Regulatory Mechanisms for Modulation of Signaling through the Cell Integrity Slt2-mediated Pathway in *Saccharomyces cerevisiae* 

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Signal transduction mediated by the mitogen-activated protein kinase (MAPK) Slt2 pathway is essential to maintain the cell wall integrity in *Saccharomyces cerevisiae*. Stimulation of MAPK pathways results in activation by phosphorylation of conserved threonine and tyrosine residues of MAPKs. We have used an antibody that specifically recognizes dual phosphorylated Slt2 to gain insight into the activation and modulation of signaling through the cell integrity pathway. We show that caffeine and vanadate activate this pathway in the absence of osmotic stabilization. The lack of the putative cell surface sensor Mid2 prevents vanadate- but not caffeine-induced Slt2 phosphorylation. Disruption of the Rho1-GTPase-activating protein genes *SAC7* and *BEM2* leads to constitutive Slt2 activation, indicating their involvement as negative regulators of the pathway. MAPK kinases also seem to participate in signaling regulation, Mkk1 playing a greater role than Mkk2 in signal transmission to Slt2. Additionally, one of the phosphatases involved in Slt2 dephosphorylation is likely to be the dual specificity phosphatase Msg5, since overexpression of *MSG5* in a *sac7Δ* mutant eliminates the high Slt2 phosphorylation, and disruption of *MSG5* in wild-type cells results in increased phospho-Slt2 levels. These data present the first evidence for a negative regulation of the cell integrity pathway.

The PKC1-mediated MAPK¹ pathway is one of the signal transduction pathways that operate in *Saccharomyces cerevisiae* to control yeast cell biology. This signaling pathway is essential for the maintenance of cell integrity in a variety of environmental conditions and during morphogenetic events, through the regulation of cell wall and actin cytoskeleton dynamics (1, 2). The so-called cell integrity pathway is induced in periods of polarized growth during budding and mating (3) and in response to environmental conditions that jeopardize cell wall stability, such as high temperature (4), hypotonic shock (5), or interference with wall synthesis (6). Accordingly, lack of functionality of the pathway leads to cell lysis when yeast cells are exposed to these inducing conditions. This lysis defect can be prevented by osmotic stabilization, indicating a failure in the maintenance of a functional cell wall structure (7, 8). Recently, two different types of putative cell surface sensors for cell integrity signaling have been described. Hes77/Slg1/Wsc1 belongs to the family of four transmembrane proteins encoded by *WSC* genes, and their involvement in heat shock activation of the Pkc1-mediated pathway has been reported (9–11). Mid2 has been described as another putative sensor for this pathway in response to pheromone, high temperature, and cell wall disturbances (6, 12). Mll1 seems to have a partially redundant function with its homologue Mid2 (6, 12).

Although the molecular mechanisms by which these sensors transmit the signal to downstream components are unknown, the Rho1-GDP/GTP exchange factor Rom2 has been reported to mediate the activation of Rho1 by cell wall alterations (13). Rho1 is a small GTPase that functions as a binary switch between two interconvertible GTP-bound active and GDP-bound inactive forms. The switch is up-regulated by the GDP/GTP exchange factors Rom1 and Rom2 (14) and down-regulated by the GTPase-activating proteins (GAPs) Sac7 and Bem2 (15, 16). Among other functions, Rho1 is known to bind and activate Pck1 (17, 18). This protein kinase in turn activates a MAPK cascade that is composed of the MAPKK kinase Bck1/Slk1 (19, 20), the redundant MAPKKs Mkk1 and Mkk2 (21), and the MAPK Slt2/Mpk1 (7, 22). Signaling is transmitted through this protein kinase cascade by sequential phosphorylation. MAPKs are dual specificity protein kinases that catalyze the phosphorylation of the MAPK on both tyrosine and threonine conserved residues in subdomain VIII, leading to the activation of this last element of the cascade. Transcription of a number of genes involved in cell wall biosynthesis has been shown to be dependent on this pathway (23, 24), indicating the importance of a functional pathway to ensure cell integrity.

Hyperactivation of MAPK pathways is known to lead to severe growth defects. Therefore, activation of MAPKs needs to be tightly controlled by mechanisms that inhibit the activity of the pathways when there is no activating input and that rapidly down-regulate this activity following stimulation. Protein phosphatases that negatively regulate the HOG and the mating pathways have been characterized. The tyrosine phosphatases Ptp2 and Ptp3 operate in both pathways, playing a role in maintaining a low basal level of tyrosine-phosphorylated Hog1 and Fus3 and in the adaptation to osmotic shock and pheromone treatment (25–27). An additional phosphatase has been shown to act in the mating pathway, the dual specificity phosphatase Msg5, but it only participates in adaptation by inactivating Fus3 following pheromone stimulation (27, 28). *MSG5* has also been identified as a multicopy suppressor of a hyperactive *MKK1* allele (29). Additionally, a genetic interaction of the type 2C serine threonine phosphatase gene *PTC1*...
with the PKC1 pathway has been reported (30). However, mechanisms responsible for the negative modulation of Slt2 remain largely unknown.

In this study, we have used an anti-active MAPK antibody raised against the dually phosphorylated region (Thr202/Tyr204) within the catalytic core of p44/42 MAPKs to specifically detect the active form of the MAPK Slt2. Using this “read-out” for signaling through the cell integrity pathway, we have identified caffeine and vanadate as novel compounds that lead to Slt2 activation and have studied how cells sense these stimuli. We report that Sac7 and Bem2 are negative regulators of this pathway. Additionally, we found that Mkk1 plays a more important role than Mkk2 in signaling to Slt2. Finally, evidence for a role of Msg5 in Slt2 dephosphorylation is also presented. Described by Soler and Wileman (32).

**TABLE I**

| Strain      | Relevant genotype                                                                 | Source or reference                     |
|-------------|------------------------------------------------------------------------------------|-----------------------------------------|
| BJ5464      | MATa, ura3–52, leu2Δ1, trp1, his3Δ200 pep4::HIS2, pbr1Δ1.6R, can1                  | Yeast Genetic Stock Center              |
| BJ5464-DK   | MATa, ura3–52, leu2Δ1, trp1, his3Δ200 pep4::HIS2, pbr1Δ1.6R, can1, slt2Δ::URA3   | This work                               |
| TD28        | MATa, ura3–52, inos1–151, can1                                                    | Ref. 7                                  |
| TD28-F54    | MATa, ura3–52, inos1–151, can1, slt2-F54                                         | Ref. 34                                 |
| 1753        | MATa, ura3–52, his4, trp1–4, trp1–1, leu2–3,112, can8                             | Ref. 22                                 |
| DL454       | MATa, ura3–52, his4, trp1–1, leu2–3,112, can8                                      | Ref. 22                                 |
| DL43        | MATa, slt2D35, trp1–1, leu2–3,112, ura3–52                                       | Ref. 27                                 |
| OHNY1       | MATa, ura3, his3, trp1, leu2, ade2                                                | Ref. 17                                 |
| HNY21       | MATa, ura3, his3, trp1, leu2, ade2, ade2, rho1–104                                | Ref. 17                                 |
| Y806        | MATa, ura3, leu2, ade2, ade3, lys2, p0                                            | Dr. Alan Bender                         |
| Y807        | MATa, ura3, leu2, ade2, ade3, lys2, p0, benz2::URA3                               | Dr. Alan Bender                         |
| JVG987      | MATa, ura3–52, his3Δ200, leu2Δ1, ade2–101, lys2–801, barΔ1::LEU2                  | J. V. Gray                              |
| JVG1079     | MATa, ura3–52, his3Δ200, leu2Δ1–1, ade2–101, lys2–801, his7Δ7::LEU2               | J. V. Gray                              |
| DDI–2B      | MATa, ura3, his2, trp1, leu2, ade1                                               | Ref. 28                                 |
| DDI–2D      | MATa, ura3, his2, trp1, leu2, ade1, msg5–1::LEU2                                  | Ref. 28                                 |
| TB50α       | MATα, ura3, his3, trp1, leu2                                                    | Dr. Michael N. Hall                     |
| AS171–3a    | MATα, sou7–kanMX                                                                  | Dr. Michael N. Hall                     |
| AS169–2α    | MATα, bag7::HIS3                                                                  | Dr. Michael N. Hall                     |
| 3233–1C     | MATα, ura3, his3, trp1, leu2                                                     | Ref. 21                                 |
| 3233–1D     | MATα, ura3, his3, trp1, leu2, mkk2Δ::HIS3                                         | Ref. 21                                 |
| 3233–1B     | MATα, ura3, his3, trp1, leu2, mkk2Δ::HIS3                                         | Ref. 21                                 |
| YLG3        | MATα, ura3, his3, trp1, leu2, ade8, can1, pde1::LEU2, pde2::URA3                   | Dr. Steven Oliver                       |
| W303–1A     | MATa ade2–1 can1–100 trp1–1 ura3–1 his3–11,15 leu2–2,112                          | Ref. 39                                 |
| W303–1Ams1(mid2)Δ | MATa ade2–1 can1–100 trp1–1 ura3–1 his3–11,15 leu2–2,112 ssa1::URA3              | Ref. 39                                 |

**Culture Conditions**—YEPD (1% yeast extract, 2% peptone, and 2% glucose) broth or agar was the complete medium used for growing yeast strains. YEPG was YEPD with 2% galactose instead of glucose. Synthetic minimal medium (SD) contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose and was supplemented with appropriate amino acids and nucleic acid bases (31). SG and SR were SD with 2% galactose or raffinose, respectively, instead of glucose. Where indicated, n-sorbitol was added to the media to a final concentration of 1% and 2% caffeine and sodium orthovanadate (Sigma) were dissolved in sterile water just before use and added to the medium to the desired concentration. Galactose induction experiments in liquid were performed by growing cells in SR minimal selective medium to log phase and then by adding galactose to 2% for 8 h.

**Preparation of Yeast Extracts and Immunoblot Analysis**—Yeast cells were grown overnight to mid-log phase in the appropriate medium. The cultures were then diluted to an OD600 of 0.2 and grown for 1 generation prior to collection, shifting to 39 °C, or treatment with the desired compound for the duration of the experiment. Cells were collected on ice by adding 20 ml of the culture to an equal volume of ice in a Falcon centrifuge tube and pelleted in a refrigerated centrifuge. Cells were then transferred with 1 ml of ice-cold water to an Eppendorf tube, pelleted, and immediately broken or frozen in dry ice. Cells were lysed in 120 ml of cold lysis buffer (50 mM Tris-Cl (pH 7.5), 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and the protease inhibitors tosylphenylalanine chloromethyl ketone, tosyllysine chloromethyl ketone, leupeptin, pepstatin A, antipain, and aprotinin each at 25 μg/ml) by vigorous shaking with 0.45-mm glass beads in a fast prep cell breaker (Bio 101; level of 5.5 for 20 s). Cell extracts were separated from glass beads and cell debris, collected in a new Eppendorf tube by centrifugation, and further clarified by a 13,000 × g spin for 15 min at 4 °C. The protein concentration of the supernatants was measured at 280 nm and normalized with lysis buffer. Then 2× SDS-polyacrylamide gel electrophoresis sample loading buffer was added, and samples were boiled for 5 min. Protein samples (50 μg) were fractionated by SDS-polyacrylamide gel electrophoresis using 8% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond; Amersham Pharmacia Biotech). Membranes were probed with either anti-
phospho-p44/42 MAPK (Thr202/Tyr204) antibody (New England Biolabs) as a tool to easily monitor the phospho-Slt2 signal in Western blots. The substitution of amino acid 398 of Pkc1, located in the pseudosubstrate site, leads to the incapacitation of this site and thereby to the constitutive activation of the kinase (17, 41). We used the plasmid pEX-PKC1ΔA398A405A406, bearing a dominant constitutively activated allele of PKC1 in which the arginine 398 and the basic conserved residues of the pseudosubstrate site arginine 405 and lysine 406 have been changed to alanine (PKC1ΔA398A405A406), placed under the control of the strong GAL1 promoter in the multicopy vector Yeplac112.2 As shown in Fig. 1, induction of PKC1ΔA398A405A406 expression in the strain BJ5464 led to a dramatic increase in the phospho-Slt2-dependent signal in Western blots. These results confirmed the effectiveness of the assay to monitor the level of activation of the pathway and indicated that only a fraction of cellular Slt2 is phosphorylated under mild heat shock treatment.

The presence of 1 m sorbitol in the medium did not reduce the level of Slt2 phosphorylation in cells overexpressing the hyperactive PKC1 allele (Fig. 1). This is consistent with the currently accepted model in which the osmotic stabilization of the medium prevents Slt2 activation by physical stabilization of the cell surface. In this case, activation of the pathway would not be triggered from the cell surface but as a consequence of the constitutive activation of an upstream element of the MAPK cascade.

In accordance with a previous work using other hyperactive PKC1 alleles (41), overproduction of Pkc1ΔA398A405A406 completely inhibited the growth of wild type strains. However, the deleterious effect on growth does not seem to be only a consequence of the strong Slt2 activation, since overexpression of this PKC1 allele in a \( slt2 \Delta \) mutant also led to cell lethality (data not shown). This result provides additional evidence that Pkc1 acts through a bifurcated pathway.

Caffeine and Vanadate Activate the Slt2 Pathway—We exploited the anti-phospho-p44/42 MAPK antibody in an attempt to determine conditions that lead to Slt2 activation. Because sensitivity to caffeine and vanadate is a characteristic phenotype in mutants affected in the Pkc1 pathway (20, 42, 43), it indicated that only a fraction of cellular Slt2 is phosphorylated under mild heat shock treatment.

The presence of 1 m sorbitol in the medium abolished Slt2 activation in response to both compounds as happened under thermal stress (Fig. 2B). Kamada et al. (4) have shown that prevention of heat-induced Slt2 activation by osmotic stabilization was not a consequence of activating the HOG pathway. Therefore, we determined the level of phospho-Slt2 in the \( hog1 \) mutant JBY10 under caffeine and vanadate exposure. Fig. 2C shows that in this mutant the induced Slt2 phosphorylation was also inhibited by 1 m sorbitol, indicating that high osmolarity prevents the pathway activation in response to both agents by mechanisms independent of Hog1. These data suggest that all of these stimuli trigger the activation of the cell

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integrity pathway by a common mechanism that can be inhibited by osmotic stabilization.

Role of Cell Surface Sensors on Vanadate and Caffeine Activation of the Cell Integrity Pathway—The ability of sorbitol to rescue the sensitivity of mutants affected in the cell integrity pathway to caffeine (42) and vanadate (data not shown) and to prevent Slt2 phosphorylation induced by these agents suggests that these compounds could be perturbing cell wall stability. In this case, activation of the pathway in response to these stimuli would be mediated by the previously identified cell surface sensors. Mid2 has been shown to be required for induction of Slt2 tyrosine phosphorylation during exposure to high temperature, mating pheromones, and Calcofluor White (6). Calcofluor White interferes with cell wall assembly; therefore, a role for this protein as a cell wall stress sensor has been suggested (6). Hcs77/Wsc1/Slg1 has only been implicated in sensing thermal stress (9, 10). Cells of wild type and isogenic hcs77 and mid2 strains were exposed to high temperature, caffeine, and vanadate, and the increase in the level of phospho-Slt2 was determined. Fig. 3A shows that the absence of Hcs77 only results in a weak reduction of heat shock-induced Slt2 phosphorylation. However, we found that Slt2 phosphorylation was severely impaired in mid2 cells in response to vanadate as well as under thermal stress. This, therefore, indicates a role for Mid2 in sensing the vanadate insult and suggests an effect of this compound on the cell surface. In contrast, no significant reduction of caffeine-induced Slt2 activation was detected in any of these putative cell wall integrity sensor mutants.

The SNO2 gene was cloned in a screening for genes that when overexpressed were able to complement the caffeine sen-
FIG. 4. Involvement of the Rho1 switch in transmission of the signal to the Slt2 MAPK cascade. A, inactivation of Rho1 results in the block of induced Slt2 phosphorylation. Exponential cultures of the mutant HNY21 (rho1–104) and the isogenic wild type OHNY1 strains (WT) growing in YEPD at 24 °C were shifted to 39 °C, and after 10 min of incubation, cells were further incubated at this temperature in the absence of any chemical or exposed to 12 mM caffeine (Caf) or 5 mM sodium orthovanadate (Van) for an additional 45 min. B, Sac7 and Bem2 down-regulate the cell integrity pathway. Exponential cultures of the AS171-3a (sac7Δ), AS169-2a (bem2Δ) and the isogenic wild type TB50a, and Y807 (bem2Δ) and the isogenic wild type Y806 strains were grown in YEPD to mid-log phase at 24 °C (top row) or at 30 °C (bottom row). Where indicated, 1 M sorbitol was present in the medium. Cells were collected, protein extracts were prepared, and anti-phospho-Slt2 immunoblot analysis was performed as in Fig. 1. Anti-Slt2 immunoblot analysis of similar samples was performed to verify that a similar amount of Slt2 was present in each lane (not shown).

sitivity of an slt2–D35 mutant.3 SNQ2 encodes for a multidrug ATP-binding cassette transporter of the yeast plasma membrane (44, 45). Overexpression of SNQ2 was able to reverse the caffeine but not the vanadate hypersensitivity of a slt2Δ strain (Fig. 3B). Therefore, assuming that it might be due to Snq2 pumping caffeine out of the cell, this result suggests that this compound is not directly acting on the cell wall.

Since caffeine has been shown to inhibit cAMP phosphodiesterases in eukaryotic cells (46), the possibility of this chemical activating the Slt2 pathway as a result of the inhibition of Pde1 and Pde2, the two phosphodiesterases involved in the yeast cAMP-dependent protein kinase A (PKA) pathway (47), was examined. If caffeine is activating the Pkc1-mediated pathway via this inhibitory mechanism, a lack of these enzymes should lead to increased levels of phospho-Slt2 in the absence of caffeine. Therefore, we assayed a pde1Δ pde2Δ strain in the presence and absence of caffeine. The mutant strain displayed a wild type behavior consisting of a low level of phospho-Slt2 when growing at 24 °C without the drug and an increased Slt2 phosphorylation after treatment with caffeine or shifting to 39 °C (Fig. 3C). These results indicate that phosphodiesterases are not involved in the effect of caffeine on activation of the cell integrity pathway.

The GAPs Sac7 and Bem2 Negatively Modulate Signaling through the Cell Integrity Pathway.—We sought to examine the role of the Rho1 GTPase switch in the transduction of the vanadate and caffeine-induced signaling to the MAPK cascade compared with that induced by heat shock and cell wall perturbation. It has been shown that Rho1 binds and activates Pkc1, and therefore,rho1 mutants are impaired in the Slt2 kinase activity when exposed to thermal stress (17, 18). Consistent with this data, our studies revealed that heat shock–induced Slt2 phosphorylation was blocked in a rho1–104 strain (Fig. 4A). Moreover, the level of Slt2 phosphorylation at 39 °C was even lower than the basal level at 24 °C, reflecting the lack of functionality of Rho1–104 at the nonpermissive temperature. Mutant cells did not show any induction of Slt2 phosphorylation after 10 min of incubation at 39 °C (data not shown), indicating a rapid loss of function of the mutant protein. We exploited this feature of Rho1–104 to analyze the effect of caffeine and vanadate on Slt2 activation in cells lacking a functional Rho1. rho1–104 mutant and isogenic wild type cells were grown at 24 °C, shifted to 39 °C for 10 min, and then treated with these compounds for an additional 45 min. Fig. 4A shows that induction of Slt2 phosphorylation was blocked in the mutant cells in response to every stimulus. Since 75% of the Rho1–104 cells were viable after 1 h at 39 °C, the lack of Slt2 phosphorylation was not due to loss of viability. This indicates that Rho1 plays a key role in activation of the cell integrity pathway in response to these different stimuli.

Whereas the involvement of Rom2, a GDP/GTP exchange factor for Rho1, in controlling Rho1 activity in response to cell wall alterations has been shown recently (13), very little is known about how negative regulators of this small GTPase modulate signaling to the Slt2 MAPK cascade. Bem2 and Sac7 have been identified as GAPs for Rho1 (15, 16). In addition, Bag7 shows a high identity to Sac7 and also contains a Rho-GAP domain (16). Therefore, we analyzed the level of Slt2 phosphorylation in mutants affected in these proteins. As shown in Fig. 4B, only the lack of Sac7 led to a strong increase in the level of phosphorylated Slt2 when cells were grown at 24 °C. The presence of 1 M sorbitol in the medium did not reduce the level of Slt2 phosphorylation in sac7 mutants, which is consistent with Slt2 being activated as a consequence of the constitutive activation of Rho1. Disruption of Bem2 also gave rise to an increase in Slt2 phosphorylation (Fig. 4B). However, in this case, the difference between the wild type strain and the bem2 mutant was only seen when cells were grown at 30 °C but not at 24 °C. Therefore, both Sac7 and Bem2 down-regulate signaling to Slt2, although Bem2 presumably does not play a relevant role at 24 °C.

The MAPK Kinase Mkk1 Plays a More Relevant Role than Mkk2 in Slt2 Phosphorylation.—The existence of two redundant Slt2 activators (MAPKKs) suggests that a regulatory mechanism might be operating to modulate signaling through the kinase cascade. To begin exploring the role of each MAPKK on Slt2 phosphorylation, we assayed extracts from mkk1Δ, mkk2Δ, the double mkk1Δ mkk2Δ mutants, and the isogenic wild type strain after thermal stress and 12 mM caffeine treatment. Hyperactive BCK1–20 (19) and PKC1A398A405A406 alleles were also expressed in these strains in order to constitutively activate the pathway. Fig. 5A shows that, as expected, in wild type cells all stimuli resulted in a strong increase of Slt2 phosphorylation, and in the double mkk1Δ mkk2Δ null mutant neither the basal nor induced Slt2 signal was detected. However, whereas the level of Slt2 phosphorylation after cell stimulation in the mkk1Δ mutant was strikingly low, extracts from the mkk2Δ mutant showed an Slt2 signal slightly higher than that observed in the wild type strain in all assayed conditions (Fig. 5A). These results suggest that Mkk1 is likely to be a stronger Slt2 activator than Mkk2. Thus, Mkk2 might be considered to play a role in attenuation of the signaling at this level.

To test the possibility of a lower efficiency of Mkk2 at activating Slt2, we determined the effect of MKK1 and MKK2 overexpression on Slt2 phosphorylation in a wild type strain. Overproduction of both GST-Mkk1 and GST-Mkk2 led to a similar strong increase in the level of phospho-Slt2 signal at 24 and 39 °C (Fig. 5B). Anti-GST-Slt2 antibodies were used to ensure not only that equal amounts of Slt2 were present in all samples but also to confirm that GST-Mkk1 and GST-Mkk2 were overproduced (Fig. 5B). These results indicate that, when overexpressed, both kinases are able to activate Slt2 in the absence of inducing conditions to a similar extent, suggesting that the main role of Mkk1 in phosphorylating Slt2 might be due to a favored activation of Mkk1.

Slt2 Is Rapidly Dephosphorylated after Cessation of the Stimulus.—Although information regarding negative regulation of other yeast MAPKs is increasing, the mechanisms involved in

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the same phosphorylation pattern as Slt2 F54 (Fig. 6A). To test this possibility, mutant strains TD28-F54, DL443 and TD28-F54 strains growing at 24 °C were shifted to 39 °C for 2 h and then shifted back to 24 °C, and Slt2 phosphorylation was analyzed during the experiment. As shown in Fig. 6A, the phospho-Slt2 signal rapidly decreased (10 min) to the prestimulated level after cessation of the stimulus.

We wished to determine whether this rapid dephosphorylation could be dependent on Slt2 protein activity. To this end, we analyzed the dephosphorylation kinetics of the catalytically inactive protein Slt2F54, in which the conserved lysine within the ATP binding site has been mutated (36). We used the TD28-F54 strain, in which Slt2 has been replaced by the mutant allele slt2Δ. Immunoblots of extracts from slt2F54 mutant cells revealed several striking differences from those of isogenic wild type cells (Fig. 6A). First, the basal level of Slt2F54 phosphorylation at 24 °C was higher than in the case of the wild type protein. Second, heat treatment did not give rise to a significant increase in the phospho-Slt2 signal. Third, Slt2F54 phosphorylation remained with the high prestimulated level once the stimulus had finished. Since cells survived the heat treatment (82% viable cells), the lack of dephosphorylation was not due to loss of viability. In order to test whether Slt2F54 behavior was a particular feature of this mutant protein or due to the TD28 background, we repeated similar experiments with the DL443 strain, bearing a different inactive allele, namely slt2Δ35, in another genetic background (42). However, since this strain displays a strong lytic phenotype at 39 °C, the exposure time was lowered to 1 h in order to avoid high loss of viability. Slt2F54 exhibited the same phosphorylation pattern as Slt2F54 (Fig. 6A). To test whether the introduction of wild type Slt2 reverted this altered pattern, the mutant strain DL443 was transformed with the centromeric plasmid pHR0, bearing Slt2Δ, thus, transformants harbored both Slt2 and Slt2Δ proteins. Immunoblots of extracts from these transformants displayed a wild type Slt2 phosphorylation pattern (Fig. 6A). These observations suggested that a negative feedback mechanism, dependent on functional Slt2, might operate to regulate the level of Slt2 phosphorylation. However, although slt2 mutants do not show a lytic phenotype at 24 °C, the possibility of cells having cell wall alterations that trigger activation of the pathway could not be ruled out. This could be the reason for the increased Slt2 basal phosphorylation and the absence of dephosphorylation after cessation of the stimulus displayed by these mutants. To test this possibility, mutant strains TD28-
cies that do not jeopardize cell integrity but lead to the activation of the pathway. The presence of a constitutive activation prevents us from determining whether the Slt2 dephosphorylation after finishing the thermal stimulus depends on functional Slt2.

The Dual Specificity Protein Phosphatase Msg5 Regulates the Level of Slt2 Phosphorylation—The above results indicate that dephosphorylation of Slt2 is an important mechanism in modulating the functionality of the pathway. Since the overexpression of MSG5, a gene encoding a dual specificity phosphatase (28), has been reported to suppress the lethality caused by the hyperactivated allele MKK1^{23Sc} (29), we explored the potential role of Msg5 in mediating the down-regulation of Slt2 phosphorylation. First, we tested the effect of MSG5 overexpression from a 2µ plasmid on Slt2 phosphorylation in the wild type strain TB50α and in the isogenic SAC7-deleted strain AS171–3 at 24 °C. As shown in Fig. 7A, no phospho-Slt2 was observed in any of these MSG5-overexpressing strains, suggesting that Msg5 is involved in the regulation of Slt2 phosphorylation. Furthermore, overexpression of MSG5 partially suppressed the cold sensitivity of the sac7Δ strain (Fig. 7B).

Next, the effect of Msg5 on Slt2 phosphorylation was further analyzed by testing the MSG5-deleted strain DD1-2D. Cells grown at 24 °C were shifted to 39 °C for 2 h and then shifted back to 24 °C. Fig. 7C shows that the level of phospho-Slt2 is much higher in the deleted strain than in the isogenic wild type both at 24 and 39 °C. However, in cells lacking Msg5, the pattern of Slt2 phosphorylation/dephosphorylation in response to thermal stress and after cessation of the stimulus was similar to that of the wild type. Transformation of the msg5-deleted strain with the centromeric plasmid YCpMSG5, harboring the gene MSG5, lowered the amount of phospho-Slt2 to wild type levels in both induced and noninduced conditions (Fig. 7C). Taken together, these results indicate that Msg5 negatively modulates the activity of the pathway by down-regulating Slt2 phosphorylation.

DISCUSSION

MAPKs require phosphorylation on conserved Thr and Tyr in subdomain VIII to be active (40). Phosphorylation of both sites is essential for different aspects of MAPK functionality, not only for the catalytic kinase activity but also for proper protein movement and localization in the cell (48–51). Not surprisingly, the corresponding residues in Slt2, Thr^{192} and Tyr^{199}, have been shown to be essential for its function (22). Therefore, the amount of dually phosphorylated Slt2 in the cell could be considered as a good reporter for the activation of the Pkc1/Slt2 pathway. Herein, we describe the use of a combination of an antibody raised against dually phosphorylated p44/42 MAPK and a specific anti-Slt2 antibody as a versatile system to detect dual Slt2 phosphorylation. The use of anti-Slt2 antibodies allowed us to monitor the levels of total Slt2 in different strains without the need of any tag. Both antibodies recognize native levels of protein; thus, overexpression of SLT2 is not necessary. Dephosphorylation of either Thr or Tyr of the activation loop is sufficient for MAPK inactivation (40). Additionally, MAPKs have a propensity to autoprophosphorylate on the Tyr but not the Thr, leading to inactive monoautophosphorylated enzymes (55). For these reasons, the use of an anti-dually phosphorylated MAPK antibody appears to be a more precise means of monitoring the level of stimulation of the pathway than the one that recognizes phosphotyrosine. In this way, we have gained insight into different aspects of signal transduction through the cell integrity pathway.

In this study, we have identified caffeine and vanadate as novel stimuli that trigger activation of this pathway. This is consistent with the observation that mutants affected in the Slt2 pathway display a vanadate and caffeine-sensitive growth phenotype that is remedied by osmotic stabilization of the cells (20, 42, 43). The fact that the presence of 1 M sorbitol also prevents induced activation of the cell integrity pathway by both agents suggests that these might alter the cell surface. Several additional observations support a model in which vanadate acts directly or indirectly on the cell wall. First, the lack of Mid2, a putative cell wall integrity sensor, reduces this activation. Second, vanadate has been shown to cause cell wall alterations in the yeast Hansenula polymorpha (54). Third, some vanadate-resistant mutants of S. cerevisiae are defective in protein glycosylation, showing altered secretion and detergent resistance (55, 56). Although caffeine sensitivity has been
shown to be a common phenotype among cell wall-related mutants (57, 58), the mechanism by which caffeine activates the cell integrity pathway remains obscure. \( wsc1/slg1/hcs77 \) mutants display caffeine sensitivity (10, 11). However, neither this mutant nor \( mid2 \) cells present a significant reduction in the caffeine-induced Slt2 phosphorylation. In \( mid2 \) mutant cells, not only heat shock but also Calcofluor- or vanadate-induced activation of the pathway is severely reduced (Fig. 4) (6, 12). Therefore, the absence of reduction in the caffeine-induced Slt2 phosphorylation in cells lacking Mid2 suggests that caffeine activates the cell integrity pathway by means other than those stimuli. Consistently, the kinetics of caffeine-induced Slt2 phosphorylation is different from that of heat shock or vanadate. However, the possibility of Wsc2, Wsc3, or Wsc4, proteins belonging to the same family as Wsc1 (10), or Mtl1, a homologue of Mid2 (6, 12), sensing the caffeine effect cannot be ruled out. In fact, deletion of \( WSC2 \) or \( WSC3 \) in a \( wsc1 \) background or deletion of \( MTL1 \) in \( mid2 \) strains increases the sensitivity of the cells to caffeine (6, 10). The finding that \( SNQ2 \) overexpression suppresses the caffeine sensitivity of \( slt2 \) mutant suggests that the lethal effect of caffeine in these mutants is not caused by a direct action on the cell wall. Therefore, a caffeine-mediated effect on an intracellular event resulting in alteration of the cell wall or a direct disorganization of the cell membrane caused by this drug is an attractive hypothesis. The fact that this compound activates Slt2 phosphorylation in \( pde1 \) \( pde2 \) mutants eliminates the possibility of caffeine-induced Slt2 phosphorylation mediated by inhibition of phosphodiesterases, one of the putative targets of caffeine.

In view of the results presented here, Mid2 seems to play a more relevant role than Hcs77 in sensing different stimuli (Fig. 4). Although a significant reduction in Slt2 kinase activity in response to heat shock in \( hcs77 \) mutants has been reported (9), these authors carried out the experiments in diploid mutant strains, in which the phenotype is more severe. Since we have obtained similar results in haploid \( hcs77 \) mutants of different backgrounds (data not shown), the ploidy variation might be the reason for this difference. Nevertheless, the presence of a number of proteins with homology to these receptors suggests the idea of a very complex system operating at the cell surface to ensure precise detection of a wide range of cell wall stresses. In contrast, inactivation of Rho1 appears to block Slt2-induced phosphorylation in response to all assayed stimuli. These findings are consistent with previous data (13) indicating that cell wall defects activate Rho1 and suggest that this GTPase plays an essential role in transmitting the signal induced not only by heat shock, but also by many other different stimuli, to the Pkc1-mediated cell integrity pathway. The fact that overexpression of Rho1 from its own promoter results in increased Slt2 phosphorylation (data not shown) suggests that changes in Rho1 activity bring about similar modifications in MAPK cascade activation.

Despite its importance in the upstream regulation of signaling to the Pkc1 pathway, data on the regulation of Rho1 in this role are very limited. Our experiments provide considerable evidence that Sac7 and Bem2 are negative regulators of the cell integrity pathway. Since both proteins have been shown to be GAPs for Rho1 (15, 16), their down-regulating effect on Slt2 phosphorylation is likely to be through Rho1. Our observations also suggest that each GAP controls Rho1 in a temperature range. The function of Sac7 in a wide range of temperatures would explain both the cold sensitive phenotype of the \( sac7 \) mutants and their ability to suppress Ts\(^-\) defects (16, 59).

Signaling through the MAPK cascade has also been studied. Very recently, an example of a regulatory role for a MAPK kinase has been described. The MAPKK Pek1 of \( Schizosaccharomyces pombe \) has been shown to play a dual stimulatory and inhibitory function that depends on its phosphorylation state (60). Since overexpression of either MAPK activator Mkk1 or Mkk2 leads to a similar amount of phospho-Slt2, the different MAPKK activities observed under stimulation conditions might be due to a different activation by the MAPKK kinase Bck1. Although the experiments presented here offer no obvious evidence about how it functions, they suggest the existence of a novel modulation mechanism of signaling through this pathway.

Together with the light shed on mechanisms down-regulating upstream components of the pathway, the experiments shown in this work provide insights about Slt2 dephosphorylation. Unlike the osmosensing and the mating pathways, there is no evidence for the existence of a mechanism for adaptation to signal in the cell integrity pathway, since the activation is maintained as long as the inducing stimulus is present (4). One explanation could be the need of a continuous cell wall remodeling while the cell wall alteration is present. The constitutive activation of the Slt2 pathway displayed by \( slt2 \) mutants in the absence of osmotic stabilization supports this notion. Nevertheless, at least two other types of mechanisms down-regulating the pathway by Slt2 dephosphorylation could be envisioned. One of them would be responsible for maintaining a low basal level of phospho-Slt2, whereas the other one would rapidly operate in the recovery process after cessation of the stimulus. Our results suggest the existence of both mechanisms, with the dual specificity protein phosphatase Msg5 involved in the first but not in the second proposed mechanism. Interestingly, this phosphatase also acts in the mating pathway, but playing a different role. Whereas Msg5 appears to be constantly down-regulating the level of phospho-Slt2, it seems to participate in the adaptive response by inactivation of the MAPK Fus3 following pheromone stimulation (27). As the amount of phospho-Slt2 declined to the prestimulated level in the absence of Msg5, other phosphatases are assumed to be inactivating Slt2 after cessation of the stimulus. The fact that the tyrosine phosphatases Ptp2 and Ptp3 are known to function both in the mating and HOG pathways raises the possibility of all of these protein phosphatases being versatile negative modulators of MAPK pathways. Furthermore, the expression of \( PTP2 \) has been shown to be dependent on the Slt2 pathway (30). The possibility of Ptp2 and Ptp3 as well as serine/threonine protein phosphatases like Ptc1 playing a role in the inactivation of this signaling pathway remains to be established.

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