Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in *FKS2-lacZ* expression, glucanase resistance and thermotolerance

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The protein kinase C (*PKC1*) pathway is essential for maintaining cell integrity in yeast. Here it is shown that various forms of cell wall damage result in activation of the downstream MAP kinase Slt2/Mpk1. Several cell wall mutants displayed enhanced *FKS2-lacZ* expression, a known output of Slt2 activation. A similar response was obtained with wild-type cells grown in the presence of the cell wall perturbants Calcofluor white and Zymolyase. Upregulation of *FKS2-lacZ* in response to sublethal concentrations of these agents fully depended on the presence of Slt2. The same cell wall stress conditions resulted in dual threonine and tyrosine phosphorylation of Slt2. Both Slt2 phosphorylation and *FKS2-lacZ* induction could be largely prevented by providing osmotic support to the plasma membrane. Interestingly, Slt2 phosphorylation in response to cell wall damage required the putative plasma-membrane-located sensor Mid2 but not Hcs77/Wsc1. Finally, cell wall perturbation gave rise to cells with increased resistance to glucanase digestion and heat shock. These responses depended on the presence of Slt2. These results indicate that weakening of the cell wall activates the Slt2/Mpk1 MAP kinase pathway and results in compensatory changes in the cell wall.

Keywords: wall damage, wall integrity, Slt2, Mid2, thermotolerance

INTRODUCTION

The yeast cell wall is an essential organelle that, due to its mechanical strength, is able to resist internal turgor pressure and consequently prevents cell lysis. Synthesis and assembly of 1,3- β -glucan are required for the construction of a strong and functional wall. *FKS1* encodes a subunit of the yeast 1,3- β -glucan synthase (Mazur *et al.*, 1995) and Gas1 is involved in remodelling of 1,3- β -glucan (Popolo & Vai, 1999). Loss of either of these two plasma-membrane-localized proteins results in significantly reduced levels of 1,3- β -glucan in the wall and in the formation of viable but swollen cells (Ram *et al.*, 1995; Popolo & Vai, 1999). Both *fks1* Δ and *gas1* Δ

cells also display an increase in chitin content and in the expression of the alternative subunit of the yeast 1,3- β -glucan synthase, *Fks2* (Mazur *et al.*, 1995; Ram *et al.*, 1998). These changes have been proposed to be part of a set of compensatory reactions to ensure cell wall integrity. Simultaneous depletion of protein kinase C in these mutants is lethal, suggesting that their viability depends on signalling through the *PKC1*-controlled cell integrity pathway (Garrett-Engle *et al.*, 1995; Popolo *et al.*, 1997).

Protein kinase C activates a linear MAP kinase cascade that consists of a MAPKK kinase, Bck1/Slk1; a pair of redundant MAPK kinases, Mkk1 and Mkk2; and a MAP kinase, Slt2/Mpk1 (Gustin *et al.*, 1998; Mellor & Parker, 1998; Heinisch *et al.*, 1999). Signalling through MAP kinase cascades results in activation of MAP kinases by dual phosphorylation of the conserved threonine and tyrosine residues in subdomain VIII

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Abbreviation: CFW, Calcofluor white.

(Cobb & Goldsmith, 1995). Activation of MAP kinases typically leads to the activation of downstream transcription factors, and consequently to changes in gene expression patterns. Known outputs of Slt2 activation are increased expression of *FKS2* and several other genes encoding cell-wall-related proteins (Zhao *et al.*, 1998; Jung & Levin, 1999), signalling to the actin cytoskeleton (Helliwell *et al.*, 1998) and increased thermotolerance after preceding mild heat treatment (Kamada *et al.*, 1995). Loss of any of the components of the *PKC1*-MAPK cascade results in osmotic-remedial cell lysis and downregulation of a subset of cell wall genes, demonstrating a regulatory role for this pathway in cell wall construction (Igual *et al.*, 1996; Gustin *et al.*, 1998; Mellor & Parker, 1998).

Activation of the *PKC1* pathway seems to be mediated through a family of plasma membrane-localized sensors, Wsc1-4, Mid2 and its homologue Mtl1 (Verna *et al.*, 1997; Gray *et al.*, 1997; Jacoby *et al.*, 1998; Rajavel *et al.*, 1999; Ketela *et al.*, 1999). Cell wall damage and growth at elevated temperatures generate a signal that is transduced from Hcs77/Wsc1 via the GTPase Rho1 to Pkc1 and results in depolarization of the actin cytoskeleton independent of the MAP kinase cascade (Delley & Hall, 1999). Several cell wall mutants show enhanced GEF activity towards Rho1 (Bickle *et al.*, 1998) and require Mid2 (Ketela *et al.*, 1999) or Pkc1 (Roemer *et al.*, 1994; Garrett-Engele *et al.*, 1995; Popolo *et al.*, 1997) for survival, suggesting that cell wall stress in these mutants is perceived and signalled through this pathway.

Here, we analyse Slt2 activation in several cell wall mutants and in cells with an altered cell wall due to treatment with Calcofluor white (CFW), a fluorescent dye that hinders normal cell wall assembly, and with Zymolyase, a cell-wall-degrading enzyme preparation. We found dual phosphorylation of Slt2 and induction of *FKS2-lacZ* expression in an Slt2-dependent manner in response to these wall-weakening conditions. Additionally, we show that Slt2 is required for increased resistance to glucanase digestion and heat shock in response to cell wall stress.

METHODS

Strains and plasmids. *Escherichia coli* strain DH5 α was used for plasmid analysis and subcloning. *Saccharomyces cerevisiae* strains *kre1::HIS3* and *kre9::HIS3*, and their isogenic wild-type SEY6210 (*MAT α leu2-3,112 ura3-52 his3- Δ 200 lys2-801 trp1- Δ 901 suc2- Δ 9*) were kindly provided by Dr H. Bussey. Strains JVG987 (*MAT α ura3-52 his3 Δ 200 leu2 Δ 1 ade2-101 lys2-801 bar1 Δ ::LEU2*) and the isogenic *hcs77::LEU2* (JVG1079) were provided by Dr J. V. Gray. Strain W303-1A (*MAT α ade2-1 can1-100 trp1-1 ura3-1 his3-11,15 leu2-3,112*) and the isogenic *sms1(mid2)::URA3* were obtained from Dr Y. Kikuchi. The other deleted genes were all replaced by *HIS3* in strain FY834 (*MAT α his3 Δ 200 ura3-52 leu2 Δ 1 lys2 Δ 202 trp1 Δ 63*) resulting in strains *fks2 Δ* (AR101), *slt2 Δ* (EV71), *pkc1 Δ* (EV68), *gas1 Δ* (AR105) and *fks1 Δ* (AR100; Ram *et al.*, 1998), and in FY1679-28c (*MAT α his3 Δ 300 ura3-52 leu2 Δ 1 trp1 Δ 63*) resulting in strain *slt2 Δ* (FYDK).

Plasmids *FKS2-lacZ* [*FKS2*(-928 to -1)-*lacZ*] and *FKS2*⁷⁰⁶-

lacZ [*FKS2*(-706 to -1)-*lacZ*] contain, respectively, 928 bp and 706 bp of the *FKS2* upstream non-coding region in front of the bacterial *lacZ* gene (Zhao *et al.*, 1998) and were kindly provided by Dr D. Levin. Plasmid *TIR1-lacZ* (pLGDSRP5'; Marguet & Lauquin, 1986) contains 2.2 kb of the *TIR1* upstream region and the first 58 codons of *TIR1* in-frame with the *lacZ* gene and was a kind gift from Dr G. Lauquin. All three reporter construct plasmids are based on the same yeast episomal plasmid containing a 2 μ origin of replication and *URA3* as selectable marker (Marguet & Lauquin, 1986; Zhao *et al.*, 1998).

Deletions. Gene deletions were essentially performed using the method described by Baudin *et al.* (1993). In short, disruption cassettes consisting of *HIS3* flanked by 50 nucleotides identical to the targeted genes, allowing homologous recombination, were constructed in a PCR. The YDp-H plasmid (Berben *et al.*, 1991) was used as template in the PCR for the disruption cassettes. Oligonucleotides 5'-GTCAT-GAGTT TTTCACAATT GGAGCAGAAC ATTAAGAAAAAGATAGCCGT GAATTCCCGG GGATCCG (EV21) and 5'-TCTTCATAAA TCCAAATCAT CTGGCATAAA GGAAATCCT CTAAACTCTT AAGCTAGCTT GCCTGCAG (EV22) were used for the PCR of *pkc1::HIS3*. Oligonucleotides 5'-GAGATGGCTG ATAAGATAGA GAGGCATACT TTCAAGGTCT TCAATCAAGA GAAT-TCCCGG GGATCCG (EV25) and 5'-GTCCTAAAAA TATTTTCTAT CTAATCCAAA CTCCAGCTCT TTTTCAAGGT AAGCTAGCTT GCCTGCAG (EV26) were used for the PCR of *slt2::HIS3*. Deletion of the targeted genes in strain FY834 was confirmed by PCR using primers 5'-GGGGTACCTG TATTTATGAG GCATTGCTAT CTT (EV23) and 5'-GGGGTACCGA GGTTTTTCAT ATGC-ATGCTC C (EV24) for *pkc1::HIS3*, and 5'-GGGGTACCGT TCGAATACTT GTGAGCCTA (EV27) and 5'-GGGGT-ACCAG AGGCGATAAC AAACCTTCCG (EV28) for *slt2::HIS3*.

Growth assays. The sensitivity of yeast strains to CFW or Zymolyase-100T was assayed as growth inhibition. Cells were precultured in YEPD for 24 h at 28 °C prior to dilution to a concentration of 75 000 cells (200 μ l YEPD)⁻¹. Growth was continued in the presence of the antifungals in flat-bottom 96-well Greiner PS-microplates at 28 °C without shaking, and growth was determined as OD₅₉₅ of each culture after resuspension.

β -Galactosidase assay. Cells containing the *FKS2-lacZ* or *TIR1-lacZ* plasmids were precultured in selective medium for 24 h at 28 °C. Fresh precultures were used to inoculate liquid YEPD cultures without any addition or with CFW or Zymolyase-100T and the transformants were grown for an additional 4–7 h at 28 °C. Growth was determined as OD₅₉₅ and the cells were permeabilized immediately using chloroform/SDS as described previously (Guarente, 1983). β -Galactosidase activity was determined at 30 °C (Guarente, 1983) and was expressed in Miller units.

Detection of dually phosphorylated Slt2. Yeast cells were grown overnight to mid-exponential phase in YEPD. The cultures were then diluted in YEPD to OD₅₉₅ 0.3 and growth was continued for 4 h at 24 °C prior to collecting the cells or to treatment with CFW, Congo red or Zymolyase 100-T. Where indicated, sorbitol was added to the media to a final concentration of 1 M. Cells were collected on ice by adding 20 ml of the culture to an equal volume of ice in a Falcon centrifuge tube and pelleted in a refrigerated centrifuge. Cells were then resuspended in 1 ml ice-cold water and transferred to an Eppendorf tube, pelleted and immediately broken or frozen on dry ice. Cells were lysed in 120 μ l cold lysis buffer

(50 mM Tris/HCl pH 7.5, 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β -glycerol phosphate, 5 mM Na pyrophosphate, 5 mM EDTA, 1 mM PMSF and the protease inhibitors tosylphenylalanine chloromethyl ketone, tosyllysine chloromethyl ketone, leupeptin, pepstatin A, antipain and aprotinin, each at 25 μ g ml⁻¹) by vigorous shaking with 0.45 mm glass beads in a fast-prep cell breaker (Bio101; level 5.5 for 25 s). Cell extracts were separated from glass beads and cell debris and collected in an Eppendorf tube by centrifugation and further clarified by a 13 000 g spin for 15 min at 4 °C. The protein concentration of the supernatants was measured at 280 nm and normalized with lysis buffer. Then, 2 $\times$ SDS-PAGE sample loading buffer was added and samples were boiled for 5 min. Protein samples (50 μ g) were fractionated by SDS-PAGE using 8% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond; Amersham). Membranes were probed with anti-phospho44/42 MAP kinase (Thr202/Tyr204) antibody (New England Biolabs) to detect active Slr2 at 1/2000 dilution in the presence of 5% non-fat milk for 2 h at room temperature. The primary antibody was detected using a horseradish peroxidase-conjugated anti-rabbit antibody with the ECL detection system. To monitor the amount of Slr2, blots were stripped and reprobed with polyclonal anti-Slr2 antibodies (Martin *et al.*, 1993) at 1/1000 dilution, followed by detection as described above.

Thermotolerance. Cells pregrown in YEPD for 24 h at 28 °C were diluted in YEPD to a density of 0.5×10^7 cells ml⁻¹ (OD₅₉₅ 0.3) and growth was continued for at least 4 h at 28 °C in the presence or absence of cell-wall-destabilizing compounds. Cultures were washed and resuspended in YEPD, yielding a suspension of OD₅₉₅ 1. Following a heat shock of 0–20 min at 50 °C, cells were plated on YEPD and incubated for 2–3 d at 28 °C. Colony forming units were counted and percentage survival was determined relative to cultures that were not shocked at 50 °C.

1,3- β -Glucanase sensitivity. Cells were washed and resuspended in 50 mM Tris/HCl pH 7.4 to a concentration of 1.5×10^7 cells ml⁻¹. β -Mercaptoethanol was added to a final concentration of 40 mM and the cells were incubated at room temperature for 30 min prior to the addition of 100 U 1,3- β -glucanase per ml (Quantazyme *y*/g; Quantum Biotechnologies). The decrease in OD₅₉₅ was followed in time as a measure of cell lysis and was expressed as a percentage of the OD₅₉₅ prior to enzyme addition.

RESULTS

FKS2-lacZ expression is enhanced in cell wall mutants

Reduced levels of 1,3- β -glucan in the wall result in swollen and spherical yeast cells (Popolo *et al.*, 1993; Ram *et al.*, 1995; Kapteyn *et al.*, 1997). Two mutants with less 1,3- β -glucan in their walls, *gas1* Δ and *fks1* Δ , have previously been shown to contain increased levels of Fks2, presumably as a result of a cell wall compensation mechanism induced by these mutants to maintain cell integrity (Mazur *et al.*, 1995; Ram *et al.*, 1998). We transformed these mutants and the wild-type strain FY834 with an *FKS2-lacZ* reporter construct and determined β -galactosidase activity at different growth stages. Exponentially growing FY834 cells transformed

Fig. 1. Enhanced *FKS2-lacZ* expression in cell wall mutants. Wild-type cells and isogenic cell wall mutants (*fks1::HIS3* and *gas1::HIS3* in FY834 and *kre1::HIS3* and *kre9::HIS3* in SEY6210) were transformed with the *FKS2-lacZ* reporter plasmid. Transformants were precultured to saturation in selective medium prior to growth in YEPD at 28 °C. β -Galactosidase activity, expressed in Miller units, was determined at early-exponential phase (OD₅₉₅ 1; hatched bars) or at stationary phase (black bars). β -Galactosidase activity of FY834 [*FKS2-lacZ*] at early-exponential phase was 0.5 ± 0.1 Miller units. Values are means $\pm$ SEM ($n = 6$). Note that in several cases the standard error is too small to show in the figure.

with *FKS2-lacZ* showed a low basal level of β -galactosidase activity (about 0.5 Miller units) that strongly increased in stationary phase (Fig. 1). This is in accordance with the earlier reported responsiveness of this construct to stationary phase (Zhao *et al.*, 1998). Similarly, transformants of *fks1* Δ and *gas1* Δ both showed enhanced levels of *FKS2-lacZ* activity in stationary phase (Fig. 1). At exponential phase, however, basal β -galactosidase activities were in both mutants significantly higher than in the wild-type (Fig. 1), reflecting a constitutive induction of the *FKS2* reporter construct in these mutants, as observed previously by transcript and immunoblot analyses (Mazur *et al.*, 1995; Ram *et al.*, 1998).

Next, we used this reporter construct to follow *FKS2* induction in mutants defective in other wall components. The extracellular proteins Kre1 and Kre9 are important for synthesis of 1,6- β -glucan. Loss of either protein alone results in reduced levels of 1,6- β -glucan in the wall and concomitantly an aberrant cell wall morphology whereas simultaneous loss of both proteins is lethal (Boone *et al.*, 1990; Brown & Bussey, 1993). The wild-type SEY6210 [*FKS2-lacZ*] transformants gave a high basal level of β -galactosidase activity (about 20 Miller units) during the exponential growth phase prior to an increase in activity in stationary phase (Fig. 1). The *kre1* Δ [*FKS2-lacZ*] transformant showed an

Fig. 2. Cell wall perturbation by CFW and Zymolyase-100T induces *FKS2-lacZ* expression. (a) *FY834* [*FKS2-lacZ*] precultures were diluted to OD_{595} 0.25 in YEPD without (open symbols) or with (closed symbols) 1 M sorbitol and various amounts of CFW. Cultures were incubated at 28 °C for 6.5 h and OD_{595} , as a measure of growth (dotted lines with circles), and β -galactosidase activity (solid lines with squares) were determined. β -Galactosidase activity is expressed as a ratio relative to the activity in the absence of CFW. (b) As (a) but in the presence of various amounts of Zymolyase-100T. Values are means $\pm$ SEM ($n = 4$). Note that in several cases the standard error is too small to show in the figure.

approximate twofold increase of the basal level of activity during the exponential phase whereas in the *kre9* Δ [*FKS2-lacZ*] transformant the basal activity increased almost fourfold (Fig. 1).

When the *gas1* Δ , *fks1* Δ , *kre1* Δ and *kre9* Δ mutants and their respective wild-types were transformed with a *TIR1-lacZ* reporter construct, no significant β -galactosidase activity could be detected. *TIR1* encodes a

cell wall protein that is induced by growth at low temperatures and under anaerobiosis (Donzeau *et al.*, 1996). The *TIR1-lacZ* reporter plasmid was functional because significant β -galactosidase activity could be obtained with *FY834* and *SEY6210* transformants under inductive growth conditions (data not shown). Taken together, these results show that the *FKS2-lacZ* construct but not the *TIR1-lacZ* construct can be used as reporter for cell wall compensation reactions occurring in cell wall mutants.

Enhanced *FKS2-lacZ* expression after cell wall weakening by CFW or Zymolyase

CFW is a negatively charged, fluorescent dye that is unable to pass through the plasma membrane but preferentially binds to chitin in the yeast cell wall and interferes with normal wall assembly (Pringle *et al.*, 1989; Ram *et al.*, 1994). We tested the effect of CFW-induced cell wall perturbation on *FKS2-lacZ* expression. *FY834* [*FKS2-lacZ*] and *SEY6210* [*FKS2-lacZ*] transformants were transferred from selective medium to YEPD without or with CFW. Induction of β -galactosidase activity during a 6.5 h incubation period depended on the concentration of CFW but levelled off at concentrations above 50 $\mu\text{g ml}^{-1}$ (Fig. 2a). After prolonged incubation, the cultures entered into stationary phase and *FKS2-lacZ* expression increased independently of the presence of CFW (data not shown).

Cell wall integrity can also be challenged by Zymolyase-100T, a cocktail of 1,3- β -glucanase and protease activities that is commonly used to degrade yeast cell walls. *FY834* cells cultured in the presence of up to 2.6 U of Zymolyase-100T (ml YEPD) $^{-1}$ showed slightly impaired growth whereas a 10-fold lower concentration severely slowed growth of *SEY6210* cells (data not shown). Both *FY834* [*FKS2-lacZ*] and *SEY6210* [*FKS2-lacZ*] transformants gave clear induction of β -galactosidase activity when grown in the presence of Zymolyase (Fig. 2b and data not shown). Although the levels of β -galactosidase activity were much lower in *FY834* [*FKS2-lacZ*] than in *SEY6210* [*FKS2-lacZ*] transformants, the relative increase was similar.

Addition of 1 M sorbitol to the growth medium partially relieved growth inhibition by CFW or Zymolyase (Fig. 2 and 3 and data not shown) and was associated with reduced *FKS2-lacZ* expression (Fig. 2). Similar suppression of *FKS2-lacZ* expression was obtained when transformants were cultured in the presence of 0.8 M KCl or 1 M sucrose. The observed suppression of *FKS2-lacZ* expression in an osmotically supported medium suggests that loss of membrane support due to a loss in cell wall strength is a crucial step in sensing CFW- and Zymolyase-induced stress. Unlike the *FKS2-lacZ* transformants, cells transformed with the *TIR1-lacZ* reporter construct did not give rise to detectable β -galactosidase activity when grown in the presence of identical amounts of CFW or Zymolyase (data not shown).

Fig. 3. *slt2Δ* and *pkc1Δ* cells are hypersensitive to Zymolyase-100T. YEPD cultures in microtitre plates without (a) or with (b) 1 M sorbitol were inoculated with stationary-phase cells to a starting OD₅₉₅ of 0.025. Growth in the presence of the indicated amounts of Zymolyase-100T was determined as OD₅₉₅ after 18 h growth at 28 °C and is expressed as a percentage of the OD₅₉₅ in the absence of Zymolyase-100T (labelled as percentage of control growth). The OD₅₉₅ after 18 h of growth at 28 °C in the absence of Zymolyase-100T was (a) FY834, 7.2; *slt2Δ*, 2.5; (b) FY834, 4.0; *slt2Δ*, 2.3; *pkc1Δ*, 2.6. ■, FY834; ◆, *slt2Δ*; ●, *pkc1Δ*. Values are means ± SEM (n = 3).

Induction of *FKS2-lacZ* expression in response to cell wall perturbation fully depends on Slt2

The combined deletion of *PKC1* and several cell wall genes has been demonstrated to be lethal (Roemer *et al.*, 1994; Garrett-Engle *et al.*, 1995; Popolo *et al.*, 1997). Therefore, we further investigated the requirement for *PKC1* and *SLT2* in the response to CFW- and Zymolyase-induced cell wall stress. Loss of *SLT2* resulted in hypersensitivity to both compounds which could be completely alleviated by the addition of 1 M sorbitol. The *pkc1Δ* mutant does not grow without osmotic support but was hypersensitive to both compounds in the presence of 1 M sorbitol (Fig. 3 and

data not shown). One explanation for these findings is that an important response triggered after cell wall perturbation is mediated through the *PKC1* pathway.

The *FKS2-lacZ* reporter construct has been shown to respond to a variety of signals transduced by several different pathways, amongst others the *PKC1* pathway, through spatially separated elements within the *FKS2* promoter. The *FKS2*⁷⁰⁶-*lacZ* reporter, for example, contains a shorter fragment of the *FKS2* promoter region and is therefore no longer responsive to calcineurin-transduced signals or stationary phase but remains responsive to high temperature, a known Slt2-transduced signal (Zhao *et al.*, 1998). Exponentially growing SEY6210 cells transformed with the *FKS2*⁷⁰⁶-*lacZ* reporter construct showed a much reduced β -galactosidase activity under non-inducing conditions compared to SEY6210 [*FKS2-lacZ*] transformants (0.5 and 20 Miller units, respectively; Figs 1 and 4a). However, a more than sixfold induction of β -galactosidase activity was observed when SEY6210 [*FKS2*⁷⁰⁶-*lacZ*] cells were grown in the presence of CFW (Fig. 4a), similar to that observed in SEY6210 [*FKS2-lacZ*] transformants under the same conditions (data not shown). These results are consistent with the proposed role for the Slt2-mediated pathway in up-regulating *FKS2-lacZ* expression in response to cell wall perturbation. Next, we examined *FKS2-lacZ* expression in an *slt2Δ* strain. Stationary-phase induction of *FKS2-lacZ* occurred to the same extent in both *slt2Δ* and FY834 transformants (data not shown). However, unlike FY834 [*FKS2-lacZ*] transformants, the isogenic *slt2Δ* [*FKS2-lacZ*] showed no increased β -galactosidase activity when grown in the presence of sublethal concentrations of CFW or Zymolyase that allowed growth of both strains (Fig. 4b). These data demonstrate that induction of *FKS2-lacZ* in response to cell wall perturbation depends completely on Slt2, which is not the case for induction by stationary phase.

Slt2 is activated in response to cell wall damage

To confirm the direct involvement of Slt2 in *FKS2* induction following cell wall perturbations, Slt2 phosphorylation in response to similar stimuli was examined. Recently, tyrosine phosphorylation of an overexpressed HA-epitope-tagged version of Slt2 in response to CFW-induced cell wall stress has been shown (Ketela *et al.*, 1999). However, MAPK activation requires phosphorylation of both conserved tyrosine and threonine residues within the activation loop (Cobb & Goldsmith, 1995). Therefore, to follow Slt2 activation we used an anti-phospho-p44/p42 MAP kinase antibody, which recognizes the dually phosphorylated form of Slt2. Previous work using other inducing conditions has shown the specificity of this antibody in detecting the dually phosphorylated form of Slt2 by Western blotting (Verna *et al.*, 1997; Martin *et al.*, 2000).

Consistent with the above-described enhanced *FKS2* expression in cell wall mutants, *gas1Δ*, *fkp1Δ* and *kre9Δ*

Fig. 4. *FKS2-lacZ* expression in response to cell wall perturbation depends on Slt2. (a) SEY6210 cells were transformed with a shortened (*FKS2*⁷⁰⁶-*lacZ*) reporter construct that is unresponsive to stationary phase or calcineurin-mediated signals (Zhao *et al.*, 1998). Precultures of the transformants were diluted to OD₅₉₅ 0.24 in YEPD with the indicated amounts of CFW. Cultures were incubated at 28 °C for 5 h, and OD₅₉₅ (○) and β -galactosidase activity, expressed as Miller units (●), were determined. (b) FY834 and *slt2* Δ cells were transformed with the *FKS2-lacZ* reporter plasmid. Two independent transformants of each were precultured to saturation in selective medium prior to dilution in YEPD to yield an OD₅₉₅ of 0.3. Cultures were incubated for 6 h at 28 °C without addition (white bars), or in the presence of Zymolyase-100T (0.26 U ml⁻¹; hatched bars) or CFW (10 $\mu\text{g ml}^{-1}$; black bars). The OD₅₉₅ of all cultures at least doubled during the incubation period. β -Galactosidase activity was determined and is expressed in Miller units. Values are means $\pm$ SEM ($n = 4$).

mutants displayed a much higher level of dually phosphorylated Slt2 than their wild-type strains (Fig. 5a). However, whereas the strong Slt2 phosphorylation shown by *gas1* Δ mutants was prevented when cells were grown in medium with 1 M sorbitol, osmotic

stabilization of the medium greatly reduced but did not totally eliminate the increase in phospho-Slt2 signal shown by *fkS1* Δ and *kre9* Δ mutants (Fig. 5a). In contrast to these mutants, lack of Kre1p did not result in a detectable increase of Slt2 phosphorylation, possibly because cell wall damage in *kre1* Δ cells was relatively mild (Boone *et al.*, 1990; Brown & Bussey, 1993; see also Fig. 1).

When wild-type cells were grown in the presence of CFW (10 $\mu\text{g ml}^{-1}$) a progressive increase in the level of phospho-Slt2 was detected, starting after a 10 min exposure (Fig. 5b). The addition of 1 M sorbitol to the medium initially completely suppressed the activation of the pathway, but at longer incubation times the phosphorylation was not totally prevented (Fig. 5b). These data suggest that osmotic stabilization only fully suppresses the Slt2-mediated response when the cell wall is not severely stressed. In accordance with this explanation, prevention of Slt2 activation lasted longer when cells were treated with lower CFW concentrations (data not shown). In addition, treatment of wild-type cells with either Congo red, another compound that interferes with cell wall construction (Roncero & Duran, 1985), or with Zymolyase also resulted in a strong induction of Slt2 phosphorylation (Fig. 5c, d).

Two putative cell surface sensors, Hcs77(Wsc1/Slg1) and Mid2, have been reported to mediate the activation of the cell integrity pathway (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998; Ketela *et al.*, 1999; Rajavel *et al.*, 1999). As shown in Fig. 5(e), deletion of *MID2* strongly reduced dual phosphorylation of Slt2 in response to CFW-induced stress. This is consistent with recent data showing the requirement of Mid2 for CFW-induced tyrosine phosphorylation of overexpressed Slt2-HA (Ketela *et al.*, 1999). In contrast, Hcs77(Wsc1/Slg1) seems not to be required for signalling cell wall stress to the Slt2 pathway since loss of this protein did not affect CFW-induced Slt2 activation (Fig. 5e).

Cell wall perturbation induces thermotolerance in an Slt2-dependent manner

One reported output of Slt2 activation is the acquisition of thermotolerance. Growth at slightly elevated temperatures (37 °C) results in a cellular response that depends in part on Slt2 activation and allows the cells to survive an otherwise lethal heat shock at 50 °C (Kamada *et al.*, 1995). We determined whether Slt2 activation in response to cell wall perturbation also resulted in the acquisition of thermotolerance. Loss of *FKS1* resulted in a 7-fold, and loss of *GAS1* in a 200-fold higher survival after a 15 min heat shock at 50 °C compared to the wild-type strain FY834 (Fig. 6a). Similarly, enhanced thermotolerance was observed for the *kre1* Δ (2-fold) and *kre9* Δ (200-fold) mutants compared to their wild-type SEY6210 (data not shown). Growth of FY834 and SEY6210 in the presence of the cell wall perturbants CFW or Zymolyase also resulted in cells that were significantly more resistant to heat than mock-treated cells (Fig. 6b and

Fig. 5. Perturbation of the cell wall induces Slt2 phosphorylation. (a) Slt2 phosphorylation in cell wall mutants. Mid-exponential phase cells of the mutant strains *slt2Δ* (background FY1679-28c), *fks1Δ* and *gas1Δ* (background FY834), *kre1Δ* and *kre9Δ* (background SEY6210), and the isogenic wild-type strains FY834 and SEY6210 (WT), were grown in YEPD or YEPD plus 1 M sorbitol at 24 °C. (b) Time course of Slt2 phosphorylation after exposure to CFW. Exponential cultures of the wild-type strain FY834 growing in YEPD (left) or YEPD plus 1 M sorbitol (right) at 24 °C were treated with 10 µg CFW ml⁻¹. Aliquots of the same culture were withdrawn before (zero time point) and at the indicated times (in min) following exposure to CFW. (c) Treatment of wild-type cells with Congo red. Mid-exponential phase cells of strain FY834 growing in YEPD or YEPD plus 1 M sorbitol at 24 °C were incubated for 2 h without addition or in the presence of 10 µg Congo red ml⁻¹. (d) Treatment of wild-type cells with Zymolyase-100T. Mid-exponential phase cells of strain SEY6210 growing in YEPD were incubated for 30 min without addition or in the presence of 2.6 U Zymolyase-100T ml⁻¹. (e) Slt2 phosphorylation in the absence of cell surface sensors. Exponential cultures of the mutant strains *hcs77Δ* and *mid2Δ*, and the respective control strains JVG987 and W303-1A (WT) were incubated in the absence or presence of 10 µg CFW ml⁻¹ for 2 h. In all cases, cells were rapidly cooled and collected, cell extracts were prepared and equal amounts of total protein in each sample were separated on an SDS-polyacrylamide gel and immunoblotted with anti-phospho-p44/42 MAP kinase antibodies (top panel of each pair). Anti-Slt2 immunoblot analysis was performed on the same membrane to verify that similar amounts of Slt2 were present in every lane (bottom panel of each pair). The absence of signal in the *slt2Δ* strain and the estimated mobility of the protein migrating in this band (64 kDa), as expected for Slt2, identify the detected protein as Slt2.

Fig. 6. Cell wall perturbation induces thermotolerance in an Slt2-dependent manner. (a) FY834 (■), *gas1*Δ (◆) or *fks1*Δ (●) cells were grown in YEPD to an OD_{595} of 1.5 prior to heat shock at 50 °C. Cells were harvested and prepared for heat treatment as described in Methods. Survival was determined as colony formation after different heat-shock periods and is expressed as percentage survival of cells that were not heat-shocked. (b) YEPD cultures were inoculated with FY834, *slt2*Δ or *fks2*Δ cells to yield a starting OD_{595} of 0.45. Cultures were further incubated at 28 °C for 4.5 h without addition (white bars), or after addition of Zymolyase-100T (0.52 U ml⁻¹; hatched bars) or CFW (10 µg ml⁻¹; black bars). The OD_{595} of all cultures at least tripled during the incubation period. Cells were then heat-shocked for 10 min at 50 °C; the percentage of surviving cells was determined and is expressed as a ratio relative to the percentage survival of heat-shocked cells that had been incubated without cell-wall-perturbing agents. Values are means $\pm$ SEM ($n = 6$).

data not shown). This response to cell wall perturbants depended on Slt2 because an *slt2*Δ mutant in the FY834 background did not acquire thermotolerance (Fig. 6b). Although upregulation of Fks2 is one of the outputs of Slt2 activation, it is not essential for the concomitant

Fig. 7. Cells cultured in the presence of CFW or Zymolyase-100T display Slt2p-dependent resistance to 1,3-β-glucanase digestion. YEPD cultures were inoculated with FY834 (a), *slt2*Δ (b) or *fks2*Δ (c) cells to yield a starting OD_{595} of 0.25. Cultures were incubated at 28 °C for 4 h (FY834 and *fks2*Δ) or 6 h (*slt2*Δ) without addition (■), or in the presence of CFW (10 µg ml⁻¹; ◆) or Zymolyase-100T (0.26 U ml⁻¹; ●). The OD_{595} of all cultures at least doubled during the incubation period. Cells were washed and digested with a recombinant 1,3-β-glucanase (Quantazyme *ylg*) as described in Methods. The decrease in OD_{595} represents cell lysis and is expressed as a percentage of the starting OD_{595} . Values are means $\pm$ SEM ($n = 6$).

induction of heat resistance because cells lacking Fks2 still became thermotolerant in response to cell wall perturbation (Fig. 6b).

Increased resistance to cell wall degradation in response to cell wall perturbation depends on Slt2

In addition to *FKS2* induction, an increase in chitin content also seems to be part of a general compensatory response to cell wall defects (Popolo *et al.*, 1997; Ram *et al.*, 1998). Such changes in the composition and the architecture of the cell wall (Kapteyn *et al.*, 1999a) should affect its properties. Therefore, we examined cells with a perturbed wall for their resistance to a glucan-degrading enzyme and analysed the dependence of this on Slt2. Exponentially growing FY834, *slt2Δ* and *fks2Δ* cells were sensitive to Quantazyme, a recombinant 1,3- β -glucanase (Fig. 7), but became resistant after entry into the stationary phase (data not shown) reflecting cell wall alterations upon diauxic shift (De Nobel *et al.*, 1990). Exponentially growing *gas1Δ*, *fks1Δ* and *kre9Δ* but not *kre1Δ* cells also became more resistant to digestion by 1,3- β -glucanase (data not shown). Likewise, both wild-type strains became significantly more resistant to Quantazyme digestion during exponential growth in the presence of CFW or Zymolyase (Fig. 7 and data not shown). This increased Quantazyme resistance depended to a large extent on Slt2 because an *slt2Δ* mutant remained much more sensitive when grown under the same conditions (Fig. 7). Fks2, which is upregulated after Slt2 activation, was not required for the increased Quantazyme resistance (Fig. 7). In summary, cell wall perturbation by CFW or Zymolyase provokes an Slt2-mediated response that confers increased resistance to further cell wall degradation, probably as a result of cell wall modifications. Similar cell wall alterations seem to occur when cells enter stationary phase but these are mediated by an Slt2-independent mechanism.

DISCUSSION

Many *S. cerevisiae* mutants defective in the synthesis of particular cell wall components, such as 1,3- β -glucan, 1,6- β -glucan or mannoproteins, show a characteristic set of alterations in the composition and architecture of their walls. Chitin deposition is strongly increased (Dallies *et al.*, 1998; Kapteyn *et al.*, 1999a) and becomes directly linked to 1,6- β -glucan (Kapteyn *et al.*, 1997). In addition, the expression levels of several cell wall proteins and of the alternative subunit of 1,3- β -glucan synthase, Fks2, are upregulated. It has been proposed that these changes are part of a compensation mechanism in response to cell wall weakening (Popolo *et al.*, 1997; Ram *et al.*, 1998; Kapteyn *et al.*, 1999a; Smits *et al.*, 1999). Such a mechanism might also protect fungal cells against cell-wall-degrading enzymes, which are frequently present in their natural environment. For example, mycoparasites and plants infected with fungi secrete large amounts of glucanases and chitinases (Lamb *et al.*, 1989; Lorito *et al.*, 1998).

The *PKC1* pathway is believed to regulate cell integrity because loss of components in this pathway results in cell lysis that can be prevented by providing osmotic support (Gustin *et al.*, 1998; Mellor & Parker, 1998). This notion is further supported by the observation that the expression of several genes involved in cell wall assembly seems to be controlled by this pathway (Igual *et al.*, 1996; Jung & Levin, 1999). Specifically, the temperature-induced expression of Fks2 has been shown to be one of the outputs of Slt2 activation (Zhao *et al.*, 1998). Activation of the cell integrity pathway has been reported in response to heat and hypotonic stress and during polarized growth (Levin *et al.*, 1994; Gustin *et al.*, 1998; Mellor & Parker, 1998). These conditions might activate the *PKC1* pathway by challenging cell wall integrity indirectly. Furthermore, enhanced GDP/GTP exchange activity towards Rho1, an activator of Pkc1 (Nonaka *et al.*, 1995; Kamada *et al.*, 1996) has been found in cell wall mutants (Bickle *et al.*, 1998). In agreement with this, Hcs77, Rho1 and Pkc1 are required for depolarization of the actin cytoskeleton in response to heat stress and cell wall degradation (Delley & Hall, 1999). These observations suggest that a decrease in cell wall integrity activates Pkc1. Since activation of Slt2 depends on Pkc1 and loss of Slt2 results in fragile cells (Torres *et al.*, 1991; Lee *et al.*, 1993; Martin *et al.*, 1993), a role for Slt2 in transducing the signal elicited by cell wall stress could also be envisioned. However, at present it is unclear whether all the reported outputs generated by cell wall perturbation depend on Slt2. In fact, although stress-induced actin depolarization depends on Pkc1, it does not require the downstream MAP kinase cascade (Delley & Hall, 1999). In addition, activation of Slt2 as a consequence of cell wall weakening has not been demonstrated unequivocally. For example, Ketela *et al.* (1999) recently found increased tyrosine phosphorylation of an overexpressed HA-tagged form of Slt2 in cells grown in the presence of CFW. However, no readily apparent changes in the amount of phosphotyrosine of native Slt2 were detected under those conditions. Furthermore, MAP kinase activation requires dual phosphorylation of threonine and tyrosine residues and the occurrence in yeast of tyrosine-phosphorylated but nevertheless inactive Slt2 has been reported (Buehrer & Errede, 1997).

Here, we used two readouts for Slt2-mediated signalling: antibodies that detect the dually (threonine and tyrosine) phosphorylated form of Slt2 and *FKS2-lacZ* reporter constructs. Increased dual phosphorylation of Slt2 and Slt2-dependent *FKS2-lacZ* expression were found in *gas1*, *fks1* and *kre9* which are cell wall mutants, and in cells treated with cell-wall-destabilizing agents. This response was partially or totally prevented when 1 M sorbitol was added to the medium.

Taken together, these results clearly show the following. (i) Slt2 is activated in response to defects in the cell wall; this activation is likely to mediate the compensation reactions occurring in damaged cells to ensure their integrity. (ii) Osmotic stabilization of the plasma membrane in the presence of a defective cell wall can

prevent stimulation of the pathway. This effect depends on the extent of the cell wall damage and indicates that at least a mild cell wall stress is not sensed under such conditions. These conclusions are consistent with *gas1* and *fks1* mutants requiring a functional Pkc1 pathway for survival (Garrett-Engle *et al.*, 1995; Popolo *et al.*, 1997; Turchini *et al.*, 2000) and with the hypersensitivity of *slt2* mutants to cell wall perturbing agents (Fig. 3).

Previously, Ketela *et al.* (1999) reported that tyrosine phosphorylation of overexpressed Slt2-HA in response to CFW-induced cell wall perturbation depends on the putative cell surface sensor Mid2. Our results confirm the role of Mid2 in sensing cell wall stress since we found no activation of Slt2 in *mid2Δ* cells stressed with CFW. In contrast, the putative cell integrity sensor, Hcs77, seems not to have a role in sensing cell wall defects and activating the MAP kinase cascade (Fig. 5e). Interestingly, Hcs77 but not Mid2 is required for Slt2-independent actin depolarization in response to heat or cell wall damage (Delley & Hall, 1999). These results suggest that cell wall damage can be sensed by at least two different cell surface proteins each triggering a different response. The Hcs77 homologues, Wsc2 and Wsc3 (Verna *et al.*, 1997), might also be involved in sensing cell wall damage.

The Slt2-mediated increases in thermotolerance and glucanase resistance displayed by cells with an altered cell wall reflect how cells rely on a Slt2-mediated compensation mechanism to face wall-related stress. This also indicates the significance of the modifications in the cell wall architecture of cells in which this mechanism has been triggered. The exact changes in the wall responsible for altered sensitivity to 1,3- β -glucanase digestion are at present unknown. Induction of Fks2 is not essential because an *fks2Δ* strain gave a similar response after cell wall perturbation. Possibly, the strongly increased deposition of chitin in the lateral walls that has been reported for many cell wall mutants, including *gas1Δ* and *fks1Δ* cells (Dallies *et al.*, 1998; Kapteyn *et al.*, 1997, 1999b) and for cells stressed with CFW (Roncero & Duran, 1985; Ketela *et al.*, 1999) is responsible for the increased glucanase resistance. In addition, growth in the presence of CFW reduces the permeability of the yeast wall (De Nobel *et al.*, 1990), suggesting enhanced incorporation of cell wall manno-proteins that could also account for this resistance. Further study is also required to determine whether increased thermotolerance in these cells depends on concurrent cell wall alterations. Unlike induction by cell wall perturbation, enhanced resistance to degradation by 1,3- β -glucanase at stationary phase is independent of Slt2. This indicates that alternative signal transduction pathways might be responsible for cell wall alterations induced at this stage.

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