the Use of the Anti-phospho-p44/42 and Anti-Swi6—These analyses were performed as described previously (27). The anti-HA antibody was used at a dilution of 1:1000 in TBST buffer (20 mM Tris-HCl, pH 8, 0.125 M NaCl, 0.1% tween 20) in the presence of 0.25% milk fat, and the corresponding horseradish peroxidase-linked anti-mouse secondary antibody, at a dilution of 1:10.000 in TBST containing 0.25% milk fat.

Sulfhydryl Determination with DTNB (Ellman's Reagent)—Yeast cells were grown in SC medium at 25 °C (to an initial A_{600} = 0.6). The assay was essentially conducted as described before (29). Aliquots of 30 ml of culture were used for each treatment. They were centrifuged at 3,000 rpm for 5 min and resuspended in 2 ml of Tris-HCl buffer (10 mM, pH 7.4), and then treated and incubated at 4 °C for 30 min as follows: (i) control without treatment; (ii) 20 mM dithiothreitol (DTT); (iii) 1 mM H_2O_2; (iv) 2 mM H_2O_2; and (v) 4 mM diamide. After that, samples were washed three times with the same buffer. Next, they were resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 20 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), and incubated for 10 min at room temperature. Finally, samples were centrifuged at 10,000 rpm for 3 min, and the supernatants were used to measure the optical density at 412 nm. The amount of free SH residues was quantified at this wavelength. The reducing agent DTT was used as a control to quantify the maximum amount of free SH residues on cell surfaces. Osmotic stabilization was achieved in *pkc1* and wild type cell cultures by adding sorbitol to a final concentration of 0.8 M.

Zymolyase Digestions—Cells were exponentially grown in SC medium at 25 °C and subsequently treated with 4 mM diamide and 1 mM hydrogen peroxide for 30 and 60 min, respectively. Mock treated cells were used as controls. After the treatments, 10 ml of each cell culture were centrifuged at 2,500 rpm for 5 min. The resulting pellets were washed first with 10 ml of sterile Milli Q water and then with 5 ml of Tris-HCl (50 mM, pH 9.4). The final pellet was resuspended in the last buffer. Samples of 1 ml of volume of each culture were added to 0.2 or 0.5 units zymolyase. Cell lysis was spectrophotometrically quantified at 600 nm.

Actin Staining—Cells growing exponentially at 25 °C were stained with rhodamine-phalloidin as described before (3).

Electron Microscopy—Log phase cells were treated, or mock treated, with 1 mM diamide for 9 h, then fixed with 2.5% glutaraldehyde for 1 h in 0.1 M phosphate buffer (pH 7.4) at 4 °C. Samples were then washed three times in the same buffer followed by a second fixation in osmium tetraoxide 1% in H_2O for 2 h at 4 °C. After washing, the pellets were processed by dehydration using a series of acetones from 30–100% followed by propylene oxide treatment. Samples were then incubated in a resin (Durcupan ACM Epoxy Resin, Fluka): propylene oxide 3:1 for 45 min, 1:1 for 45 min, and 1:3 for 45 min, before embedding in resin and curing. Sections were cut and treated with lead citrate before viewing under a Zeiss 910 transmission electron microscope.

RESULTS

Oxidative Stress Provoked by Diamide and Hydrogen Peroxide Activates the Cell Integrity Pathway—Given the role that the cell integrity pathway plays in the response to a wide variety of stresses, we decided to investigate whether this pathway was also involved in the oxidative stress response. Exponentially growing cells were treated either with diamide or hydrogen peroxide for the indicated times (Fig. 2*A*). Samples were taken for Western blot to detect activated Slt2, using the anti-phospho-p44/42 MAPK antibody raised against dually phosphorylated Thr^{202}/Tyr^{204}-p44/42 MAPK. It is generally accepted that detection of the doubly phosphorylated form of Slt2 provides a measure of kinase activation. The concentrations of the two oxidizing agents chosen for this study were sublethal and did not cause cell death during the course of the experiment. We had previously tested the following concentrations for the two agents: 0.5, 1, 2, 2.5, 3, and 5 mM in the case of hydrogen peroxide, and 1, 2, and 4 mM for diamide (not shown). During the first 3 h of treatment, we observed that concentrations of hydrogen peroxide of $<$1 mM failed to provoke a higher induction in Slt2 activity, whereas concentrations of $>$2 mM caused no detectable activation whatsoever. Increasing the concentrations of diamide had a different effect in Slt2 activity. Greater concentrations of diamide provoked higher inductions of Slt2 double phosphorylation, although these were concomitant with an increase in cell lethality (results not shown in this study).

TABLE I
Yeast strains used in this work

Strain	Relevant genotype	Reference
AN3–5D	*MAT?*, *ura3–52*, *trp1*, *leu2–3,113*, *sec1ts*	Obtained from L. Castillo
CML125	*MATα leu2–3,112 ura3–52 trp1 his4 can1^{y}*	de la Torre, *et al*. (27)
CML128	*MATa as CML125*	Gallego, *et al*. (44)
CML399	*MATa slt2*::*URA3*	de la Torre, *et al*. (27)
DL2357	*MATa mtl1*::*HIS4 mid2*::*URA3*	Obtained from D. Levin
MML200	*MATα bck1*::*kan*Mx4	de la Torre, *et al*. (27)
MML304	*MATα pkc1*::*LEU2(pBCK1–20)*	de la Torre, *et al*. (27)
MML344	*MATa pkc1*::*LEU2*	de la Torre, *et al*. (27)
MML357	*MATa wsc1*::*kan*Mx4	de la Torre, *et al*. (27)
MML363	*MATα rom2*::*kan*Mx4	This work
MML384	*MATa wsc2*::*nat*Mx4	This work
MML387	*MATa mid2*::*kan*Mx4	de la Torre, *et al*. (27)
MML392	*MATa wscs1*::*URA wsc2*::*natMx4*	This work
MML393	*MATa mid2*::*kanMx4 wsc1*::*URA*	This work
MML411	*MATa Galpkc1*::*kan*Mx4	This work
MML429	*MATa wsc3*::*kan*Mx4	This work
MML431	*MATa wsc4*::*nat*Mx4	This work
MML513	*MATa mtl1*::*kan*Mx4	This work
MML550	*GFPPkc1*	This work
X2180–1A	*MATa SUC2 gal2 CUP1 ma10*	ATCC 204504

Diamide and peroxide treatments both significantly induced Slt2 dual phosphorylation, although the kinetics of activation differed, with activation occurring considerably sooner with diamide (Fig. 2*A*). The addition of the osmotic stabilizer sorbitol almost totally abolished Slt2-dependent diamide activation, suggesting that diamide might affect the cell wall. In contrast, sorbitol did not significantly affect Slt2 induction of activity provoked by peroxide. This seems to indicate that the mechanism that regulates the induction of the PKC1-MAPK pathway mediated by peroxide takes place inside the cells. Interestingly, when we used other peroxides such as *t*-butyl hydroperoxide we obtained similar results to those observed with H_2O_2 (not shown).

Activation of the PKC1-MAPK pathway and the Slt2 kinase was correlated to two different processes: phosphorylation of Swi6 and transcriptional induction of genes regulated by Rlm1. To check whether the increase in Slt2 phosphorylation dependent on oxidative stress could be correlated with the activity of the kinase and the pathway, we tested both processes in cells treated with either diamide or hydrogen peroxide. We thereby confirmed this hypothesis by means of two results. One consisted on the observation that Swi6 became hyperphosphorylated upon both peroxide or diamide treatments as shown in Fig. 2*B*. In this figure we observe a slower mobility hyperphosphorylated Swi6 band detected with a polyclonal antibody (Dr. Noel Lowndes gift). And the second that the transcriptional levels of *SLT2* and *PST1* (both genes are transcriptionally regulated by Rlm1) increased concomitantly with increases in Slt2 phosphorylation (Fig. 2*B*). From these results we conclude that when cells sense the oxidative stress provoked by diamide and hydrogen peroxide, Slt2 becomes activated.

Mtl1 Senses and Transduces the Oxidative Effect Caused by Diamide—We tested *mid2*, *mtl1*, *wsc1*, *wsc2*, *wsc3*, and *wsc4* single mutants together with *wsc1wsc2*, *mid2wsc1*, and *mtl1mid2* double mutants for Slt2 phosphorylation due to the addition of diamide or hydrogen peroxide to check whether any of the known cell receptors related to the cell integrity pathway were involved in sensing and signaling the oxidative stress input. In this study we decided to perform a quantitative analysis of Slt2 activation by using the following criteria: we calculated the value called induction -fold, as the ratio between quantitative levels of Slt2 phosphorylation at either 30 or 60 min and Slt2 phosphorylation level measured at time 0 (untreated cells). All of the single quantified values were also normalized with respect to their respective loading controls (Fig. 2*C*). Therefore, the numerical values shown in this study correspond to relative levels of Slt2 activation in samples treated with oxidative agents after different times of exposure. In some of the mutants used in this study the basal levels of Slt2 activity were significantly lower than those estimated for wild type cells. For this reason and to avoid possible misinterpretations we show the numerical values corresponding to Slt2 relative induction for each experiment. Relative induction of Slt2 phosphorylation was partly defective in *mid2* mutant cells treated with diamide, whereas it was almost completely eliminated in *mtl1* cells (Fig. 2*C*). The latter result led us to consider the possibility of Mtl1 and Mid2 being partly redundant for sensing the oxidative effect caused by diamide. To test this, we checked the status of Slt2 activity in the *mtl1mid2* double mutant and obtained that for this function Mid2 and Mtl1 did not constitute additive sensors of oxidative stress. The other cell wall receptor mutants assayed displayed no significant differences with respect to their respective single mutants and wild type cells (not shown). However, different results were obtained for hydrogen peroxide: none of the mutants showed marked deficiency in the relative induction of Slt2 phosphorylation with respect to wild type levels, although a partial reduction in Slt2 phosphorylation was observed in *mid2* and *mid2mtl1* mutants upon 2 h of exposure to hydrogen peroxide (Fig. 2*C*). To further characterize the role of Mtl1 and Mid2 as sensors of oxidative stress caused by diamide, we tested cell viability in the presence of diamide. We observed that only the absence of Mtl1 significantly impaired cell viability in response to diamide (Fig. 2*D*). Moreover, overexpression of the constitutive active Pkc1 allele under the Gal1 promoter (named Gal-Pkc1*, a gift from Dr. M. Hall, for details see Ref. 12) rescued both cell viability in the *mtl1* mutant (Fig. 2*E*). These results indicate that Mtl1 functions in the cell integrity pathway by sensing and transmitting the oxidative signal. Our results indicate that both Mtl1 and, at least in part, Mid2 are sensors for the diamide-mediated oxidative effect at the cell surface and play a role in the cell integrity pathway for this response. These findings are also consistent with the results shown above and suggest that hydrogen peroxide activates the pathway at the intracellular level.

Pkc1, Rom2, and Mtl1 Are Required for Transmitting the Oxidative Signal to Slt2 and for Cell Viability in Response to Oxidative Stress—Once demonstrated that oxidative stress activates Slt2 mediated by cell wall receptors, we investigated which elements of the cell integrity pathway were involved in this activation. To do this, we treated *rom2* mutant cells with

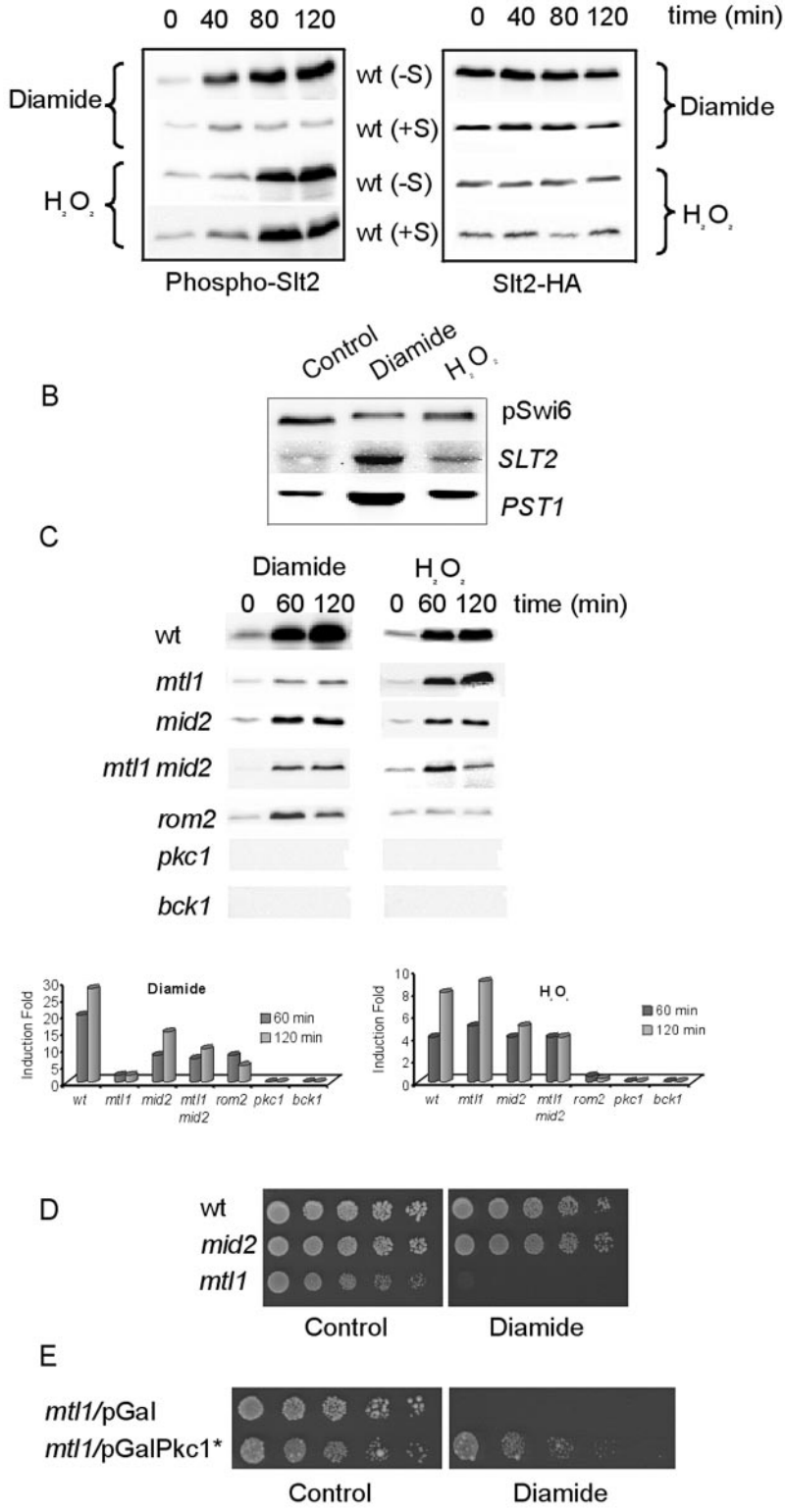

FIG. 2. **Slt2 kinase activity is induced upon oxidative stress via Pkc1.** *A*, in all the experiments, cells were grown exponentially at 25 °C in SC medium plus amino acids and treated either with 4 mM diamide or 1 mM hydrogen peroxide. Cells harboring the YEP352-Slt2-HA plasmid (see "Materials and Methods") were treated either with diamide or hydrogen peroxide for the indicated times. "*S*" refers to 0.8 M sorbitol added to the culture medium. Double phosphorylation of Slt2 was detected using the p42/44 antibody (*left panel*), whereas total Slt2 was detected using the anti-hemagglutinin monoclonal antibody (*right panel*), which was used as a loading control in this study. *B*, samples from the previous experiment were collected after 30 min and processed for Northern (using *SLT2* and *PST1* as probes) and Western blotting (using the polyclonal anti-Swi6 antibody). *C*, exponentially growing cells from CML128 (wild type), *mtl1*, *mid2*, *rom2*, *pkc1*, and *bck1* strains were treated either with diamide or hydrogen peroxide, and samples were collected for Western blot after 1 and 2 h of treatment. Double phosphorylation of Slt2 was detected using p44/42 antibody. Induction of Slt2 activity was quantified and normalized with respect to the loading control (band quantified with p44/42 *versus* band quantified with anti-HA antibodies) and represented in the form of a histogram. *D*, wild type and *mtl1* mutant cells were exponentially grown in SC plus 2% glucose medium at 25 °C and then plated onto SC plates containing or not 0.75 mM diamide. They were subsequently incubated at 25 °C for 3 days. *E*, *mtl1* cells were transformed with the GalPkc1* plasmid (Delley and Hall (12)) and with the empty vector YEplac112. Cells were grown in SC plus 2% galactose for 9 h at 25 C°. 4 mM diamide was then added for 4 h, and cultures were serial diluted and spotted on plates containing SC plus 2% glucose. The plates were incubated at 25 °C for 3 days.

either diamide or hydrogen peroxide, and upon both treatments a marked defect in Slt2 phosphorylation was observed, with respect to wild type cells (Fig. 2*C*).

We also confirmed that Slt2 activation was totally eliminated in both *pkc1* and *bck1* mutants following treatments with both peroxide and diamide (Fig. 2*C*). These results demonstrate that upstream Pkc1 elements are required to transduce the activating signal to Slt2 upon oxidative treatment. However, at present we still cannot rule out the possibility of oxidants directly activating elements downstream of Pkc1. We tested cell viability in various mutants after diamide and peroxide treatment to gain an understanding of the physiological consequences of the involvement of the cell integrity pathway in oxidative stress response (Fig. 3). We observed that *rom2* and *pkc1* deletions were significantly sensitive to the oxidizing agents, whereas *bck1* and *mpk1* cell growth was indistinguishable from that of wild type cells (Fig. 3, *A* and *B*). It is relevant to note that we obtained the same results (qualitatively speaking) when checking with different concentrations of the two agents: 0.5, 1, 1.5, 1.5, 2, 4, and 6 mM

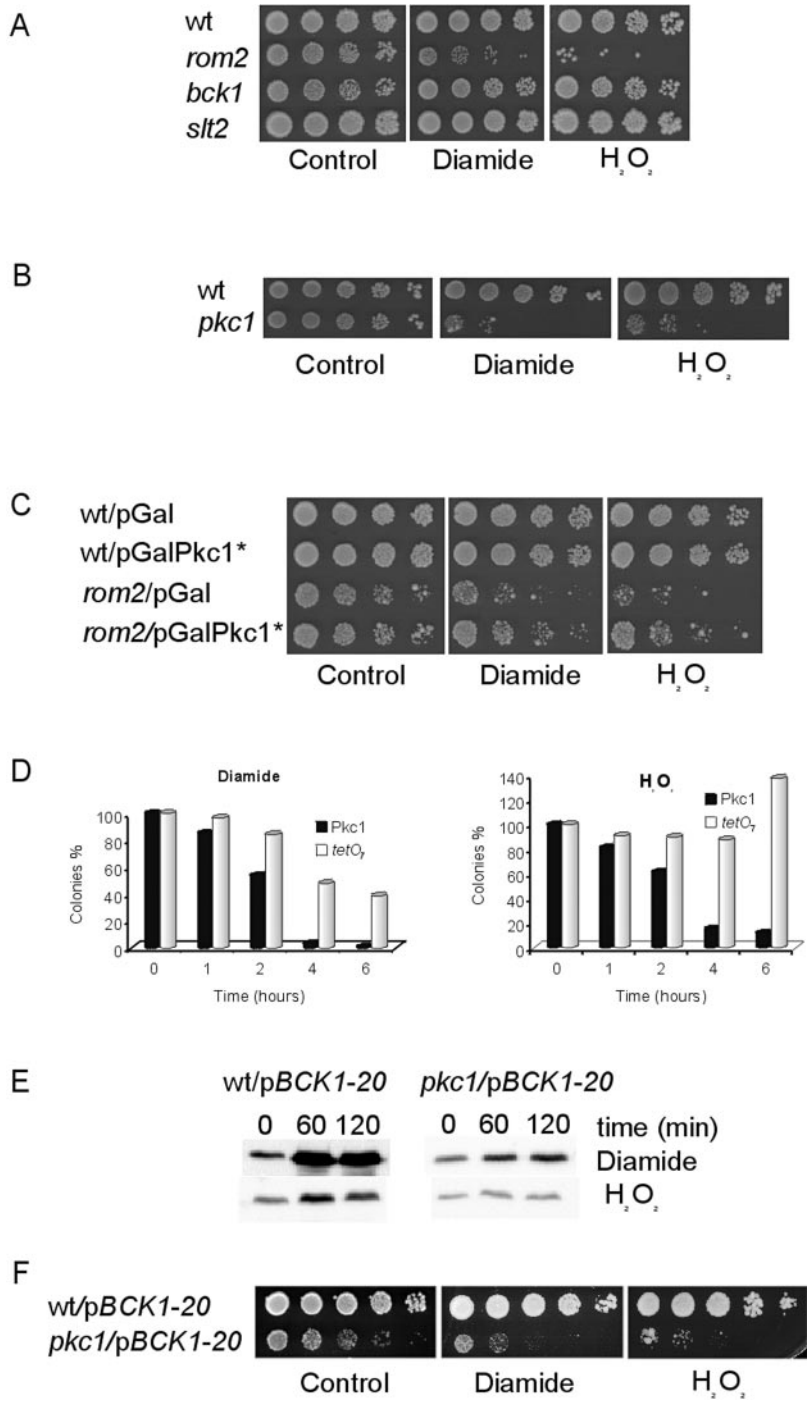

FIG. 3. **Rom2 and Pkc1 are directly involved in cell survival after oxidative stress.** *A*, cells from CML128, *rom2*, *bck1*, and *slt2* strains were grown at 25 °C to logarithmic phase, and serial dilutions were performed and subsequently spotted onto SC plates, SC plus 1 mM hydrogen peroxide, and SC plus 0.75 mM diamide. Plates were incubated for 4 days before taking photographs. *B*, both CML128 and *pkc1* strains were exponentially grown and plated on SC plates plus 0.8 mM sorbitol containing either 1 mM hydrogen peroxide or 0.75 mM diamide. *C*, wild type and *rom2* cells transformed with the GalPkc1* plasmid, or alternatively with empty plasmids, were treated as in Fig. 1*E*. *D*, wild type cells and wild type cells transformed with the centromeric plasmid pMM126 harboring the *PKC1* gene under the inducible *tet*O7 promoter, tagged in the N terminus with the HA epitope, were exponentially grown in SD medium, and then treated with either 1 mM peroxide or 0.75 mM diamide for the indicated times. About 500 cells were plated on minimum medium plates without the oxidizing agent to count the number of viable cells. These results represent the average of three independent repetitions. *E*, wild type and *pkc1* cells, each harboring the p*BCK1–20* plasmid, were treated and processed for Western blot with the p44/42 antibody as in Fig. 1*C*. *F*, wild type and *pkc1* cells, each harboring the p*BCK1–20* plasmid, were treated and processed as in *A*.

in the case of hydrogen peroxide, and 0.5, 0.75, 1, 1.5, 2, 3, 4, and 6 mM in the case of the diamide (data not shown). It is important to note that lack of viability of *pkc1* due to the presence of the oxidizing agents was not totally rescued by sorbitol (Fig. 3*B*). This strongly suggests that Pkc1 function is essential for cell viability under oxidative conditions on despite of the presence of sorbitol as osmotic stabilizer and cell wall protective agent. We next performed cell viability studies in a *rom2* mutant that had been transformed using the plasmid GalPkc1*. We observed that its overexpression compensated for *rom2* cell death caused by diamide and peroxide (Fig. 3*C*). These data support that the Rom2 and Pkc1 functions are needed for cell survival against oxidation. The MAPK module downstream of Pkc1 (see Fig. 4*A*, *bck1* and *mpk1* viability) is dispensable for this cellular response.

We overexpressed Pkc1 under the *tet*O_7 promoter (30) and subsequently performed cell viability analysis upon addition of diamide or peroxide, to ascertain whether Pkc1 has a direct role in cell viability when subjected to oxidative treatment. In both cases, overproduction of Pkc1 clearly provoked a marked increase in cell viability in wild type cells exposed to either of both oxidizing agents. Nevertheless, we observed that overproduction of Pkc1 was significantly more efficient at rescuing cell viability upon peroxide treatment than it was upon application of diamide (Fig. 3*D*).

The MAPK Module Downstream of Pkc1 Is Dispensable for Cell Viability Upon Oxidative Stress—Overexpression of Slt2 or the constitutively active allele *BCK1–20* did not significantly rescue lethality associated with the oxidative treatment observed in wild type cells and *pkc1* and *rom2* mutants (not

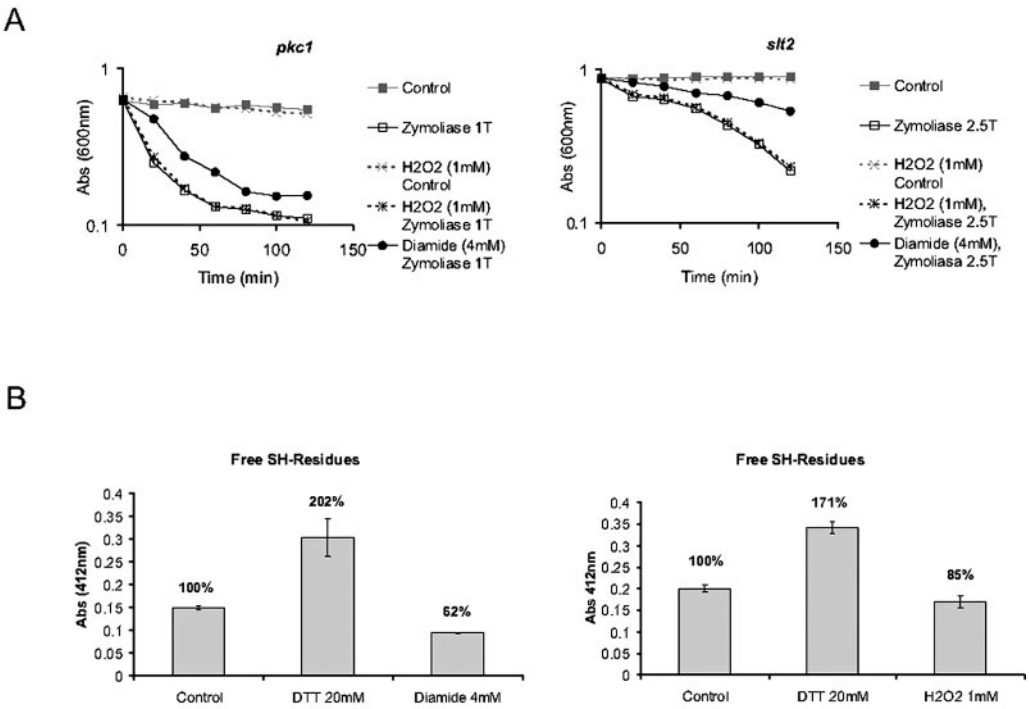

FIG. 4. **Diamide modifies the cell outer layer structure conferring greater resistance to zymolyase digestion and oxidating free-SH residues in cell-wall proteins.** *A*, exponentially growing *pkc1* and *slt2* mutant cells in SC medium at 25 °C were treated with either diamide 4 mM for 30 min or with H_2O_2 1 mM for 1 h. Subsequently, 10 ml of culture at A_{600} of 0.6 was first washed with H_2O and then with 50 mM Tris-HCl, pH 9.4. Cell pellets were then resuspended in 1 ml of this buffer prior to the addition of either 0.2 or 0.5 units zymolyase. Cell lysis was estimated by measuring at A_{600} for the indicated periods. The *pkc1* mutant was grown in the presence of the osmotic stabilizer sorbitol 0.8 M. *B*, when quantifying free-SH residues with DTNB (see "Materials and Methods"), the A_{412} was determined in intact cells treated with DTT, diamide, or hydrogen peroxide.

shown). These results reinforce the model that suggests that the Pkc1 function plays an essential role in the cellular response induced by oxidative stress.

We next decided to investigate whether the oxidizing signal could induce the pathway by activating some element of the MAPK module downstream of Pkc1, regardless of the activity of the upstream elements. To check this, we used a strain in which the *PKC1* promoter was substituted by the GAL1 promoter and which contained a plasmid carrying the constitutively activated *BCK1–20* allele. In the presence of glucose, the GAL1 promoter was repressed and the strain behaved like a *pkc1*mutant. The presence of *BCK1–20* prevented cell lethality due to the absence of the Pkc1 function in non-osmostabilizing conditions, as described before (27). The main reason for not using the *pkc1* null strain transformed with *BCK1–20* in these experiments was the relative high frequency of intragenic suppressors. Upon adding diamide or peroxide to the cell cultures, we observed that the induction of Slt2 in wild type cells carrying the *BCK1–20* allele was similar to that found in normal wild type cells. The only exception was that Slt2 phosphorylation was higher at time 0, due to Slt2 constitutive activity conferred by the presence of the *BCK1–20* allele. However, in *pkc*1/*BCK1–20* cells growing in glucose, and unlike non-treated cells, no Slt2 activation was detected upon hydrogen peroxide treatment. Only residual activation was detected after the addition of diamide: this may have been due to the function of the repressible GAL1 promoter (Fig. 3*E*). These results indicate that Slt2 activation upon oxidative stress is almost entirely dependent on components of the cell integrity pathway that lie upstream of Pkc1.

The *BCK1–20* allele partly rescues cell lethality that is specifically due to the lack of Pkc1. In Fig. 3*F* we observe that *pkc1* cells were capable to survive in minimum medium without the osmotic stabilizer sorbitol, due to the presence of the *BCK1–20* allele. However, the *BCK1–20* allele is unable to suppress lethality in a *pkc1* background associated with either the diamide or the hydrogen peroxide treatment. Thus, *pkc*1/*BCK1–20* cells lose viability in the presence of each diamide or hydrogen peroxide agents, similarly to what it was observed in *pkc1* cells (Fig. 3, compare *B* and *F*). This indicates that there is a Pkc1 function, essential for the oxidative stress response, that is different from the activation of the MAPK module.

Diamide and Not Hydrogen Peroxide Treatment Increases the Resistance to Zymolyase Digestion by Oxidating Sulfhydryl Groups in Cell Wall Proteins—The results shown above suggest that diamide carries out its action at the level of the cell outer layer. Diamide causes oxidation of free sulfhydryl residues giving rise to the formation of disulfide bonds (31). An increase in free sulfhydryls in the yeast cell wall caused by DTT significantly diminished resistance to zymolyase digestion (29). Because disulfide bridge formation reduces cell porosity and the accessibility of zymolyase to its β-glucan substrate, these authors proposed the use of zymolyase to measure cell wall porosity. In view of the above results, we wondered whether oxidative treatment could affect cell wall integrity. To avoid the effect of the compensatory mechanism associated with members of the PKC1-MAPK pathway (21, 32), we used *pkc1* and *slt2* mutants and monitored cell wall resistance to zymolyase digestion in samples treated and untreated with either diamide or hydrogen peroxide (Fig. 4*A*). We observed that, although hydrogen peroxide did not cause any significant differences between treated and untreated cells, diamide increased resistance to zymolyase digestion in both *slt2* and *pkc1* mutant cells. It therefore seems evident that diamide caused structural modifications to the cell wall: a function that was not shared with hydrogen peroxide.

To check whether or not the increase in zymolyase resistance detected in *slt2* and *pkc1* mutants subject to diamide treatment was due to the formation of disulfide bridges in cell wall proteins, we used DTNB to determine the number of reduced

sulfhydryls in intact cells (29). In cultures treated with diamide for 30 min, a significant reduction (35–40%) in the number of free sulfhydryls was observed as compared with wild type cells. The value obtained with diamide was similar to that presented by (29) when treating intact cells with the oxidizing agent sodium tetrathionate. It was considered statistically significant in terms of the number of new disulfide bridges formed by the oxidizing agent (see *error bars* in Fig. 4*B*). However, in the case of hydrogen peroxide, the reduction of free sulfhydryls was of the order of 10–12% (a value that was not statistically significant, as observed in Fig. 4*B*). The reducing agent DTT was used as a control to quantify the total quantity of free sulfhydryls in cell surface proteins. Our results therefore indicate that diamide affects the cell wall structure by inducing the formation of disulfide bonds in cell wall proteins and thereby changes the structural properties of the cell surface. This effect is not exerted (or at least was not at a significant enough level to be detected in this particular assay) by other oxidizing agents such as hydrogen peroxide. This raises the question as to whether different oxidizing agents activate the PKC1 pathway in different ways.

A Blockage in Secretion Abolishes Slt2 Activation upon Diamide Treatment—In view of the results shown above, we propose that the major cellular effect caused by diamide occurs in the cell outer layer and is a consequence of the oxidation of free sulfhydryl residues. In this context, it would be predictable to obtain a relatively rapid activation of the Pkc1-MAPK pathway, as occurs by MAPK pathways. Nevertheless, upon diamide addition Slt2 activity increases gradually, reaching its maximum levels after 1–2 h of exposure to the chemical agent. One possible interpretation to this result could be that newly synthesized proteins integrant of a cell wall in a constant remodeling process could also be susceptible of being oxidized and modified by diamide thus signaling to the cell integrity pathway. In an attempt to understand this, we designed the next two experiments: For the first one, we blocked secretion by using the *sec1–1* mutant. This thermosensitive mutant is reversibly impaired in the final stages of exocytosis when grown at 38 °C. Following a shift to 38 °C for 3 h, the secretory machinery of these cells is completely blocked. Under these circumstances, Slt2 was found to be phosphorylated by the effect of the temperature in wild type and *sec1–1* cells (Fig. 5, *A* and *B*, time 0). We next treated both cultures with diamide and observed a relatively early but transient (after 10–15 min) additional increase in Slt2 activity in *sec1–1* cells as compared with the gradual increase in Slt2 activation caused by diamide in wild type cells, peaking later in time (30–60 min, Fig. 5*B*). Therefore one speculative interpretation to the early and rapid Slt2 phosphorylation detected in *sec1–1* cells could be the modification, namely oxidation, of pre-existing proteins, components of the cell surface in a system in which secretion was blocked. This supports the previous model by which diamide alters mainly the cell outer layer and indicates that this oxidant does not affect the secretory machinery. In a parallel experiment, we blocked protein synthesis by adding cycloheximide to wild type cells and then treated with diamide or shifted to 38 °C for 30 min (Fig. 5, *C* and *D*). We could observe that in these conditions Slt2 did not become activated in response to diamide treatment, although it does as a consequence of the heat shock. We conclude from these observations that the activation of the cell integrity pathway caused by diamide requires protein synthesis.

Diamide Treatment Induces the Localization of Pkc1 to the Cell Periphery and Provokes an Increment in Cell Wall Thickness—It has been demonstrated that, in exponentially growing cells, Pkc1 localizes to sites of polarized growth at pre-bud

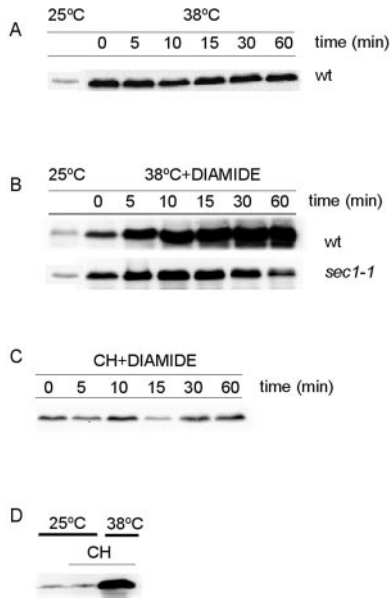

FIG. 5. **The slow rate of Slt2 activation upon diamide treatment requires an intact secretory machinery and protein synthesis.** *A*, wild type cells growing exponentially at 25 °C in SC culture medium were shifted to 38 °C for 3 h, after that samples were collected at the indicated times for protein extraction and Western blot analysis was performed using the p44/42 antibody to detect the Slt2-activated form. *B*, wild type and *sec1–1* cells were grown at 25 °C, then shifted to 38 °C for 3 h to be subsequently treated with 4 mM diamide for the indicated times. *C*, wild type cells were added to cycloheximide 100 mM (*CH*) for 30 min to block protein synthesis. Then 4 mM diamide was added, and samples were collected for the indicated times. *D*, wild type cells were grown to logarithmic phase (first line) to be subsequently treated with cycloheximide as in *C*. These cultures were shifted to 38 °C in the presence of CH. All the samples taken from *A* to *D* were processed for protein extraction and Western blot to detect the phosphorylated form of Slt2. All the *lanes* were evenly loaded upon detection of total Slt2 (not shown).

sites, bud tips and around mother-daughter bud-neck prior cytokines (33). However, when the cell wall is damaged, Pkc1 localizes to specific regions beneath the cell surface, apparently to target proteins involved in repairing the damage. We considered that studying Pkc1 localization could contribute toward a better understanding of the cytological effects of oxidative stress. For this reason, we tagged Pkc1 with GFP in its C-terminal domain and treated cells with either hydrogen peroxide or diamide. We observed that, during a 30-min treatment with diamide, Pkc1 rapidly delocalized through the cell and that after 30–60 min of treatment, Pkc1 delocalized to the cell periphery. Up to 5 h later, Pkc1 then relocated to the cell neck. This relocation was concomitant with renewed cell growth (Fig. 6). Electron microscopy analysis revealed that cells treated with diamide had significantly thicker cell walls (Fig. 7). When Slt2 activity was tested, we observed that this progressively increased and accumulated with treatment time, which was consistent with the mechanism of cell wall damage signaling and the induction of a compensatory mechanism (not shown). These results are consistent with the previous ones and fit into a model in which diamide altered the cell wall, and therefore Pkc1 rapidly localized to the cell periphery to repair or target the reparation of oxidative damage caused in the cell outer layer. Simultaneously, and probably as a consequence of a compensatory mechanism, Slt2 activity increased and the cell wall became thicker (Fig. 7).

Diamide and Hydrogen Peroxide Activation of the Cell Integrity Pathway Both Provoke Transient Actin Cytoskeleton Depolarization—It is well known that different types of stress, such as heat shock (34, 35), osmotic shock (36), and cell wall stress in

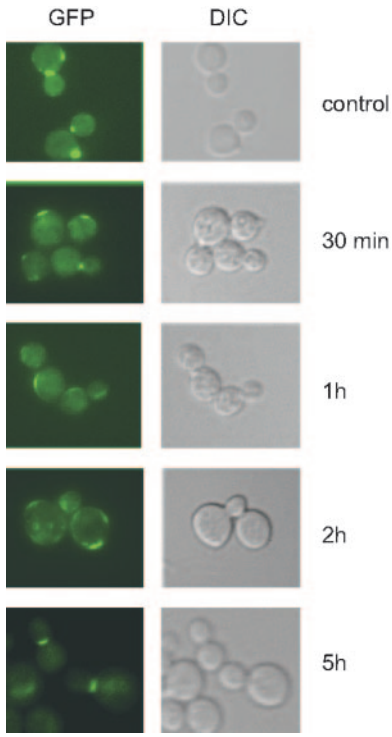

FIG. 6. **Pkc1 cellular localization upon diamide treatment.** Wild type cells in which Pkc1 was tagged with GFP epitope were grown to logarithmic phase (*control*) and treated with diamide 4 mM for the indicated times.

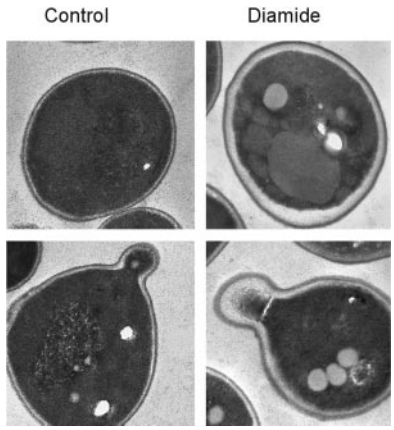

FIG. 7. **Ultrastructural modifications in the cell outer layer caused by diamide.** Cells were exponentially grown in SD medium at 25 °C in the presence, or absence, of 1 mM diamide for 10 h and subsequently processed for electron microscopy. Images have been enlarged 20,000 times with respect to the real size.

general (3, 12), induce a transient depolarization of the actin cytoskeleton. Because both diamide and hydrogen peroxide provoke another type of environmental stress in cells, oxidative stress, we decided to investigate their potential effect on the organization of the actin cytoskeleton. We observed that both hydrogen peroxide and diamide induced a transient depolarization of actin (visualized as randomly distributed actin patches, Fig. 8) followed by a subsequent repolarization after 3 h of treatment. This effect was dosage-dependent, because increasing the doses of diamide or peroxide caused a non-reversible depolarization of actin and consequently cell death (data not shown).

We studied the kinetics of acting polarization in *mtl1*, *pkc1*,[2] and *rom2* mutants after both diamide and hydrogen peroxide treatments to determine whether the elements of the cell integrity pathway involved in cell survival upon oxidative stress also played a role in the mechanism driving actin polarization. After the two treatments, actin was visualized in depolarized patches in all the mutants tested except in *mtl1* cells. In the case of the *mtl1* mutant, the majority of the actin cables escaped depolarization after the addition of diamide. This supports the putative function of Mtl1 as a cell wall sensor for diamide damage. Interestingly, the absence of Rom2 and Pkc1 (not shown) provoked a marked descent in the time with respect to actin repolarization from patches to cables, compared with wild type cells, concomitantly with a notable descent in viability (Figs. 8*A* and 3, *A–E*). These results indicate that Mtl1, Rom2, and Pkc1 proteins are needed to repolarize and restore the actin cytoskeleton in response to oxidative stress.

Pkc1 Overexpression Induces a Rapid Restoration of Actin Cables Upon Oxidative Stress Caused by H_2O_2 but Not When It Is Caused by Diamide—To determine whether the Pkc1 function is directly involved in actin repolarization and filament formation, we overproduced Pkc1 under the control of the $tetO_7$ promoter and chased protein expression by Western blot. We observed that, under conditions of Pkc1 overproduction, hydrogen peroxide also induced actin depolarization (Fig. 8*B*) during the first hour of treatment. However, actin repolarization occurred earlier and more efficiently. After 1 h of treatment, the percentage of depolarized budded cells was significantly lower in cells overexpressing Pkc1 than in wild type cells. Interestingly, after 4–6 h of treatment, actin cables were clearly observed not only in mother cells, but also in cell buds (Fig. 8*B*). This suggests that, upon oxidative treatment and consequent actin depolarization, Pkc1 is required to induce actin cable formation and restore normal cell polarity and growth. However, this mechanism seems to be specifically induced by the oxidative stress caused by peroxide, because Pkc1 overexpression did not restore actin cable formation upon treatment with diamide (not shown).

DISCUSSION

Here we report that the activity of Pkc1 and the upstream elements of the cell integrity pathway (Rom2 and Rho1) are required for cell survival following oxidative treatment, whereas the elements integrating the MAPK module are dispensable. This suggests that a specific function shared by the upstream Pkc1 elements is essential for the oxidative stress response.

Two different oxidizing agents were used: diamide and hydrogen peroxide. The two agents affect different targets in the cell and induce different responses. Both have two important properties in common with respect to the cellular responses they induce: Pkc1 function is essential for cells to overcome cellular damage, and the organization of their respective actin cytoskeletons is the main cellular function affected by oxidative stress, although, as described below, this is the result of different mechanisms.

We also describe a new function for the protein Mtl1, based on several lines of evidence: (i) after diamide treatment only a very small percentage of *mtl1* cells present actin depolarization in comparison with wild type cells; (ii) the induction of Slt2 phosphorylation is severely impaired in the mutant in comparison with wild type cells; (iii) *mtl1* mutant cells are sensitive to diamide; and (iv) *mtl1* cell viability is rescued by increasing the level and activity of the Pkc1 protein. In this work, Mtl1 is therefore characterized as a cell membrane sensor of oxidative stress in the cell integrity pathway upstream of Pkc1. The fact that the actin cytoskeleton becomes depolarized in the absence of Mtl1 (although only in a very small percentage of cells in the

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

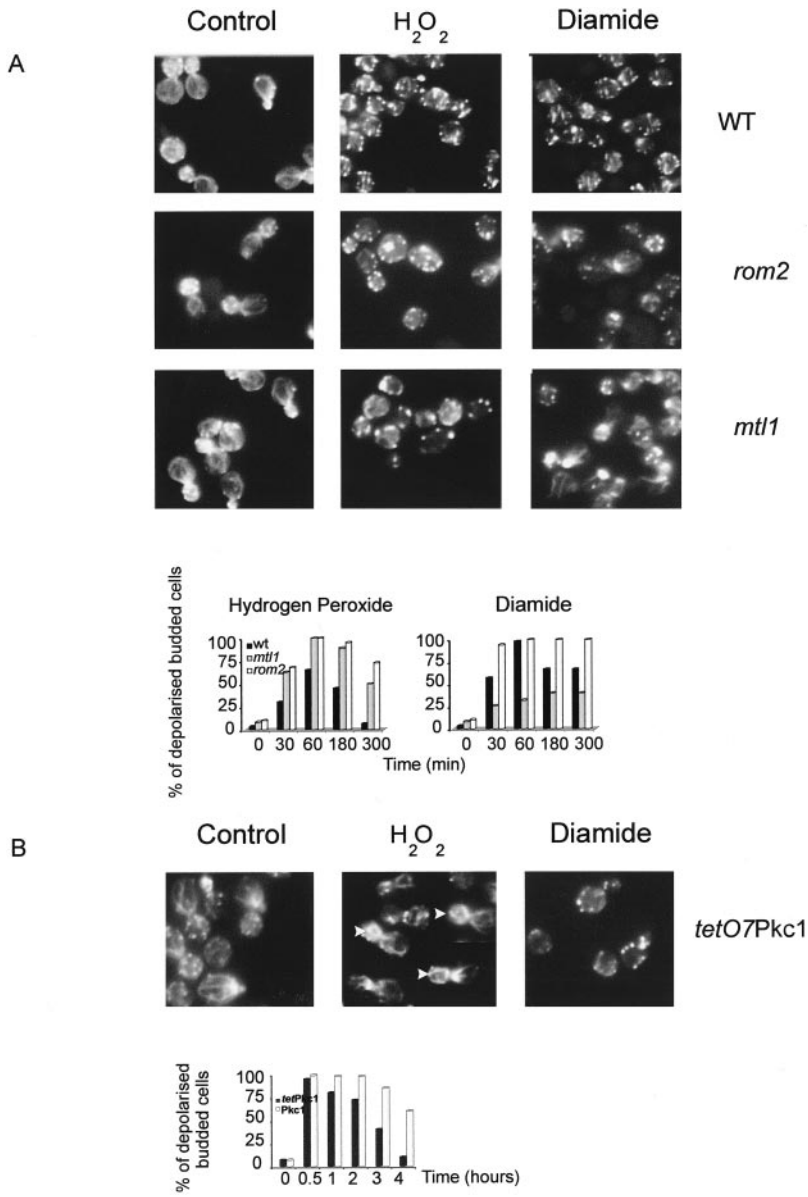

FIG. 8. **Actin cytoskeleton organization in different cell integrity mutants upon oxidative treatment.** *A*, actin staining of samples taken from wild type, *rom2*, and *mtl1* cells after 1 h of treatment with either hydrogen peroxide or diamide. Histograms represent the percentage of depolarized budded cells counted during the indicated periods of incubation for each of the oxidizing agents. *B*, actin staining of wild type cells overexpressing Pkc1, after 1 h of treatment with either hydrogen peroxide or diamide. Histograms are as in *A*. The *intensity* of images, representing filament stains in buds and mother cells in cells overexpressing Pkc1 upon hydrogen peroxide treatment, was markedly more intense than the rest of the images obtained in this study. Therefore, to optimize the quality of the figures, their intensity has been reduced. This is clearly discernible by comparing controls using the naked eye.

culture, see Fig. 8), and the fact that a slight induction of Slt2 phosphorylation is still detectable upon diamide treatment, could be explained by the functional redundancy of other cell wall receptors (such as Mid2) that could possibly replace the Mtl1 function.

Some authors (37) have recently reported that oxidative stress caused by linoleic acid hydroperoxide induces a short, fast induction of Mpk1/Slt2 activity. This differs from our results in which the response to hydrogen peroxide and diamide occurred later and was associated with a recovery process. The authors propose that Mpk1 is required for cell viability upon linoleic acid hydroperoxide treatment, although in our study, Pkc1 and Rho1 functions determined cell viability upon peroxide and diamide treatment. It is possible that different oxidizing agents operate on different targets, inducing specific cellular responses involving the cell integrity pathway. Linoleic acid hydroperoxide is likely to react with membrane components, whereas hydrogen peroxide is highly diffusible and could enter cells passively and act on internal targets. On the other hand, diamide is a potential oxidant of thiol groups.

The results presented in this work indicate that diamide treatment provokes a significant increase in resistance to zymolyase digestion in *pkc1* and *slt2* mutant cells due to it having a direct effect on the cell wall, where it is probably responsible for a reduction in cell porosity, a consequence of the oxidation of sulfhydryl groups in cell surface proteins (29). Therefore, structural changes in the cell wall, caused by a reduction in the number of free sulfhydryls, affect cell integrity in a way that makes receptor proteins (such as Mtl1 and Mid2) sense these changes and transduce the signal to the elements of the PKC1-MAPk pathway, which, in turn, activates the Slt2 kinase. This mechanism resembles that widely described for cell wall damage. We have observed that protein synthesis is not needed to activate Slt2 in response to cell surface stress induced by heat-shock. However, in the absence of protein synthesis Slt2 did not became phosphorylated as a consequence of diamide treatment. This suggests that the activation of the cell integrity pathway mediated by diamide could be reflecting an adaptive process more than an immediate response driven by an MAPK pathway in response to an external stimulus. We reasoned that, if diamide were to continuously damage the cell wall, even in the absence of secretion, we would expect Slt2 activation to gradually increase with exposure to diamide. The slow rate of Slt2 phosphorylation can also be explained by the fact that new cell surface protein components are continuously synthesized and excreted *de novo* in a cell system whose cell wall is continuously

remodeled. At the time these proteins are secreted to the cell periphery, they could be susceptible to being oxidized by diamide, present in the culture medium during the course of the experiment. This could provoke an accumulative cell wall modification (presumably oxidation of thiol residues present in cell surface proteins) that activates the signaling cascade mediated by the cell integrity pathway.

The observation that, upon blockage of secretion and subsequent treatment with diamide, Slt2 becomes rapidly and transiently phosphorylated is also consistent with the second model. Furthermore, the observation that diamide, in the experimental conditions we use, does not affect the secretory machinery constitutes another novel finding of this study.

The localization of Pkc1 to the cell periphery upon diamide addition could be attributable to a response to cell wall damage, as previously reported (33), although we cannot rule out the possibility of diamide provoking cell wall damage. In fact, the walls of cells treated with diamide become more resistant to enzymatic digestion and significantly thicker than those of untreated cells. More specifically, diamide is responsible for an increase in the amount of glucane (Fig. 7, *light layer* around the plasmatic membrane), which makes the cell walls thicker. Consequently, the final effect observed upon diamide treatment could be the result of the activation of the compensatory mechanism upon cell wall damage.

Pkc1 localization to the cell periphery is dependent on Rho1 activity (33). We therefore inferred that the Pkc1 localization to the cell periphery that takes place upon diamide treatment must also depend on Rho1 activity and consequently on the cell integrity pathway. This means that the Pkc1 function is required at the cell surface upon diamide treatment. Activation of Rho1 and Pkc1 is known to cause depolarization of the actin cytoskeleton (12). In our system, the actin depolarization that we observed upon oxidative stress was not directly due to induction of the activity of the previously mentioned protein members of the cell integrity pathway, because the same actin cytoskeleton disorganization effect was observed in the absence of their function. Interestingly, it has been reported that a point mutation in the Rho1 protein might also provoke depolarization of the actin cytoskeleton into patches (16). We cannot discard the possibility of oxidative stress damaging one of the proteins studied in this report (Rho1 or Pkc1) and this damage producing changes in actin organization: this is one hypothesis currently being studied by our laboratory.

One conclusion that we draw from this study is that diamide (and also hydrogen peroxide, as discussed below) provokes a clear depolarization of the actin cytoskeleton, which is independent of the activity of the protein components of the upper part of the cell integrity pathway. However, even more relevant is the observation that Mtl1, Rho1, and Pkc1 are required to overcome and survive this oxidative stress. In summary, we believe that constant exposure to diamide causes increasingly greater structural changes to the cell wall, which in turn activate the pathway. This is reflected in Slt2 phosphorylation (which is dosage-dependent and increases with exposure to the oxidizing agent), depolarization of the actin cytoskeleton, Pkc1 cell surface localization, and increases in cell wall thickness, in samples treated with diamide.

The mechanism by which hydrogen peroxide oxidizes cellular elements and induces a response from the cell integrity pathway is different to that described for diamide. According to our results, hydrogen peroxide does not cause clear, major damage to the cell wall. Hydrogen peroxide treatment did not render clear differences between wild type cells and each of the *mtl1*, *rom2*, and *slt2* mutants with respect to the expression of genes regulated by Msn2/4, Yap1, or Skn7 (not shown). The cellular function most severely impaired by this oxidizing agent is the organization of the actin cytoskeleton, which implies that hydrogen peroxide mainly operates at an intracellular level. Pkc1 activity is required to overcome the effect of oxidative stress caused by hydrogen peroxide. Actin depolarization mediated by hydrogen peroxide occurs in the absence of Rom2 and Mtl1. However, Rom2, Pkc1, and Mtl1 play an essential role in repolarizing the actin cytoskeleton, and adapting to and recovering from peroxide treatment. Overproduction of Pkc1 significantly rescues cell viability in response to hydrogen peroxide treatment. This is more efficient than in response to diamide, which again points to different cellular targets and repair mechanisms associated with the different oxidizing agents. The observation, that high levels of Pkc1 protein activate the repolarization of actin and induce the formation of actin cables upon hydrogen peroxide treatment, suggests a further activation of the proteins involved in this process. We speculate that candidates could be formins and consequently prophylin (13, 14). However, this hypothesis is currently under study in our group. We are currently developing experimental designs to further characterize a model for oxidative stress caused by hydrogen peroxide. One possible explanation for why overexpression of Pkc1 does not visibly induce actin cable repolarization upon diamide treatment would be that the signal that triggers depolarization of the actin cytoskeleton is more intense than the one mediated by Rho1-Pkc1-formins-profylin to restore actin cable formation.

In the course of submission of this report, another relevant report was published (38). The authors make a series of observations coincidental with ours; they observed that, upon hydrogen peroxide treatment, Slt2 becomes phosphorylated and that transcription dependent of Rlm1 is induced. However, they also report several conclusions that are not in accordance with ours. They observed an early and transient Slt2 phosphorylation upon treatment with 5 mM hydrogen peroxide. In our study we tested several concentrations of this agent and different times of exposure to the oxidizing agent (as described previously under "Results"), including that of 5 mM. We reproduced these experiments in two independent backgrounds. We could not observe a significant Slt2 activation during the first 30 min of treatment with hydrogen peroxide in any of the conditions tested. Another result shown by Ref. 38 is that a *slt2* mutant is sensitive to hydrogen peroxide. Upon testing cell viability in response to hydrogen peroxide treatment in *slt2* mutant cells (we also performed this experiment in two independent backgrounds and obtained identical results), we could not observe any detrimental effect as compared with wild type cells (data not shown). This is not in accordance with the results published in Ref. 38. However, the fact that we performed the experiments in exponentially growing cells, whereas these authors used saturated cultures, could contribute to the dissimilarities observed between the results of both studies. Finally, we would like to note that, in a previously published article (3), we reported that the absence of *SLT2* seriously affects cell viability when cells enter the stationary phase.

Genome-wide analyses indicated that diamide treatment induced the activation of a number of genes whose expression depends on Rlm1 (22). This induction reflects activation of the pathway, again supporting the model by which diamide mainly affects the integrity of the cell outer layer. The fact that Rom2, Rho1, and Pkc1 functions are all essential for overcoming the effects of diamide upon cell viability, whereas the downstream elements of the pathway are not, suggests that they all share a common cell integrity function required for this response. Gene expression (reviewed by Ref. 18) and "*de novo*" protein synthesis (39) are required for adaptation to oxidative stress. It is well

characterized that Skn7, Yap1, and Msn2/Msn4 transcriptional factors are involved in the oxidative stress response (18). However, their upstream elements and the factors responsible for transmitting the oxidative signal to them and thereby activating them, have yet to be identified. It has been recently demonstrated that the Ras-cAMP pathway negatively affects the hydrogen peroxide stress response through Msn2/4 (40). Interestingly, genome-wide studies published previously (21) also show a functional connection between Msn2/4 and the PKC1-MAPK module, because both seem to contribute to the cell wall compensatory mechanism. Our study represents another contribution that helps to explain the complex signaling processes, which take place upon oxidative damage, and gives added relevance to the role of the cell integrity pathway in cellular responses to oxidants.

Studies involving human cells describe the role of certain PKC isoforms in the protection of epidermal intestinal cells against oxidative stress by stabilizing F-actin (41). F-actin turnover is required for the correct maturation of macrophages and for the activation of PKCα (42). Thus, PKCα inhibition is related to the establishment of the infection caused by *Leishmania donovani* through the inhibition of macrophage maturation (43).

PKC involvement in actin organization may therefore be related to defense against infections. All these studies, including the present one, underlay the biological relevance of Pkc1 involvement in actin organization upon oxidative stress.

Further studies will be required to ascertain which molecules are most essential in the response to oxidative stress, what the targets for the different oxidative agents are, and what molecular mechanism is responsible for connecting proteins in the Pkc1 pathway with the actin cytoskeleton in response to particular oxidative stresses.

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