Modulation of Yeast Sln1 Kinase Activity by the Ccw12 Cell Wall Protein*

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The yeast Sln1p sensor kinase is best known as an osmosensor involved in the regulation of the hyperosmolarity glycerol mitogen-activated protein kinase cascade. Down-regulation of Sln1 kinase activity occurs under hypertonic conditions and leads to phosphorylation of the Hog1p mitogen-activated protein kinase and increased osmotic stress-response gene expression. Conditions leading to kinase up-regulation include osmotic imbalance caused by glycerol retention in the glycerol channel mutant, fps1 (Tao, W., Deschenes, R. J., and Fassler, J. S. (1999) J. Biol. Chem. 274, 360–367). The hypothesis that Sln1p kinase activity is responsive to turgor was first suggested by the increased Sln1p kinase activity in mutants lacking Fps1p in which glycerol accumulation leads to water uptake. Also consistent with the turgor hypothesis is the observation that reduced turgor caused by treatment of cells with nystatin, a drug that increases membrane permeability and causes cell shrinkage, reduced Sln1p kinase activity (Tao, W., Deschenes, R. J., and Fassler, J. S. (1999) J. Biol. Chem. 274, 360–367). Reiser, V., Raitt, D. C., and Saito, H. (2003) J. Cell Biol. 161, 1035–1040). The turgor hypothesis is revisited here in the context of the identification and characterization of the cell wall gene, CCW12, as a determinant of Sln1p activity. Results of this analysis suggest that the activity of the plasma membrane localized Sln1p is affected by the presence or absence of specific outer cell wall proteins and that this effect is independent of turgor.

Little is known about how cellular osmosensors monitor changes in the osmolarity of the environment. Yeast sensors of hyper-osmotic stress are located in the plasma membrane and include the histidine kinase, Sln1p (3, 4), as well as the four transmembrane domain protein, Sho1p (5, 6). These proteins have distinct relationships to the osmotic response HOG1 MAP2 kinase pathway that they regulate. Sho1p is involved in recruiting the Pbs2p MAPK kinase to the cell surface (6), whereas Sln1p is the initiating member of a two-component type phospho-relay cascade that negatively regulates the HOG pathway (3). Msb2p, a putative third osmosensor, encodes a protein with a single transmembrane domain and a large putative extracellular domain that works in parallel with Sho1p (7).

The Sln1p sensor kinase has three activity states. Under normal growth conditions the activity of the Sln1p kinase is modest. Moderate activity is required for viability, because loss of Sln1p kinase activity (as in the null mutant) leads to inappropriate and lethal activation of the HOG pathway (8). Hyper-osmotic conditions shift the kinase to a low activity state. As Sln1p and other members of the SLN1-SKN7 pathway accumulate in the dephosphorylated form, the HOG pathway becomes activated, ultimately increasing expression of the osmotic response genes that are required for survival during osmotic stress (3). In addition to inactivation by hyper-osmotic conditions, the Sln1p kinase can also be stimulated. An increase in kinase activity leads to shutdown of the HOG pathway and activation of the Skn7p transcription factor (9). Sln1p kinase activation is apparent by the increase in expression of SLN1-SKN7 response genes such as OCH1 (10) and NCA3.

The role of the SLN1-SKN7 pathway is not well understood. Although the sensitivity of skn7Δ mutants to oxidative stress reflecting a separate SLN1-independent role for this transcription factor in the oxidative stress-response has been reported (11), cells lacking SKN7 are neither sensitive to hyper- nor hypo-osmotic conditions. A role for the SLN1-SKN7 pathway in cell wall integrity was suggested by the original isolation of skn7 mutants on the basis of their resistance to killer factor (kre, for killer resistance) (12). A large body of data link Skn7p to the PKC cell wall integrity pathway (10, 13–15) and suggest that the SLN1-SKN7 and the PKC pathways may function in parallel to protect cells from lysis because of the build up of turgor pressure.

The role of turgor pressure in Sln1p kinase regulation was first suggested by the increased Sln1p kinase activity in mutants lacking the major glycerol efflux channel, Fps1p (1). The absence of the glycerol channel leads to osmotic imbalance because of the accumulation of intracellular glycerol and the subsequent uptake of water (1, 16–18). Also consistent with a mechanical signal involving the presence or absence of pressure against the wall is the observation that reduced turgor caused by treatment of cells with reagents such as nystatin, which increases membrane permeability and causes cell shrinkage, caused a reduction in Sln1p kinase activity and a consequent increase in Hog1p activity in the absence of an osmotic stimulus (2).
In a recent screen of the yeast deletion collection for mutants with increased SLN1-SKN7 pathway activity, we identified a specific component of the cell wall that affects the activity of the SLN1-SKN7 pathway. Although genes involved in cell wall integrity had previously been identified as targets of the pathway (10), the role of specific wall components in osmotic stress sensing and signaling has not received much attention. Here we find that the relative abundance in the wall of the abundant GPI-anchored wall mannoprotein, Ccw12p, affects SLN1-SKN7 pathway activity. A working model in which the Ccw12 protein plays a special role in maintaining the Sln1 kinase in the repressed state is proposed. Conditions leading to reductions or loss of the Ccw12 protein from the cell wall are predicted to cause activation of the SLN1-SKN7 pathway.

**EXPERIMENTAL PROCEDURES**

**Strains—**Strains were constructed for these experiments or are from the Fassler laboratory collection (Table 1). Disruption of CCW12, SED1, and YLR111W and PPZ1 and PPZ2 in the S288C background (JF1565) was accomplished by PCR-mediated one-step gene disruption with a kanamycin (CCW12 and YLR111W) or HIS3 (SED1) cassette flanked by homologous tails. CCW12-F–300 (5‘-ATAGGATCCCATTTCCGCGCCAC-3‘) and CCW12-R+1000 (5‘-CCTCGAATTCGACACACACACATCACGTTT-3‘) primers were used to amplify a CCW12-KanR PCR fragment; YLR111W-F (5‘-ACGCGATCCAGTGGATAGGTGACAGTGG-3‘) and YLR111W-R (5‘-GATGAGCTAAGTGAACAGTTGG-3‘) primers were used to amplify a YLR111W-KanR PCR fragment; PPZ1-F–242 (5‘-AAGGACCACCATCAA-ACTGCTTCCA-3‘) and PPZ1-R+2291 (5‘-GTGAGGAGCAGATGAGCATTCA-3‘) primers were used to amplify a PPZ1-KanR PCR fragment; and PPZ2-F–202 (5‘-TGGCTAAGAA-TACACATATAGT-3‘) and PPZ2-R+2231 (5‘-CTATAACGACAACCTTATCGCAG-3‘) primers were used to amplify a PPZ2-KanR PCR fragment. Templates were genomic DNA prepared from the appropriate deletions from the Research Genetics disruption collection in strain BY4742 (19), SED1-1p-RS (ACTACAAGAACAAGCAAATAAAATACGTTGCTCTATTAGCGTGGCGGTATTTCACCAAG) and SED1+1017-RS (GAAGACATTAAAGAAGGCGGTAGTGTCAAACACACCCGATAGTGTGAGTGCAC) primers were used with pRS313 (Strategene) template to generate a SED1-HIS3 PCR fragment. Disruptions were confirmed by genomic PCR. The ppz1 and ppz2 disruption strains were crossed to generate the double mutant.

**Plasmids—**Plasmids were constructed for these experiments or are from the Fassler laboratory collection (Table 2). pHJ1470 was constructed by introduction of a 2.73-kb PCR product containing the complete AHP1, CCW12, YLR111W, and partial YLR112W open reading frames into Cln1- and EcoR1-digested pRS313 (CEN HIS3) (20). pHJ1497 and pHJ1498 are subclones of pHJ1470 in pRS313. A 1.6-kb NotI fragment encompassing the open reading frames of CCW12 and the adjacent hypothetical open reading frame YLR111W was cloned into Litmus28 (New England Biolabs) to generate plasmid pHJ1558. The 1.6-kb fragment was isolated from Xhol and Stul digestion and introduced into Xhol-SmaI cut pRS313 vector to generate pSS1559. pSS1560 was generated by site-directed mutagenesis of the start codon of YLR111W in pSS1558 using primers YLR111W-12F (5‘-GTTGGGCGCCGATATCGGGAAATTCAAAAACAAAAGC-3‘) and YLR111W-12R (5‘-CCGTTTTTGTGGAATTCCGGATATCGGGC-3‘) and pRS426-PTP2

**TABLE 1**

| Genotype                                      | Derivation                        |
|-----------------------------------------------|-----------------------------------|
| MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | Ref. 1                           |
| MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | JF2154:Δ::LEU2 derivative of JF1565 (1)               |
| JF1904 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | Ref. 45                           |
| JF1919 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | Also known as BJY1428 (21)         |
| JF2007 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | JF2007:Δ::LEU2 derivative of JF2007; one-step replacement |
| JF2008 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pRS426-PTP2                       |
| JF2030 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF1659; one-step replacement |
| JF2154 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF1904; one-step replacement |
| JF2181 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | ccw12Δ::KanMX derivative of JF2181; one-step replacement |
| JF2278 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF1919; one-step replacement |
| JF2297 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF1659; one-step replacement |
| JF2300 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF1904; one-step replacement |
| JF2336 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF2337; one-step replacement |
| JF2337 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF2007; one-step replacement |
| JF2338 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF2007; one-step replacement |
| JF2368 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF2007; one-step replacement |
| JF2343 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF2007; one-step replacement |
| JF2441 MATα can 6 cyc 6 his 3.2Δ200 leu 2Δ1 ura 3-2 trp 1Δ63 lys 2Δ202 | pccw12::KanMX derivative of JF2007; one-step replacement |

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The mutagenized 1.6-kb fragment was then cloned into pRS313 to generate pSS1560. pCLM1774 is a derivative of pCLM994 using EcoRI and StuI sites. Primer sequences for deletion were sequenced, and suppressor genes were identified by comparison of the sequence with the yeast genome data base.

**Tests of SLN1-SKN7 Activation by Hypotonic Conditions and by Zymolyase Treatment**—For hypotonic conditions, log phase cultures grown at 30 °C in YPD were diluted 1:1 with water. Aliquots removed at various times following dilution were subjected to filtration and the filters immediately frozen at −20 °C. Cells from frozen filters were resuspended in water and collected by low speed centrifugation for RNA or protein extraction. For zymolyase treatment, 1 unit/ml zymolyase was directly added to log phase cultures growing in YPD after adjusting the pH of the culture with Na2HPO4 to a final concentration of 20 mM (2). Zymolyase negative controls were also adjusted to 20 mM Na2HPO4. Reducing agents were not added. Following incubation at 30 °C for 60 min, cells were harvested by centrifugation and the supernatant was used to determine glycerol levels as per the manufacturer's instructions. Cell dry weight was determined by filtration and the filters immediately placed on dry ice. Cells were washed in 10 mM Tris, pH 8, 1 mM EDTA, pelleted, and disrupted in 16% trichloroacetic acid plus glass beads. Following centrifugation of the lysate, the pellet was resuspended in loading buffer (150 mM Tris/HCl, pH 7.4, 5% 2-mercaptoethanol, 15% glycerol), boiled 10 min, and subjected to electrophoresis on 10% SDS-polyacrylamide gels.

### Intracellular Glycerol Measurements

Intracellular glycerol was assayed enzymatically with a commercial glycerol determination kit (Roche Applied Science). Glycerol levels were normalized to cell dry weight. Extracts were prepared from log phase cultures grown in selective media. Pelleted cells were washed once with chilled selective media and once with 2 ml of 0.5 M Tris-HCl, pH 7.4, and resuspended in 2 ml of 0.5 M Tris-HCl, pH 7.4. 500 μl of the cell suspension was heated at 100 °C for 10 min and pelleted at 5000 rpm for 5 min, and the supernatant was used to determine glycerol levels as per the manufacturer's instructions. Cell dry weight was determined by filtering 1.5 ml of the cell suspension through pre-weighed 0.45-μm cellulose nitrate filters (Whatman). After drying at 70 °C for 2 h, filters were weighed, and the difference in weight was used to calculate cell dry weight. Intracellular glycerol levels were expressed as the average of six different assays using at least three different colonies per strain.

### Plate Assays

Log phase cultures (~10⁷ cells/ml) were diluted in YPD, and 10⁶, 10⁵, 10⁴, 10³, and 10² dilutions were spotted on plates containing YPD or YPD plus hygromycin B (15–50 μg/ml), Calcofluor White (30 μg/ml), or Congo Red (30 μg/ml).
micrograms per milliliter). Survival following hypo-osmotic stress was performed using log phase cells grown in YPD plus 1 M sorbitol. Cultures were diluted in the same medium and spotted on YPD with (iso-osmotic) or without sorbitol (hypo-osmotic) and incubated for 48 h at 30 °C.

**β-Galactosidase Assays**—Cultures were grown selectively at 30 °C and harvested at 10^7 cells/ml. Yeast protein extracts were prepared by glass bead lysis and extracts cleared by centrifugation. Activities were calculated in Miller units (22), normalized to protein, and expressed as the average of four to six assays using at least three independent colonies or transformants.

**Northern (RNA) Hybridization Analysis**—Total RNA was prepared using the hot acidic phenol method (23) from cultures grown to ~10^7 cells/ml in suitable medium. 20 μg of total RNA was loaded per lane of a 1% agarose gel and electrophoresis was performed at 75 V for 4 h. Hybridization was performed with PerfectHyb hybridization buffer (Sigma) as directed by the manufacturer. 32P-Labeled probes were prepared by using random primers with Prime-It random priming labeling kit (Stratagene) as directed by the manufacturer. 32P-Labeled probes were diluted in the same medium and spotted on YPD with 1 m DTT and resuspended in 1:10 volume of the same buffer. 1 m DTT was added to the cell suspension to a final concentration of 5 mM and incubated at 4 °C with shaking for 2 h. Cells were collected by low speed centrifugation, and the supernatant was analyzed. Cell pellets from DTT-extracted cells were used in preparing the “post-DTT” cell extract.

**Fluorescence Microscopy**—Log phase cultures expressing GFP fusions were pelleted and resuspended in residual culture medium. Cells were observed with a Leica DM RBE microscope and a Leica ×100 PL Fluotar 1.3 NA objective lens. Fluorescence images were captured using a Photonic Science digital charge-coupled device camera system. Images were processed and edited using Adobe Photoshop.

## RESULTS

**CCW12 Involvement in SLN1-SKN7 Signaling**—A screen of the yeast deletion collection (Research Genetics) based on increased expression of a lacZ reporter driven by the promoter of the SLN1-SKN7 target gene, OCH1 (P_{OCH1 lacZ}) (10), led to the identification of the YLR111W open reading frame and the CCW12 gene (YLR110C) as potential regulators of SLN1-SKN7 pathway activity. The short distance (330 bp) between ATGs of the two divergently transcribed open reading frames suggested that one deletion might eliminate the regulatory region required for expression of the other. To determine the open reading frame responsible for normal SLN1-SKN7 pathway activity, subclones were tested for complementation (Fig. 1). A subclone containing CCW12 and associated upstream regulatory sequence partially complemented the elevated reporter gene expression seen in the ccw12 and ylr111w mutants (vector); however, the same was not true of YLR111W. Because full complementation of the phenotype required both the CCW12 and YLR111W open reading frames, the pertinence of the YLR111W open reading frame was more directly tested by introducing a mutation into its predicted start codon (Fig. 1). The ATG mutant (pSS1560) complemented the ccw12 deletion phenotype to the same extent as the equivalent construct lacking the mutation (pSS1559), confirming that activation of SLN1-SKN7 signaling in the YLR111W deletion mutant was indirect.

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**FIGURE 1. Involvement of the CCW12 locus in SLN1-SKN7 pathway activation.** Subclones containing portions of the left arm of chromosome XII were tested for complementation of the pathway activation phenotype of the ccw12 and ylr111w deletion mutants from the Research Genetics Collection. The effects of subclones on pathway activity was measured using the pathway-responsive P_{OCH1 lacZ} reporter, pPJ1320 (10). β-Galactosidase (β-gal) activity values are the average of three or more transformants and are represented as Miller units normalized to protein. Standard deviations are less than 25%. pSS1560 is identical to pSS1559, but with a mutation of the putative translational start codon of YLR111W, which is marked by the X. Solid lines, clones that complement; stippled lines, clones that complement poorly or not at all.

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3 Z. Li and J. S. Fassler, unpublished data.
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The relevance of the cwc12 mutation to Sln1-Skn7 pathway activity was further investigated by RNA analysis of the Sln1-Skn7 target genes, Och1 and Och1, and its dependence on Skn7. Northern (RNA) hybridization analysis of the Nca3 and Och1 expression in CWC12 skn7Δ (JF1904, lane 1) and cwc12Δ skn7Δ (JF2279, lanes 2-4) strains containing different alleles of skn7 carried on CEN plasmids (pSL232 [SKN7-]), pSL237 [skn7ΔD427N], or pRS315 [skn7Δ]). DED1 hybridization is shown as a normalization control.

| TABLE 3 | SKN7 and SLN1 dependence of cwc12 activation of the OCH1 SLN1-SKN7 pathway target gene |
|-----------------|---------------------------------|-------------------------------|
| Genotype | β-Galactosidase activity (S.D.) | Induction (cwc12Δ/CWC12) |
| SKN7 CWC12Δ | 320.8 (25.8) | 1.0 |
| SKN7 cwc12Δ | 538.9 (72.4) | 1.7 |
| skn7ΔD427N cwc12Δ | 123.5 (30.1) | 0.4 |
| skn7ΔD427N cwc12Δ | 26.5 (10.2) | 0.08 |
| SLN1 SSK1 CWC12Δ | 360.8 (71.2) | 1.0 |
| SLN1 SSK1 cwc12Δ | 743.7 (59.9) | 2.1 |
| SLN1 ssk1Δ CWC12Δ | 136.6 (30.1) | 1.0 |
| SLN1 ssk1Δ cwc12Δ | 474.9 (44.3) | 3.5 |
| sln1Δ ssk1Δ CWC12Δ | 40.3 (13.9) | 1.0 |
| sln1Δ ssk1Δ cwc12Δ | 19.1 (0.8) | 0.5 |

| a | β-Galactosidase activities are given in Miller units normalized to protein concentration and represent the averages of three measurements. Standard deviations are given in parentheses. |
| b | Each strain is transformed with the CEN-based P_{OCH1-lacZ}, pZL1320 (10). |
| c | CWC12 skn7Δ, JF1904; cwc12Δ skn7Δ (JF2279) strains carry the wild type (pSL232) or D427N (pSL237) alleles of SKN7 on CEN plasmids, and strains lacking SKN7 carry the empty pRS315 vector. |
| d | Strains include CWC12 SSK1 SLN1, JF1565; CWC12 skn7Δ SLN1, JF1919; cwc12Δ SSK1 SLN1, JF2276; cwc12Δ ssk1Δ SLN1, JF2301; CWC12 skn7Δ sln1Δ, JF2181; cwc12Δ ssk1Δ sln1Δ, JF2300. |

Note: Changes in turgor are not sufficient for Sln1-Skn7 pathway activation—The possibility that up-regulation of the Sln1 kinase is attributable to increased intracellular pressure in the cwc12 mutant as it appears to be in the fps1 mutant (1) was evaluated by testing the response of the Sln1-Skn7 pathway to other treatments known to affect turgor. The PKC cell wall integrity MAPK pathway is activated in response to increased turgor caused by a wide variety of conditions (27, 28). Two of these, hypo-osmotic shock and the ppz1 ppz2 double mutant which accumulates intracellular K+, were used in evaluating the response of the Sln1-Skn7 pathway to turgor.

Cultures grown in rich media (YPD) were diluted 1:1 with water. This treatment has been shown to activate the PKC pathway very quickly (27). Phosphorylation of the Slt2p/Mpk1p MAP kinase in the PKC pathway within 2 min of exposing cultures to a dilute environment confirms that this treatment does cause an increase in turgor (Fig. 3C). Because the SLT2/MPK1 gene is also a target of the PKC pathway (29), its expression was examined as a base line for normal PKC pathway target gene induction kinetics (Fig. 3A, bottom panel). SLT2/MPK1 expression was elevated 2-fold in one experiment (Fig. 3A) and 3.5-fold in a replicate experiment at the 10-min time point.

To determine the effect of an increase in turgor sufficient to activate the PKC pathway on Sln1-Skn7 target genes, NCA3 and OCH1 gene expression was monitored over time. NCA3 levels were increased ~2-fold at the 30-min time point. This increase was not observed in a strain carrying the skn7ΔD427N allele indicating that the increase was SKN7 Asp-427-dependent. OCH1 levels were increased only very slightly to 1.2- or 1.3-fold and then restored to untreated levels by 30 min (Fig. 3A).

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4 J. S. Fassler, O. Carmel-Harel, G. Storz, and A. Gasch, unpublished data.
The PPZ1 and PPZ2 phosphatases are regulators of K\(^+\) and pH homeostasis (30), and the double mutant exhibits elevated intracellular K\(^+\) levels (30) leading to water uptake. An associated increase in cell size and PKC pathway activation (Fig. 3D) has been observed (29), consistent with an increase in turgor in the mutant. If SLN1-SKN7 pathway activation in ccw12 and fsp1 mutants and zymolyase-treated cells is attributable to changes in membrane tension, the ppz1, ppz2 double mutant would be expected to activate the pathway. No increase in the expression of NCA3 (Fig. 3B) or OCH1 (data not shown) was observed in the ppz1 ppz2 double mutant, despite the expected increase in NCA3 (Fig. 3B) and OCH1 (not shown) expression in the ccw12 mutant. Taken together, the absence of an effect on SLN1-SKN7 target gene expression in the ppz1 ppz2 double mutant combined with the slow and modest increases observed following hypo-osmotic stress suggests that simple elevation in turgor is not sufficient for Sln1 kinase stimulation.

Ccw12p Does Not Regulate the Fps1p Glycerol Channel—To test the possibility that Ccw12p might regulate activity of the Fps1p glycerol channel, loss of which activates the SLN1-SKN7 pathway (1), intracellular glycerol levels were measured in the ccw12 mutant. In the absence of the glycerol channel, accumulated glycerol cannot be released to the medium, and the resulting increase in turgor causes a 100-fold decrease in viability upon hypotonic shift (16, 18). The ccw12 mutant likewise exhibited loss of viability upon hypotonic shock. A shift from growth media containing 1 M sorbitol to media lacking sorbitol led to comparable losses in viability in both the ccw12 and the fsp1 mutants (Fig. 4). Unlike the fsp1\(\Delta\) mutant, which exhibits a 4-fold increase in intracellular glycerol relative to wild type (16), the ccw12 mutant showed no elevation in glycerol levels despite activating the SLN1-SKN7 pathway to an extent equivalent to fsp1\(\Delta\) (Table 4). These results suggest that activation of the SLN1-SKN7 pathway in strains lacking the Ccw12 protein is not the result of changes in intracellular glycerol concentration, but it may instead be due to a weakened cell wall or to the absence of the Ccw12 protein itself.

Activation of the SLN1-SKN7 Pathway Is Not a General Feature of Cell Wall Mutants—To determine whether the SLN1-SKN7 pathway responds to all major cell wall perturbations or exclusively to the absence of Ccw12p, the effect on the SLN1-SKN7 pathway activity of wall gene mutations, mutations in genes affecting the wall, or mutations in genes encoding proteins similar to Ccw12p was evaluated. Included in the survey were genes showing partial sequence similarity to Ccw12 (FLO1, SED1, AGA1, FIG 2, PRY3, FLO10, YDR124C, SPI1, and WSC4), genes encoding proteins involved in mannosylation (MN1, MNN2, MNN9, OCH1, PMI1, PMI2, PMI3, PMI5, and PMI6), and genes encoding proteins with other cell wall functions (FKS1, β-1,3-glucan synthase subunit; KNR4, regulation of cell wall synthesis and possible coordination with cell cycle; KRE6, β-1,6-glucan synthase; and GAS1, β-1,3-glucanosyl-
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A. WT

ccw12Δ

fks1Δ

gnn1Δ

mid1Δ

kre6Δ

och1Δ

WT

ccw12Δ

pmnt1Δ

pmnt2Δ

pmnt3Δ

YPD

YPD + HB

B. β-galactosidase Activity

1.200

1.100

1.000

0.900

0.800

0.700

0.600

0.500

0.400

0.300

0.200

0.100

0.000

1.0 1.4 0.9 1.1 1.0 0.7 0.6 0.4

\( P_{OCH1}^{lacZ} \) / \( P_{OCH1(min)}^{lacZ} \)

FIGURE 5. Hygromycin B-sensitive wall mutants do not activate the SLN1-SKN7 pathway. Strains were KanMX marked deletions from the haploid Research Genetics Deletion Collection assembled based on their known functions in wall integrity and tested for hygromycin B sensitivity and activation of the SLN1-SKN7 pathway-dependent \( P_{OCH1}^{lacZ} \) reporter gene. A, hygromycin B sensitivity test. Strains were grown to log phase in YPD and 10-fold dilutions spotted on YPD or YPD containing 25 \( \mu \)g/ml hygromycin B plates, which were incubated at 30°C for 48 h and then scanned. B, β-galactosidase was measured in a at least three transformants carrying the \( P_{OCH1}^{lacZ} \) reporter, pZL1320 (diagonal striped bars), or the \( P_{OCH1(min)}^{lacZ} \) reporter, pSL1156 (dark bars). Activity is represented in Miller units and normalized to protein concentration. The number below the plot is the ratio of average activity for the mutant (\( P_{OCH1}^{lacZ} \) reporter/\( P_{OCH1(min)}^{lacZ} \) control reporter) over the average activity for a wild type (WT) strain measured in the same experiment.

interest (Fig. 5A). Fig. 5B shows all mutants with an HB\(^5\) phenotype as well as a sampling of others tested. An apparent increase in the och1 mutant was found to be nonspecific (see elevated \( P_{OCH1(min)}^{lacZ} \) and a relative OCH1/minimal reporter ratio of 0.4). These data suggest that the SLN1-SKN7 pathway activation phenotype is not a generic consequence of reduced wall integrity but rather a specific consequence of the loss of the Ccw12 protein.

Suppression of ccw12 Wall and Activation Phenotypes by SED1 Overexpression—Insight into the role of Ccw12p in SLN1 signal transduction was sought by looking for genes that are able to complement the HB\(^5\) phenotype of the ccw12 mutant. The strongest high copy suppressors carried the SED1 gene. Subclones containing only the SED1 gene partially suppressed the HB sensitivity and reporter gene activation phenotypes of the ccw12 mutant (Fig. 6A). The lower SED1 dosage (centromere (CEN)) was somewhat more effective than high dosage (2\( \mu \)m) suggesting that there is an optimal stoichiometry for SED1 in the wall. SED1 encodes a highly glycosylated, glucanase-extractable, GPI-anchored cell wall protein that is most abundant in the walls of stationary phase cells (32). To investigate the role of SED1 and CCW12 in wall integrity and SLN1-SKN7 pathway regulation, the effects of single and double deletions were compared. Resistance to hygromycin B and SLN1-SKN7 pathway activity measured by the \( P_{OCH1}^{lacZ} \) reporter was normal in the sed1Δ strain (Fig. 6B). In contrast, ccw12Δ phenotypes, including HB\(^5\) and SLN1-SKN7 pathway activation, were exacerbated in the absence of SED1 (Fig. 6) consistent with previous reports that elevated SED1 expression in mutants lacking GPI-anchored wall proteins such as Ccw12p has a compensatory effect (33).
were treated with low levels of zymolyase (1 unit/ml zymolyase (JF1565)) (JF1565) and skn7D427N (JF2336) cultures after 60 min of treatment with 1 unit/ml zymolyase (+) or without treatment (−). OCH1 and NCA3 expression was normalized to expression of the SLN1-SKN7-independent CDC33 gene.

**FIGURE 7. Activation of the SLN1-SKN7 pathway by zymolyase treatment.** Northern (RNA) hybridization analysis of RNA was prepared from wild type (wt) (JF1565) and skn7D427N (JF2336) cultures after 60 min of treatment with 1 unit/ml zymolyase (+) or without treatment (−). OCH1 and NCA3 expression was normalized to expression of the SLN1-SKN7-independent CDC33 gene.

**Perturbation of the Wall with Low Level Zymolyase Treatment Causes SLN1-SKN7 Pathway Activation**—To investigate whether more general cell wall insults such as enzymatic weakening of the cell wall might affect the pathway, wild type cells were treated with low levels of zymolyase (β1–3-glucanase with trace amounts of alkaline protease and mannanase). Treatment was kept well below concentrations needed to digest the wall, and reducing agents that are normally added to stimulate zymolyase activity were omitted (34). There was no loss of light scattering at A<sub>600</sub> consistent with minimal damage to the wall. These zymolyase concentrations nonetheless caused induction of SLN1-SKN7-dependent target genes, NCA3 and OCH1, to an extent comparable with that seen in the ccw12 mutant. Induction by zymolyase was dependent on SKN7 and on the phosphorylatable aspartate, Asp-427. skn7ΔΔ mutants did not exhibit an increase in NCA3 expression after zymolyase treatment (Fig. 7).

**Zymolyase-treated Cells and fps1 Mutants Have Reduced Levels of Wall-associated Ccw12**—Because zymolyase is an impure glucanase preparation with a contaminating protease component, it has the potential to digest outer wall proteins. The effect of zymolyase on SLN1-SKN7 activity might therefore be attributable to loss of the Ccw12 protein from the cell wall. To test this possibility, wall-associated Ccw12p levels in zymolyase-treated cells were compared with levels in untreated cells.

The GPI anchor of the Ccw12 (covalent cell wall) protein is believed to be responsible for its association with the wall (35). Consistent with this, the protein can be extracted using glucanase with a contaminating protease preparation (34). There was no loss of light scattering at A<sub>600</sub> consistent with minimal damage to the wall. Levels of Wall-associated Ccw12p were compared with untreated cells. To test this possibility, wall-associated Ccw12p levels in zymolyase-treated cells were compared with levels in untreated cells.

**FIGURE 8. Zymolyase treatment and the fps1 mutation cause reductions in wall-associated Ccw12p.** Anti-HA Western analysis of Ccw12 protein levels in strains carrying the CCW12-HA expression plasmid pSS1564. A, levels of cell wall-associated Ccw12p in various strains were compared by Western analysis following recovery of surface proteins upon (5 min) DTT treatment and in extracts prepared from the DTT-treated cell pellet. The α-Hog1 Western and Ponceau S staining show even loading of protein extracts in each lane. Equivalent volumes of the DTT supernatants were loaded in the top panel. Wild type (WT), ccw12Δ (JF2278) carrying pSS1564 (CCW12-HA); ccw12Δ, JF2278 carrying vector; fps1Δ, ccw12Δ fps1Δ (JF4442) carrying pSS1564 (CCW12-HA). Cells were treated with 1 unit of zymolyase 100T (US Biological) prior to DTT extraction in lane 6. B, strains were grown to log phase, and 10-fold dilutions were spotted on YPD + 0.5 μM sorbitol plates and the same type of plate but containing 25 μg/ml HB or 30 μg/ml CF. Plates were incubated at 30 °C for 48 h prior to scanning.

The effect of zymolyase on SLN1-SKN7 activity might therefore be attributable to loss of the Ccw12 protein from the cell wall. To test this possibility, wall-associated Ccw12p levels in zymolyase-treated cells were compared with levels in untreated cells. The GPI anchor of the Ccw12 (covalent cell wall) protein is believed to be responsible for its association with the wall (35). Consistent with this, the protein can be extracted using glucanase in the form of a very large (>300 kDa) polydisperse band, which includes remnants of the glucan-chitin meshwork from the wall (33). A 58-kDa cytoplasmic Ccw12p species is observed in cleared crude cell extracts. This species is larger than the 16-kDa encoded protein because of extensive O-mannosylation and the presence of a GPI anchor (33). Unexpectedly, we found that treatment of intact cells with the reducing agent, dithiothreitol, extracted a similar ~58-kDa Ccw12p species from wild type cell walls (Fig. 8A, lanes 1 and 2) suggesting that a fraction of wall-associated Ccw12p is not actually GPI anchored but may be associated with the wall via disulfide bonds.

A major reduction in DTT-extractable Ccw12 protein was observed in cells treated with the low levels of zymolyase sufficient to activate the SLN1-SKN7 pathway (Fig. 8A, lanes 5 and 6). That the CCW12 gene is expressed normally under these conditions is shown by comparing levels of the 58-kDa Ccw12p species in cleared crude cell extracts prepared from cells of each genotype following their treatment with DTT. Protein levels in each extract were normalized using anti-Hog1p reactivity and separately by Ponceau S staining (Fig. 8A, 3rd and 4th panels). If reduced wall-associated Ccw12p were responsible for SLN1-SKN7 pathway activation, a similar reduction in wall-associated Ccw12p would be expected in the fps1 mutant. Our analysis of DTT-extractable Ccw12p shows that the fps1 mutant lacks detectable DTT-extractable Ccw12 protein (Fig. 8A, lane 3).
Although a glycerol channel defect is not expected to cause wall abnormalities, the \(fps1\) mutant does exhibit sensitivity to the chitin-binding drug, CFW (Fig. 8B). Sensitivity to CFW is indicative of increased chitin levels, a phenotype common in mutants with reduced wall integrity. Although the CFW\(^S\) phenotype in the \(fps1\) mutant differs from the HB\(^S\) phenotype of the \(ccw12\) mutant, the reduction in Ccw12 protein in walls of the \(fps1\) mutant is indicative of a correlation between activation of the pathway and reduction in Ccw12p levels in the wall.

The Sln1 Extracellular Domain Is Required for Pathway Activation by Wall Perturbants—If the state of the cell wall plays a role in Sln1p kinase regulation, detection of that signal is expected to involve the extracellular (periplasmic) domain (ECD) of Sln1p. An ECD deletion encompassing amino acids 76–305, roughly 80% of the domain, was constructed. Despite the large deletion, the ECD mutant was viable, showed partial localization to the cell periphery (Fig. 9A), and exhibited Sln1p phosphorylation kinetics similar to wild type upon treatment with salt (Fig. 9C). Because mutants lacking Sln1p kinase activity are inviable because of constitutive activation of the HOG1 pathway, the viability of the ECD mutant suggests that basal kinase levels are not substantially affected. Likewise, normal Sln1p phosphorylation kinetics in the ECD mutant suggests that Sln1p inactivation is normal. To investigate the effect of the ECD mutation on Sln1p activation, the effect of the activating \(ccw12\) mutation and of zymolyase treatment on \(OCH1\) and NCA3 levels were compared in \(SLN1\) and \(sln1\Delta ECD\) strains. Both types of activation were reduced or eliminated in the ECD deletion mutant (Fig. 9D). Although Western analysis revealed a reduction in the expression of the ECD mutant relative to wild type (Fig. 9B), there was no detectable effect on basal NCA3 or \(OCH1\) expression (Fig. 9D), suggesting that the reduced protein levels are nonetheless sufficient for normal levels of signaling. The requirement for the extracellular domain of Sln1p is consistent with models of activation involving physical contact between Sln1p and some component of the wall which remains to be defined.

Physiological Role for the Sln1 Pathway in Cells Lacking a Full Complement of Wall Proteins—Involvement of the Sln1-Skn7 pathway in wall composition is suggested by the increased resistance to hygromycin B in \(sln1^+\) mutations that activate the pathway and modest sensitivity to the drug in an \(skn7^D427^N\) mutant (10). This is consistent with Sln1-Skn7 pathway regulation of the \(OCH1\) gene encoding an \(α,1,6\)-mannosyltransferase involved in N-glycosylation of wall mannoproteins (10).

**FIGURE 9.** The extracellular domain is required for Sln1 kinase activation by wall perturbations. A, representative field showing localization of \(SLN1\)-GFP (pSS1881) or \(sln1\Delta ECD\)-GFP (pSS1904). BF, bright field. B, Western analysis showing reduced levels of \(sln1\Delta ECD\) compared with full-length \(SLN1\). \(SLN1, JF2007 (sln1\Delta)\) carrying full-length \(SLN1\)-myc (pCLM994); \(sln1\Delta ECD\), JF2007 carrying \(sln1\Delta ECD\)-myc (pCLM1774). C, comparison of Hog1 phosphorylation kinetics in \(SLN1\) JF2008 (\(sln1\Delta sho1\)) carrying full-length (pCLM994) and \(sln1\Delta ECD\) JF2008 (\(sln1\Delta sho1\Δ) carrying \(sln1\Delta ECD\) (pCLM1774)) strains following addition of 0.4 M NaCl. D, Northern (RNA) hybridization analysis of RNA prepared from wild type cultures from the \(sln1\Delta\) strain, JF2007, or \(sln1\Delta ccw12\Δ\) strain, JF2368, transformed with the \(SLN1\) expression plasmids, pCLM994 (\(SLN1\)) or pCLM1774 (\(sln1\Delta ECD\)), and treated with 1 unit/ml zymolyase (+) where indicated. NCA3 expression was normalized to expression of the \(SLN1\)-SKN7-independent CDC33 gene. The extent of induction by zymolyase is shown relative to the untreated \(SLN1\) or \(sln1\Delta ECD\) strains.
Regulation of the Sln1 Histidine Kinase

The modest hygromycin B sensitivity of the skn7D427N mutant suggests that the role of the SLN1-SKN7 pathway in the integrity of a relatively static wall is minor. To investigate whether the SLN1-SKN7 pathway may assume more importance during wall remodeling, the effect of the SLN1 phospho-relay mutation, sln1D1144N, was examined in the ccw12 mutant. The lethality of the sln1D1144N mutation was suppressed in these strains by the presence of a plasmid expressing the PTP2 Hog1p phosphatase. Like the skn7D427N mutation (10), the sln1D1144N mutation alone exhibited only modest sensitivity to hygromycin B (reflecting wall glycosylation) and Congo Red (reflecting chitin level abnormalities). In the absence of the Ccw12 protein, however, the sln1D1144N mutation caused striking wall phenotypes. The effect of the sln1 mutation was most apparent on media containing 1x sorbitol as osmotic support (Fig. 10), which corrects hygromycin B and Congo Red phenotypes attributable to the absence of the Ccw12 protein. These observations point to a specific role for the SLN1-SKN7 pathway in a compensatory wall formulation that may be elicited by the absence of Ccw12 protein.

DISCUSSION

The yeast wall determines the shape and the integrity of the organism during growth and cell division. The relatively simple composition of the yeast wall, 40% mannoproteins, 60% β-glucan (85% long chain, and 15% short chain), and 2% chitin (36), nonetheless provides the necessary stability against tension caused by high internal hydrostatic pressure. This property of the wall is presumed to be due to the formation of interlinkages between the individual wall components to create a highly branched meshwork. A structure consisting of covalently linked β-1,3- and β-1,6-glucan, chitin, and mannoproteins has been reported (37, 38), and a stable phosphodiester linkage has been proposed to connect the mannoproteins to the rest of the meshwork (39).

CCW12 encodes one of three covalently linked cell wall proteins present in the most highly phosphorylated cell wall fraction of yeast cells (35). The ccw12 mutant exhibits reduced agglutination and mating (35) possibly due to inaccessibility of agglutinins at the cell surface or to other changes in the structure of the cell wall. Increased sensitivity to Calcofluor White, Congo Red (35), hygromycin B, and low doses of zymolyase5 (40) suggest extensive alterations in cell wall composition and structure in the ccw12 mutant.

The absence of the abundant Ccw12 protein from the cell surface activates the SLN1-SKN7 pathway, causing 2–3-fold increase in transcription of the OCH1 and NCA3 target genes in a SLN1 and SKN7 Asp-427-dependent manner. The isolation of SED1 as a multicopy suppressor of the ccw12 wall and activation phenotypes was predicted from previous reports in which SED1 was found to be up-regulated in mutants with cell wall instability caused by the absence of GPI-anchored mannoproteins, including Ccw12p (33). Like Ccw12p, Sed1p is a predicted GPI-anchored cell wall mannanoprotein. In addition, conserved sequences (36% identity over 74 residues of the 133 amino acids Ccw12 (41)) in the two proteins suggest possible functional redundancy. Unlike CCW12, SED1 is most highly expressed in stationary phase (32) and is regulated by the PKC pathway (42) in response to cell wall stress (43, 44). Our finding that SED1 overexpression partially suppresses both the wall phenotypes of the ccw12 mutant and the SLN1-SKN7 pathway activation phenotype indicates that these two phenotypes are inter-dependent and that SLN1-SKN7 pathway activation may therefore be attributable to the wall defects of the ccw12 mutant. The observation that the ccw12 sed1 double deletion exhibits both enhanced sensitivity to cell wall perturbants as well as increased activation of SLN1-SKN7 target genes further supports the conclusion that pathway activation is a direct consequence of wall defects because of the absence of specific mannanoproteins on the outer surface of the cell.

Although osmotic imbalance and increased turgor in the fps1 mutant was thought to be responsible for SLN1-SKN7 pathway activation (1), more extensive testing shows that this is not a sufficient condition for Sln1p kinase activation. For example, hypotonic stress conditions sufficient to cause immediate activation of the PKC MAPK pathway cause a slow and relatively weak response in SLN1-SKN7 target gene expression. Similarly, SLN1-SKN7 target genes are not activated in the ppz1 ppz2 double mutant, in which elevated intracellular K+ levels cause increased phosphorylation of the Slt2p/Mpk1p MAPK. These observations suggest that changes in turgor are unlikely to be the proximate cause of activation.

The observation that zymolyase-treated cells and the fps1 mutant both have reduced levels of wall-associated Ccw12p leads to the alternative explanation that Ccw12p plays a specific role in regulating Sln1p kinase activity. Because soluble Ccw12p levels are unaffected in the fps1 mutant (Fig. 8A), the effect is not on expression or protein stability. The basis for the reduction in cell wall association of Ccw12p in the fps1 mutant will require further investigation.

The increase in Sln1p kinase activity in response to Ccw12p loss required the extracellular domain. Because absence of the extracellular domain did not affect viability (SLN1 is an essential gene), the kinetics of Hog1p phosphorylation in response to salt or basal activity of SLN1-SKN7-dependent genes (Fig. 9), we conclude that the ECD is required for activation of the

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5 S. S. Narang and J. S. Fassler, unpublished observations.
kinase, but it is less critical for its inactivation, which is needed to trigger Hog1p phosphorylation.

The role of the cell wall in Sln1p kinase regulation has been hinted at previously. Cells lacking walls were found to activate the HOG1 pathway (2) and thus inactivate the Sln1p kinase. Because wall removal might mimic a reduction in turgor, these results were interpreted to mean that turgor is important for Sln1 kinase activation. In this study we find that the presence or absence of the Ccw12 protein in the wall, but not changes in turgor, nor other types of alterations in the wall, affects the activation of the Sln1 kinase. Our recent observation that small deletions of specific ECD sequences reduce Sln1p kinase activation by zymolyase treatment are consistent with models in which loss of Ccw12p permits an interaction between the Sln1p ECD and one or more molecules associated with the inner face of the wall that are required for activation. Because Ccw12p is itself localized to the outer face of the wall, the interaction with Sln1p is unlikely to be direct. Consistent with the conclusion that the Ccw12p and Sln1 proteins do not interact are results of random mutagenesis of the CCW12 coding region in which 100% (20 out of 20) SLN1-SKN7 activating mutations isolated from the wall leads to the appearance of novel inner wall or periplasmic molecules that are poised to contact the Sln1 ECD thus causing its activation.

Our study of the Ccw12 wall protein also provides insight into the physiological role of the SLN1-SKN7 pathway. The mild hygromycin B phenotype seen in sdn7D427N (10) and sln1D1144N (Fig. 10) mutants suggests that the SLN1-SKN7 pathway plays a minor role in cell wall integrity under normal growth conditions. However, the pathway appears to become more important when the wall is reformulated as it is expected to be in the absence of Ccw12p or in zymolyase-treated cells. In the ccw12A background, loss of SLN1-SKN7 pathway activity because of the sln1D1144N mutation caused striking wall phenotypes, especially on media containing 1 M sorbitol as osmotic support. Sorbitol corrected the hygromycin B and Congo Red phenotypes caused by the ccw12 mutation alone but was unable to correct the growth defect caused by the sln1 mutation. Hence, SLN1-SKN7 pathway activity appears to gain importance under conditions requiring wholesale wall remodeling. We therefore propose that Ccw12p levels may be a marker for conditions requiring wall remodeling activity.

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