Alkalization of the external environment represents a stress situation for *Saccharomyces cerevisiae*. Adaptation to this circumstance involves the activation of diverse response mechanisms, the components of which are still largely unknown. We show here that mutation of members of the cell integrity Pkc1/Slt2 MAPK module, as well as upstream and downstream elements of the system, confers sensitivity to alkali. Alkalization resulted in fast and transient activation of the Slt2 MAPK, which depended on the integrity of the kinase module and was largely abolished by sorbitol. Lack of Wsc1, removal of specific extracellular and intracellular domains, or substitution of Tyr303 in this putative membrane stress sensor rendered cells sensitive to alkali and considerably decreased alkali-induced Slt2 activation. In contrast, constitutive activation of Slt2 by the bck1-20 allele increased pH tolerance in the wsc1 mutant. DNA microarray analysis revealed that several genes encoding cell wall proteins, such as *GSC2/FKS2, DFG5, SKTS*, and *CRH1*, were induced, at least in part, by high pH in an Slt2-dependent manner. We observed that *dfg5, skts5*, and particularly *dfg5 skts5* cells were alkali-sensitive. Therefore, our results show that an alkaline environment imposes a stress condition on the yeast cell wall. We propose that the Slt2-mediated MAPK pathway plays an important role in the adaptive response to this insult and that Wsc1 participates as an essential cell-surface pH sensor. Moreover, these results provide a new example of the complexity of the response of budding yeast to the alkalization of the environment.

The yeast *Saccharomyces cerevisiae*, a model organism in biology with an unquestionable biotechnological interest, grows better at acidic than at neutral or alkaline pH. Maintenace of an acidic environment is based primarily on the active proton extrusion mediated by its plasma membrane H⁺-ATPase, and this proton gradient is critical for the uptake of different nutrients, including diverse cations (1, 2). Therefore, sudden alkalinization of the environment represents a stress condition for this yeast, and to survive, *S. cerevisiae* must detect the change and react to it, triggering an adaptive response.

The response of budding yeast to alkalinization of the environment has been characterized in some detail in the last few years, in many cases through the study of mutants sensitive to high pH conditions and/or the definition of the transcriptional adjustments after exposure to alkaline pH. Interestingly, the response in *S. cerevisiae* seems to be more complex than that characterized in other fungi such as *Aspergillus nidulans*. In this fungus, the zinc finger PacC transcription factor plays a pivotal role in mediating pH regulation by directly activating pH-responsive promoters (see Refs. 3 and 4 for reviews). *S. cerevisiae* encodes a PacC homolog, Rim101, which has been shown to play a role in alkaline pH response and adaptation, although Rim101 is responsible only for a limited set of alkali-induced transcriptional responses. In addition, its activation requires a single proteolytic step, in contrast to the two steps described for *A. nidulans*, and its action on responsive promoters seems to be an indirect one, by repressing the expression of transcriptional repressors such as *NRG1* (5–8). In addition to the Rim101 pathway, adaptation to external high pH involves the activation of the calcium-activated phosphatase calcineurin (8–11). It has been shown that exposure to alkali triggers a strong and transient intracellular calcium burst (10), which results in activation of calcineurin and the calcineurin-regulated transcription factor Crz1/Tcn1. Activation of the calcineurin/Crz1 pathway has relevance in high pH adaptation, as deduced from the alkali-sensitive phenotype of *cnb1* mutants. About 10% of the alkali-induced transcriptional response is partially or fully dependent on the activation of this pathway (8, 10). Analysis of the transcriptional profile after alkaline stress also reveals that alkalization of the medium probably affects the availability of different nutrients, such as phosphate, and that of specific cations, such as copper and iron (6, 8, 12, 13). Similarly, high pH stress involves some degree of oxidative stress (13).

A recent screen for strains sensitive to high pH carried out in our laboratory using a systematic library of deletion mutants revealed 118 genes that are important to ensure normal alkaline
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tolerance (13). Interestingly, our list includes BCK1 and SLT2/MPK1, which are components of the MAPK cascade involved in cell wall remodeling and maintenance of cell integrity (see Ref. 14 for a recent review and references therein). This kinase module includes the yeast protein kinase C Pkc1, which, in response to cell wall stress, phosphorylates and activates the Bck1 kinase. Activated Bck1 can phosphorylate a pair of redundant MAPK kinases, Mkk1 and Mkk2, which will activate the Slt2 MAPK. Upstream components of this pathway are a number of presumed membrane sensors (Wsc1–4, Mid2, and Mtl1) that, in some cases, have been shown to signal through the Rho1 GTPase. Activation of Slt2 results in the phosphorylation of several nuclear and cytosolic targets. Among the nuclear targets are the transcription factors Rlm1, which seems to be responsible for most of the transcriptional effects derived from the activation of Slt2, and SCB-binding factor (Swi4 and Swi6).

The identification of bck1 and slt2 mutants as sensitive to high pH, supported by the results of an independent survey using liquid cultures (11), prompted us to consider the possibility that the Slt2 MAPK pathway might be involved in alkaline pH signaling, perhaps as a result of the generation of some sort of stress rapidly and transiently activates Slt2; that this activation might be involved in alkaline pH tolerance (13). Interestingly, our list includes SLT1, which are components of the MAPK2 cascade involved in alkaline stress, and the expression of several specific genes, some of which may be known as stress genes.

The deletion cassette used to disrupt the SKT5 gene was generated as follows. A region containing the SKT5 gene from –683 to +2143 relative to its ATG initiation codon was amplified by PCR. Genomic DNA from the wild-type BY4741 strain was used as a template, and oligonucleotides SKT5 DISR_5’ (which contains an Xba1 site) and SKT5 DISR_3’ were used as primers. The resulting 2.8-kbp fragment was digested with XbaI and HindIII and cloned into pUC19, yielding plasmid pUC-SKT5. The 1.6-kbp Sall–SmaI restriction fragment from YDPl. (19) containing the LEU2 gene was cloned into Xhol–HincII-digested pUC-SKT5, yielding plasmid pDSKT5_L. The 3.1-kbp Xbal–HindIII fragment from pDSKT5_L containing the skt5::LEU2 disruption cassette was used to transform the BY4741 strain to yield strain RSC78.

WSC1-directed mutagenesis was performed as follows. All DNA fragments used to obtain mutated versions of the WSC1 gene were amplified by PCR with High Fidelity Expand Taq polymerase (Roche Applied Science) using as a template the pRS314-WSC1 HA centromeric plasmid described previously (20). PCR amplification products were gel-purified with the Geneclean kit (Bio 101, Inc.) before use for yeast transformation. Sequences of all amplified and cloned DNA products were verified by automated DNA sequencing. Constructs expressing versions of Wsc1 containing different deletions of its extracellular domain were obtained by in vivo DNA recombination after cotransformation of yeast cells with the pRS314 vector (21) digested with KpnI and PstI and two additional overlapping DNA fragments (a and b). The forward primer used to obtain the different a-fragments for all mutations was always the standard M13 reverse primer, which hybridizes to the pRS314 plasmid. In the same way, the standard universal primer was used as a reverse primer to amplify all b-fragments. To get pWSC1(D21–110), oligonucleotides WSC1N3_REV and WSC1N4_FOR were used as the reverse primer for fragment a and the forward primer for fragment b, respectively. The pWSC1(D116–256) construct was obtained with oligonucleotides WSC1N1_REV and WSC1N2_FOR used as the reverse primer for fragment a and the forward primer for fragment b, respectively, and oligonucleotides WSC1N5_REV and WSC1N2_FOR were used to generate fragments a and b, respectively, for the pWSC1(D21–256) construct. Constructs in pRS314 expressing mutations in the cytoplasmic domain of Wsc1 (such as pRS314-WSC1-Y303A HA and pRS314-WSC1(1–300) HA), YEp352-WSC1 HA, and the bck1-20 construction have been described previously (20, 22, 23). In some cases, diverse Wsc1 versions were transferred from pRS314 (TRP1 marker) to pRS316 (URA3 marker) by digestion with KpnI and SacI and subsequent ligation.

Preparation of Yeast Extracts and Immunoblot Analysis—For immunodetection of the Slt2 MAPK, saturated cell cultures of the indicated strains in YPD medium were diluted to $A_{660}$ = 0.2 in fresh YPD medium and grown to $A_{660}$ = 1.3–1.4. Alkaline shock was performed by the addition of KOH (from a 1 M stock solution) to reach a final concentration of 35 mM (which leads to a pH value of 8.2). At the specified times after KOH addition, 5 ml of the cell culture was harvested by filtration, washed once

2 The abbreviations used are: MAPK, mitogen-activated protein kinase; HA, hemagglutinin; TAP5, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid.
with prechilled water, snap-frozen, and kept at −80 °C. Control cells received the same amount of KCl. For dose-response experiments, different final concentrations of KOH were used as indicated below. Cell wall stress was carried out by the addition of Congo red to the medium to reach a final concentration of 100 μg/ml (from a 10 mg/ml stock solution). Heat stress was accomplished by rapidly shifting the temperature of the cultures to 40 °C. When indicated, YPD medium was supplemented with 1 m sorbitol.

Total cell protein extracts were prepared basically as described previously (24). Briefly, cell pellets were resuspended in 150 μl of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 0.1% SDS) containing phosphatase and kinase inhibitors (50 mM NaF, 1 mM sodium pyrophosphate, and 0.5 mM EDTA (pH 8.0)), 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Complete EDTA-free protease inhibitor mixture tablets, Roche Applied Science). One volume of acid-washed glass beads was added, and cells were broken by vigorous shaking in a FastPrep cell breaker (Bio 101, Inc.) at setting 5.5 for 25 s. After sedimentation at 16,000 × g, the clear lysate was recovered, and the protein concentration was quantified by the Bradford assay (Sigma-Aldrich). Forty μg of total protein was fractionated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences). Membranes were incubated for 2 h with anti-phospho-p44/42 MAPK antibody (Thr202/Tyr204, New England Biolabs) at 1:2000 dilution, anti-phospho-p38 MAPK antibody (Thr180/Tyr182; Cell Signaling Technology) at 1:1000 dilution, or anti-glutathione S-transferase-Slt2 antibody (25) at 1:10,000 dilution to detect dually phosphorylated Slt2, dually phosphorylated Hog1, or total Slt2, respectively. A 1:25,000 dilution of horseradish peroxidase-conjugated anti-rabbit antibody was used to detect primary antibodies. Immunocomplexes were visualized by the ECL Advance Western blotting detection kit (Amersham Biosciences). The chemiluminescence was recorded with a Fuji LAS-3000 luminescent image analyzer, and MultiGauge Version 3.0 software was used to quantify signal intensities.

Immunodetection of HA-tagged Wsc1 versions was accomplished as follows. Yeast cells from 5 ml of exponentially growing cultures were collected by centrifugation, washed with cold water, and kept dried at −80 °C. Two-hundred μl of ice-cold lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors were added to the dried cell pellets together with 1 volume of glass beads. Cells were broken by five cycles of 1 min of vigorous shaking, and glass beads were removed by sedimentation at 750 × g. Total protein concentration was quantified by the Bradford assay, and 40 μg of protein was centrifuged at 16,000 × g for 15 min. Pellets were resuspended in SDS loading buffer, fractionated by SDS-PAGE (8%), and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp.). Anti-HA monoclonal antibody (12CA5, Roche Applied Science) was used at 1:5000 dilution. The anti-mouse secondary antibody coupled to horseradish peroxidase was diluted 1:25,000, and a SuperSignal West Dura system kit (Pierce) was used to visualize the protein-antibody complexes in the LAS-3000 analyzer.

**RNA Purification, DNA Microarray Experiments, and Reverse Transcription-PCR**—For RNA purification, 20 μl of yeast cultures was grown at 28 °C in YPD medium to $A_{660} = 1.3$. KOH or KCl was added from a concentrated stock solution (1 M) to reach a final concentration of 35 mM. Yeast cells were harvested by filtration after 15 and 30 min and washed once with cold water, and dried cells were kept at −80 °C until RNA purification. Total RNA was purified using a RiboPure-Yeast kit (Ambion, Inc.) following the manufacturer’s instructions. RNA quality was assessed by denaturing 0.8% agarose gel electrophoresis, and RNA quantification was performed by measuring absorbance at 260 nm in an Eppendorf BioPhotometer.

Transcriptional analyses were performed using DNA microarrays containing PCR-amplified fragments from 6014 S. cerevisiae open reading frames (10, 26). Fluorescent Cy3- and Cy5-labeled cDNA probes were prepared from 8 μg of purified total RNA by the indirect dUTP labeling method using a CyScribe post-labeling kit (Amersham Biosciences). Prehybridization, hybridization, and washes were carried out as recommended by the Institute for Genomic Research (www.tigr.org/tdb/microarray/conciseguide.html) with minor modifications. For hybridizations of the DNA microarrays, both dried Cy3- and Cy5-labeled probes were resuspended in 35 μl of hybridization solution (50% formamide, 5× SSC, and 0.1% SDS) and mixed. Five μg of salmon sperm DNA was added to the mixture before denaturation for 3 min at 95 °C. Hybridizations were carried out in an ArrayBooster hybridization station (Sunergia Group, Inc.) for 14 h at 42 °C. A ScanArray 4000 scanner (PerkinElmer Life Sciences) was used to obtain the Cy3 and Cy5 images at 10-μm resolution. The fluorescent intensity of the spots was measured and processed using GenePix Pro Version 6.0 software (Molecular Devices Corp.).

For each condition assayed, three independent experiments were performed, and dye swapping was carried out in each case. Data from different experiments were combined, and the mean was calculated. A given gene was considered to be induced or repressed when the mean of the ratios (alkaline stress versus no stress) was $>2.0$ or $<0.50$, respectively, and at least four valid (non-flagged) values were available. Software from the GEPAS server (gepas.bioinfo.cipf.es/) was used to carry out clustering and other data analyses (27). To confirm some of the results obtained by the microarray experiments, reverse transcription-PCR analyses were performed using a OneStep reverse transcription-PCR kit (Qiagen Inc.) and 1 μg of total RNA. Oligonucleotide pairs RT_SKT5_UP/RT_SKT5_DO, RT_DFG5_UP/RT_DFG5_DO, and RT_CRH1_UP/RT_CRH1_DO (supplemental Table 1) were used to determine, after 24 cycles, the levels of mRNA corresponding to SKT5, DFG5, and CRH1, respectively.

**Actin Staining**—Wild-type BY4741 cells were diluted in fresh YPD medium to $A_{660} = 0.2$ from a saturated culture, and growth was resumed until $A_{660} = 1.3$ was reached. Alkaline stress was initiated by the addition of KOH from a 1 M stock solution to reach a final concentration of 35 mM (which leads to a pH value of 8.2). Aliquots (100 μl) of this culture were collected at different times and processed for actin staining with...
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![Image]

**FIGURE 1. Alkaline pH sensitivity of mutants in upstream and downstream elements of the Slt2 MAPK cascade.** The indicated mutants in the BY4741 background were grown on YPD-agar plates under the specific conditions. Alkaline plates were buffered with 50 mM TAPS. Congo red was used at 100 μg/ml. Growth was monitored after 2–3 days. *wt*, wild-type.

rhodamine-phalloidin essentially as described (28). KCl instead of KOH was used for non-stressed conditions. Temperature stress was accomplished by transferring cultures to a water incubator set at 42 °C.

**Growth Tests**—The sensitivity of different yeast strains to alkaline pH, high temperature, caffeine, Congo red, and Calcofluor white was assayed by drop test on YPD plates. To test alkaline stress sensitivity, plates containing 50 mM TAPS adjusted with KOH at different pH values were used. When required, 1 M sorbitol was added to the medium prior to sterilization by autoclaving. Growth in liquid medium at high pH was performed in 96-well plates. Cultures (250 μl) at an initial _A_~660~ of 0.001 were grown for 23 h at 28 °C in YPD medium containing 50 mM TAPS buffered at the indicated pH values. Growth was monitored in a Labsystems iEMS Reader MF at 620 nm.

**RESULTS**

**Alkaline Stress Results in Transient Activation of the Slt2 MAPK**—The finding that mutation of non-redundant members of the Slt2 MAPK cascade results in a strong alkaliti-sensitive phenotype (13) prompted us to evaluate in detail high pH tolerance for upstream and downstream members of this module. As shown in Fig. 1, mutants in upstream components such as the _wsc1_ strain (but not _wsc2, wsc3, or mid2_) displayed sensitivity to alkal. _mtl1_ cells were as tolerant as wild-type cells (data not shown). Mutation of _ROM2_, encoding a GDP/GTP exchange protein for the Pkc1 activator Rho1 (data not shown), or either _MKK1_ or _MKK2_, encoding the kinases directly upstream of Slt2, did not result in altered pH tolerance, which could be explained by a redundant role of the Rom1/2 and Mkk1/2 pairs in this signaling pathway. Mutation of the transcription factor _Rml1_, an important nuclear target for Slt2, did not confer high pH sensitivity. In contrast, lack of either component of the SCB-binding transcription factor, Swi4 and Swi6, resulted in sensitivity to alkal. These strains were subjected to the same stress in the presence of 1 M sorbitol. Interestingly, the presence of the osmotic stabilizer did rescue the sensitivity of the _bck1_ and _slt2_ mutants to high pH, Congo red, or high temperature (42 °C), but in contrast, the _wsc1, swi4_, and _swi6_ strains still showed a significant growth defect at high pH or in the presence of Congo red.

The sensitivity to high pH conferred by mutation of upstream and downstream components of the Slt2 cascade supported the notion that this pathway might be activated by alkalization of the medium, thus being involved in the response to this kind of stress. To directly test this hypothesis, wild-type cells were subjected to alkaline shock, and samples were taken at different times. Extracts were prepared, electrophoresed, and immunoblotted with antibodies able to detect the active phosphorylated form of Slt2 as well as with anti-Slt2 antibody. As shown in Fig. 2A, exposure to high pH resulted in a fast and transient phosphorylation of Slt2, peaking between 5 and 15 min after the stress and declining after 30 min. Dose-response experiments (Fig. 2B) showed that Slt2 phosphorylation was already noticeable at pH ~7.5, peaked at pH ~8.2, and was not further enhanced at higher pH values. Activation of Slt2 in response to external alkalinization was fully dependent on the integrity of the MAPK cascade, as demonstrated by the absence of Slt2 phosphorylation in the _bck1_ mutant (Fig. 2C). Alkalization of the medium did not result in phosphorylation of the Hog1 MAPK during the first hour after stress, as deduced from immunoblot analyses using anti-phospho-p38 MAPK antibody (data not shown).

As the cell integrity of _slt2_ mutants after cell wall damage can be preserved if osmotically stabilized, we investigated the effect on Slt2 activation of the presence of 1 M sorbitol in the alkaline medium. As shown in Fig. 3A, the addition of sorbitol largely attenuated the fast phosphorylation of Slt2 in response to high pH, although it resulted in a pH-independent, late (30–60 min) activation of Slt2. A similar time course experiment showed that phosphorylation of Slt2 in response to alkaline stress was much faster than that in response to other stresses such as exposure to Congo red and high temperature (Fig. 3A). Because it has been documented that different stress situations lead to actin depolarization, we sought to investigate if this was the case for alkaline stress. To this end, cultures were shifted to pH 8.2, and samples were taken at different times ranging from 5 to 120 min. However, we did not observe abnormal alterations in actin distribution at any time, whereas control cells shifted to 42 °C showed a marked actin depolarization after 15–30 min (Fig. 3B).

**Identification of Wsc1 as a Signaling Component in the Alkaline pH Stress Response**—The observation that mutation of _WSC1_ resulted in increased pH sensitivity raised the possibility that the membrane protein might perceive the signal(s) generated by high pH and transmit it through the Slt2 pathway. To test this hypothesis, wild-type and _wsc1_ cells were subjected to
alkaline stress, and the phosphorylation state of Slt2 was monitored. As shown in Fig. 4A, Slt2 phosphorylation was markedly reduced in the wsc1 mutant, whereas this effect was not observed in the wsc2 and wsc3 mutants. Phosphorylation of Slt2 was affected only slightly in the mid2 strain and did not change in cells lacking Mtl1, a protein with structural and functional similarity to Mid2. Therefore, the sensitivity of the wsc1 mutant was compatible with loss of full activation of the Slt2 cascade.

We presumed that, if hypersensitivity of the wsc1 mutant could be, at least in part, the result of impaired signaling through Slt2, artificial activation of the Slt2 pathway may reduce sensitivity to high pH in the wsc1 strain. To this end, we expressed, in the wsc1 mutant strain, the bck1-20 allele, which provides constitutive activation of the Slt2 pathway, and tested the tolerance of these cells to high pH and other cell wall stresses. As shown in Fig. 4B, expression of the bck1-20 allele did improve the high pH tolerance of the wsc1 strain, but was without effect on the slt2 mutant. A similar effect was observed previously when cells were grown in the presence of caffeine (29). In contrast, activation of the Slt2 pathway did not improve tolerance to Congo red or Calcofluor white in cells lacking Wsc1 (data not shown).

Identification of Wsc1 Structural Elements Required for Signaling in Alkaline pH Stress—It has been proposed that normal Wsc1 function requires both extracellular and intracellular domains of the protein (20, 30). To test the functional relevance of these elements in alkaline stress signaling, we expressed, from a centromeric plasmid in the wsc1 mutant, an HA-tagged version of both wild-type Wsc1 and Wsc1-(1–300), which lacks the entire cytosolic region (Fig. 5A). As shown in Fig. 5B, whereas the tagged wild-type protein was able to restore normal tolerance to high pH and other cell wall-damaging agents, the deleted version was not. Interestingly, a ver-
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FIGURE 4. Lack of Wsc1 results in impaired Slt2 phosphorylation after alkaline stress. A, the indicated mutants in the BY4741 background were subjected for 15 min to alkaline stress (pH 8.2) and processed for immunoblotting as described in the legend to Fig. 1. The bar graph represents the relative activation in each case determined by the ratio of alkali-induced Slt2 phosphorylation compared with non-stressed cells once corrected for the amount of total Slt2 protein. Wild-type (WT) cells were considered to have a 100% response. Data represent the means ± S.E. of five independent experiments. **, p < 0.001; *, p < 0.05. B, the wild-type BY4741 strain (circles) or the wsc1 (squares) or slt2 (triangles) derivative was transformed with an empty plasmid (open symbols) or the bck1-20 allele (closed symbols). Cells were grown in synthetic medium to saturation as described under “Experimental Procedures” and inoculated in YPD medium buffered at the indicated pH values, and A630 was recorded after 23 h. Data are presented percent growth with respect to an equivalent culture at pH 5.5 and correspond to the means ± S.E. of three independent clones.

FIGURE 5. Mutational analysis of Wsc1 reveals intra- and extracellular regions relevant for tolerance to alkaline stress. A, schematic depiction of the structure of Wsc1. Numbers indicate amino acid positions. Arrows indicate residues or domains that have been defined as relevant for signaling to Rom2 in a previous work (20). Tm, predicted transmembrane region. B, the wsc1 mutant in the W303-1A background was transformed with the indicated plasmids (TRP1 marker) and spotted onto YPD plates at the indicated pH values or in the presence of caffeine (Caf; 7 mM) or Congo red (10 μg/ml). Growth was monitored for 3–5 days. C, the wild-type (WT) BY4741 strain and its wsc1 derivative were transformed with an empty pRS316 plasmid (URA3 marker) or the same plasmid bearing the indicated versions of HA-tagged Wsc1. Transformants were grown and subjected to pH 8.2 for 15 min before collecting the cells and processing them for immunoblot analysis using antibodies that detect the phosphorylated form of Slt2 (P-Slt2) and the total Slt2 protein (Slt2p). Extract from the slt2 strain was included as a negative control.

Mannosylation of the periplasmic ectodomains of Wsc1 has been shown to be important for function, and it has been attributed to several members of the protein O-mannosyltransferase family encoded by PMT1–6 (31, 32). We reasoned that if Wsc1 is an important element for alkaline pH tolerance, certain mannosyltransferase mutants may exhibit an alkali-sensitive phenotype. To this end, the pH sensitivity of five different mutants was compared with that of the wild-type sensitive phenotype of the wsc1 mutant. We then constructed and expressed, in the wsc1 strain, three different versions of Wsc1 carrying diverse deletions of the extracellular region (Fig. 5, A and B). The first one, lacking virtually all of the extracellular segment (A21–256) except the N-terminal signal peptide, was not functional at all. Interestingly, removal of residues 21–110, which correspond to the characteristic WSC domain, yielded a protein still able to confer a certain degree of tolerance to alkaline stress. Finally, deletion of residues 116–256 yielded a protein devoid of the large Ser/Thr-rich region that was essentially ineffective. Loss of function of the mutated versions cannot be attributed to defective expression or localization, as immunoblot analysis of cell protein extracts indicated that all versions were recovered in the particulate fractions and expressed at levels at least equivalent to that of the wild-type form (data not shown). In agreement with these observations, expression of Wsc1 forms lacking the intracellular region or bearing the Y303A mutation, which were unable to return cells to their normal pH tolerance, also failed to restore normal levels of Slt2 phosphorylation upon alkaline stress (Fig. 5C). Therefore, both extracellular structural elements and intracellular signaling components are required for Wsc1 to perform its function in high pH tolerance.

sion carrying a change in the intracellular residue Tyr303 to alanine, which has been shown to disrupt the interaction of Wsc1 with Rom2 (20), was also unable to rescue the alkali-strain (Fig. 6A). The pmt1 and pmt2 mutants displayed a significant degree of sensitivity to high pH, whereas pmt3, pmt5, and pmt6 had a wild-type phenotype. Interestingly, immunoblot
analysis of HA-tagged Wsc1 (Fig. 6B) showed that the total amount of the high molecular mass form (~100 kDa) decreased in the pmt2 mutant (and also slightly in the pmt1 strain) in concurrence with the appearance of smaller protein fragments (~40 kDa). Therefore, mutations affecting the proper O-mannosylation of Wsc1 (and probably the stability of the protein) do result in increased alkaline sensitivity.

Transcriptional Profiling in Response to Alkaline Stress in Slt2-deficient Cells—It has been documented that exposure of S. cerevisiae to high pH results in a substantial and complex transcriptional response. Activation of Slt2 after cell wall damage also gives rise to changes in the transcriptional profile. We considered that comparison of the transcriptional profiles of wild-type and slt2 cells may provide information about the relevance of this kinase in remodeling gene expression after alkaline stress and perhaps reveal new genes important for tolerance to alkaline stress. To this end, we exposed wild-type cells and slt2 mutants to pH 8.2 by the addition of the necessary amount of KOH to the medium, collected the cells after 15 and 30 min of stress, and prepared total RNA for DNA microchip analysis. Exposure to high pH resulted in an at least 2.0-fold induction of 602 genes after 15 min and 178 genes after 30 min (supplemental Table 2). Comparison of the level of induction after 15 min in wild-type cells with that in the slt2 mutant (Table 1) revealed that the induction of 18 genes was reduced by at least 25% in the mutant strain (including the gene GSC2/FKS2, encoding a subunit of the 1,3-β-D-glucan synthase). In contrast, after 30 min of exposure to alkaline stress, only four genes (DFG5, SKT5, CRH1, and IRC14) reached the indicated threshold for Slt2 dependence. Interestingly, DFG5, SKT5, and CRH1 encode cell wall proteins. The induction by high pH of three of these genes and their dependence on Slt2 were confirmed by semiquantitative reverse transcription-PCR (Fig. 7A). Phenotypic analysis of these mutants for alkaline tolerance revealed that the crh1 strain was similar to wild-type cells. In contrast, skt5 and dfg5 cells grew very poorly at pH 7.8 (Fig. 7B). A dfg5 skt5 double mutant was more sensitive to alkali than any of the single mutants. This phenotype was rather intense, but not as strong as that of the slt2 mutant.

The recent availability of transcriptional profiles in response to diverse cell wall-damaging drugs or to mutation of specific genes relevant for cell wall maintenance prompted us to construct an array of expression data that would allow us to correlate these changes with those produced by exposure to high pH. As shown in Fig. 8, cell wall damage provoked by zymolyase treatment or exposure to caspofungin or dithiothreitol resulted in a transcriptional profile rather close to the one caused by high pH stress, whereas there was almost no correlation with the profiles observed after Congo red and α-factor treatment. Similarly, comparison with changes induced by mutation in relevant cell wall components illustrates that the similarity profile followed the order fks1 > gas1 > kss1 > knr4, whereas the mnn9 profile showed no positive correlation at all.

DISCUSSION

In a recent study (10), we pointed out the striking overlap between the transcriptional profile of yeast cells subjected to severe alkaline stress and that observed after mutation of genes encoding certain cell wall components or exposure to cell wall-damaging agents (33, 34). Because mutation of a significant number of genes related to cell wall biogenesis or maintenance results in sensitivity to alkalinization (11, 13), we raised the hypothesis that alkaline pH might lead, at least to some extent, to a situation of cell wall damage. To test this hypothesis, the following questions were addressed. 1) Does exposure to high pH trigger activation of the cell wall integrity pathway mediated by the Slt2 MAPK? 2) If so, would it be possible to identify a potential upstream sensor for high pH stress? 3) Does Slt2 activation result in increased expression of cell wall components that might be relevant for adaptation to high pH?

We have shown here that alkalinization of the environment results in specific activation of the Slt2 MAPK pathway. S. cerevisiae has five different MAPK modules (35–37), and in some cases, a given stress situation results in activation of several modules. Examples are activation of both the Slt2 and Hog1 MAPKs by heat stress (38) or by hyperosmotic shock (39). However, in our case, we observed a very specific involvement of Slt2 in the response to alkaline stress: although slt2 mutants are highly sensitive to alkaline pH, hog1, kss1, or fus3 cells are not. Our data also indicate that Kss1 and Fus3 MAPKs are not phosphorylated after exposure to alkali and that FUS1, the activation of which is mediated by Fus3 and Kss1 (40), is not induced by high pH (data not shown). This is remarkable, as Kss1 has been proposed previously to be involved in maintaining cell wall integrity (36). Similarly, we observed that Hog1 is not phosphorylated by high pH stress. Therefore, high pH appears to specifically activate the Slt2 MAPK module.

A remarkable characteristic of Slt2 activation after alkaline stress is its fast (5–15 min) and transient nature. Very fast phosphorylation of Slt2 was reported some time ago as a result of hypotonic shock (41), although, in this case, it peaked after 1 min and disappeared in 10–20 min. These differential kinetics suggest that the events triggered by hypotonic shock and ambi-
ent alkalinization are not the same. The relatively fast response to high pH is in contrast with the slower kinetics observed in cells exposed to Calcofluor white (42, 43), caffeine (24), or heat shock (24, 42). A slower response was also observed in cells treated with rapamycin (28) or with oxidants such as diamide and hydrogen peroxide (44). This contrast cannot be attributed to strain background differences, as we have confirmed here and elsewhere (24, 42). It appears that external high pH is perceived faster than most situations that also result in Slt2 activation. Although, phosphorylation of Slt2 is maintained for a relatively long period in all these cases, the alkaline response is transient. This could be due to feedback regulatory mechanisms or simply explained by the progressive acidification of the medium during the experiment. In any case, these phosphorylation kinetics correlate well with the rapid Slt2-dependent transcriptional response, more important after 15 min of the stimulus than after 30 min. Therefore, in contrast to the response observed for most Slt2-inducing stimuli, yeast cells would use the cell integrity pathway to elicit an almost immediate response to the medium alkalization that would be eventually essential for their survival.

It is commonly accepted that cell wall status is monitored by a family of five cell-surface proteins (Wsc1–3, Mid2, and Mtl1). Among these, Wsc1 and Mid2 play a major role in maintaining cell wall integrity, and although they may have specific functions, their role in signaling partially overlaps (see Ref. 14 and references therein). We have shown here that wsc1 mutants are

### TABLE 1

| ORF  | Gene  | Time | WT | slt2 | slt2/WT ratio | Sit-dependent | Rlm1 site | Swi4 site | Induced by | Description                                                                                     |
|------|-------|------|----|------|---------------|---------------|-----------|-----------|------------|------------------------------------------------------------------------------------------------|
|      |       | min  | fold | fold |               |               |           |           |            |                                                                                                 |
| **Cell wall** |       |      |     |      |               |               |           |           |            |                                                                                                 |
| YGR023W | GSC2  | 15   | 6.19 | 4.37 | 0.71          | 1, 2          | Yes       | Yes       | f, g, kn, m, kr, CHI, CR | Catalytic subunit of 1,3-β-glucan synthase.                                                      |
| YBL061C | SKT5  | 30   | 2.15 | 1.42 | 0.66          |               |           |           |            | Activator of Chs3 (chitin synthase III)                                                          |
| YGR189C | CRH1  | 30   | 2.28 | 1.71 | 0.75          | 1, 2          | Yes       | Yes       | f, g, kn, m, kr, CHI, CR, CFW, CR, ZYM | Putative glycosidase of cell wall.                                                              |
| YMR238W | DFG5  | 30   | 2.12 | 1.39 | 0.66          | 1, 2          | Yes       |           | f, g, kn, m, kr, ZYM | Putative mannosidase required for cell wall biogenesis.                                         |
| **Metabolism** |       |      |     |      |               |               |           |           |            |                                                                                                 |
| YDL194W | SNF3  | 15   | 2.57 | 1.88 | 0.73          |               |           |           |            | CHI Plasma membrane glucose sensor.                                                              |
| YBR050C | REG2  | 15   | 12.55| 6.86 | 0.55          |               |           |           |            | Regulatory subunit of Glc7 involved in regulation of glucose-responsive genes.                  |
| YAR042W | SWHI  | 15   | 2.90 | 1.80 | 0.62          |               |           |           |            | Protein similar to mammalian oysterol-binding protein.                                          |
| YGL224C | SDT1  | 15   | 2.08 | 1.45 | 0.70          |               |           |           | 2          | Pyrimidine nucleotidase.                                                                         |
| YKR091W | SRL3  | 15   | 4.48 | 2.60 | 0.58          |               |           |           | Yes        | Cytoplasmic nucleotidase that suppresses lethality of rad53 null mutation.                      |
| YNL134C |       | 15   | 3.04 | 2.09 | 0.69          |               |           |           | Yes        | Putative protein with similarity to dehydrogenases.                                             |
| **Cell cycle, DNA processing, and transcription** |       |      |     |      |               |               |           |           |            |                                                                                                 |
| YEL019C | MMS21 | 15   | 2.71 | 2.03 | 0.75          |               |           |           |            | SUMO ligase involved in chromosomal organization and DNA repair.                                 |
| YHR157W | REC104| 15   | 3.18 | 2.08 | 0.65          |               |           |           | Yes        | Protein involved in early stages of meiotic recombination.                                      |
| YNL278W | CAF120| 15   | 2.86 | 2.11 | 0.74          |               |           |           | Yes        | Protein involved in controlling mRNA initiation, elongation, and degradation.                  |
| **Transport** |       |      |     |      |               |               |           |           |            |                                                                                                 |
| YLR401C | ATG23 | 15   | 2.15 | 1.59 | 0.74          |               |           |           | Yes        | Peripheral membrane protein required for cytoplasm-vacuole targeting pathway.                  |
| YOR384W | FRE5  | 15   | 4.11 | 2.32 | 0.56          |               |           |           |            | Putative ferric reductase with similarity to Fre2.                                               |
| YHR105W | YPT3S | 15   | 2.57 | 1.79 | 0.70          |               |           |           | Yes        | Endosomal protein that binds to PtdIns-3-P and to proteins involved in intracellular transport. |
| **Uncharacterized** |       |      |     |      |               |               |           |           |            |                                                                                                 |
| YMR251W | GTO3  | 15   | 4.27 | 2.26 | 0.53          |               |           |           | Yes        | Putative cytosolic omega class glutathione S-transferase.                                       |
| YOR138C | IRC14 | 30   | 2.57 | 1.47 | 0.57          |               |           |           |            | Hypothetical protein.                                                                           |
| YER188W |       | 15   | 2.55 | 1.79 | 0.70          |               |           |           | Yes        | Hypothetical protein.                                                                           |
| YFL015C |       | 15   | 6.09 | 3.72 | 0.61          |               |           |           | Yes        | Hypothetical protein.                                                                           |
| YHL012W |       | 15   | 2.55 | 1.84 | 0.72          |               |           |           | Yes        | Hypothetical protein.                                                                           |
the only mutants sensitive to high pH and that activation of Slt2 is significantly decreased in wsc1 mutants, whereas it is only marginally affected in mid2 cells. It is remarkable that the WSC1 gene was not identified as relevant for alkaline tolerance in our previous systematic screen for alkaline-sensitive mutations (13) or in a similar search using liquid cultures (11). It was reported recently that a conserved intracellular residue (Tyr303) is essential for complementation of the lytic defect at normal temperature (30 °C) of a wsc1 mid2 strain and for interaction with downstream Rom2, the GDP/GTP exchange protein for Rho1 (20). We have shown here that this residue is also crucial for tolerance to specific cell wall-damaging agents (such as Congo red) or high pH in an otherwise wild-type strain as well as for activation of the Slt2 pathway in response to ambient alkalinization (Fig. 5). This reinforces the idea that Wsc1 is stimulated by high pH and that this generates a signal that, probably through Rom2 and Rho1, activates the Slt2 MAPK module. Our data also indicate that the extracellular domain of Wsc1 is crucial for high pH tolerance and downstream signaling in response to this type of stress. Interestingly, deletion of the Cys-rich WSC domain results only in limited loss of function when cells are challenged with high pH or caffeine, similar to what was observed previously for high temperature (30), thus suggesting that, albeit characteristic of the four members of the family (Wsc1–4), the WSC motif may have little functional relevance. In contrast, deletion of the Ser/Thr-rich extracellular region, which renders the protein unable to rescue growth of the wsc1 strain at 35 °C (30), also results in inability to support growth in the presence of cell wall-damaging drugs or at high pH. This region is the target for O-mannosylation reactions that have been reported to be relevant for Wsc1 function (31, 32). Protein O-mannosyltransferases are represented in the yeast S. cerevisiae by a six-member family, Pmt1–6 (45), among which only Pmt2 and Pmt4 are believed to act on WSC and Mid2 proteins (31, 32). According to this, we observed that Wsc1 displays abnormal processing in the pmt2 mutant and that this mutant strain is sensitive to alkalinization (Fig. 6). Interestingly, we also detected altered Wsc1 processing and alkali sensitivity in the pmt1 mutant. These findings confirm that correct O-mannosylation is important for Wsc1 function and agree with the notion that the integrity of Wsc1 is essential for alkaline tolerance. It cannot be rejected, of course, that defective mannosylation of other cell-surface proteins may contribute to the observed sensitivity to high pH. In any case, our data suggest a previously unnoticed role for Pmt1 in the O-mannosylation of Wsc1.

Although our data clearly show an important role for Wsc1 in Slt2 signaling in response to high pH stress, it is also evident that activation of Slt2 by high pH is not fully abolished by the absence of Wsc1, indicating that the pathway receives alternative inputs. One possibility would be that other cell wall sensors such as Mid2 may provide such additional inputs, although our data suggest that, if so, their role certainly must be minor. Alternatively, the MAPK module might receive regulatory inputs triggered by alkalinization.
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independently of the upstream signaling elements, as has been suggested for other types of stress (46). While this manuscript was in preparation, it was reported that the Rim21/Rim101 pathway can contribute to the assembly of the yeast cell wall (47). This pathway has been shown in the past to be relevant for alkaline pH tolerance (6–8). Therefore, the Rim101 pathway appears to be a possible candidate for additional regulatory inputs to the Slt2 MAPK module under alkaline pH stress.

It is worth noting that, although osmotically protected slt2 cells are no longer sensitive to alkaline pH, wsc1 mutants still display a substantial degree of sensitivity (Fig. 1). This suggests that, in addition to impaired Slt2 signaling, cells lacking Wsc1 suffer other deficiencies that are relevant for endurance of alkaline pH stress. In any case, because our data indicate that alkaline stress does not lead to abnormal actin localization, these deficiencies cannot be attributed to the proposed role of Wsc1 in actin distribution (48, 49).

An interesting issue raised by our findings is the nature of the cell wall alterations provoked by exposure to high pH. Although this would require a specific study that is out of the scope of this work, our observation that the transcriptional profile in response to high pH stress correlates much better with certain cell-damaging agents (such as caspofungin) or mutations (fks1) than with others (i.e. Congo red) (Fig. 8) might provide some clue as to this question. As mentioned above, although Wsc1 and Mid2 serve partially overlapping functions in cell wall integrity signaling (22, 42), there are also examples of specific roles attributable to each protein. For instance, wsc1 mutants are sensitive to caspofungin, and this mutation prevents activation of Slt2 after exposure to this drug (50), whereas activation of Slt2 by exposure to Calcofluor white and α-factor involves Mid2, but not Wsc1 (42, 43). Caspofungin has been shown to inhibit the activity of β-1,3-d-glucan synthase (51), whereas Calcofluor white and Congo red interfere with chitin synthesis (52). This fits with the positive correlation between the transcriptional profile in response to high pH stress and that found in the fks1 mutant because FKS1 encodes one of the two catalytic subunits of 1,3-β-d-glucan synthase. Interestingly, the gene encoding the alternative 1,3-β-glucan synthase subunit (GSC2/FKS2) is induced by high pH, whereas the gas1 mutant (lacking β-1,3-glucanosyltransferase activity) is sensitive to high pH. In contrast, CHS1 and CHS3, which encode two of the three chitin synthases in yeast and which are responsible for most of this catalytic activity, are induced by exposure to Congo red, but not to high pH (10, 34). These results reinforce the notion that Wsc1 acts as a sensor of high pH and suggest that alkalization of the environment may cause cell wall damage by affecting glucan integrity or organization. A recent report has shown that a substantial number of genes related to cell wall construction are induced in Candida albicans by alkalization of the medium (53), suggesting that the adaptive response to high pH described here could be conserved in this pathogenic fungus. Interestingly, sudden acidification of the medium also results in activation of the Slt2 pathway in S. cerevisiae (54). However, in this case, the kinetics of the response are slower and more sustained and depend mainly on Mid2. These differences point to a specialized role for both cell membrane sensors in response to sudden environmental pH modifications.

Our results indicate that Slt2 activation represents a significant component of the yeast adaptive response to high pH. We observed that the expression level of a number of genes induced by high pH is reduced in slt2 cells (Table 1). Many of these genes fulfill one or more of the following criteria: 1) they are known to be induced by other cell wall-damaging agents; 2) their expression appears to be dependent on SLT2; and 3) they contain characterized or predicted consensus Rlm1- or Swi4-binding sites. It is worth noting that, in our case, lack of Slt2 results only in partial loss of the transcriptional response. This may be because of the complexity of the signaling pathways triggered by high pH stress, which may result in multiple positive inputs to a given gene. An example is the GSC2 gene, the induction of which can be triggered by both the Slt2 and calcineurin pathways (55), two pathways that are activated in response to high pH (this work and Refs. 8 and 10). An example of an even more complex transcriptional response to alkalization (the gene encoding the Ena1 Na+−ATPase) has been reported very recently by our laboratory (56). Another interesting point is that the number of Slt2-dependent genes is relatively small compared with other studies in which cells were subjected to cell wall damage (34). This may be related to the very transient nature of the alkaline induction of Slt2. It is remarkable, however, that, among these genes, we identified several that are related to cell wall biogenesis or maintenance (including GSC2, SKTS, CRH1, and DFG5) and that the increase in the expression of these genes is very similar to that observed after exposure of yeast cells to other cell wall-damaging agents such as Congo red and zymolyase (34). In two cases (SKTS and DFG5), we observed that mutation of the genes results in added sensitivity to alkali, thus being likely targets for the role of Slt2 in high pH tolerance. However, our observation that the double mutant is not as sensitive as the slt2 strain indicates that additional Slt2 targets must be present in the cell. In fact, the limited effect of the absence of the kinase on the transcriptional response induced by high pH points to the possibility that Slt2 targets relevant for high pH tolerance, but unrelated to regulation of gene transcription, may exist.

In conclusion, we propose that environmental alkalization generates cell wall damage that is sensed mainly by Wsc1. This cell membrane sensor triggers a signal that is transmitted to the Slt2 MAPK module, which is responsible for part of the transcriptional adaptive response of the cell to high pH.

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