Mutational analysis of the cytoplasmic domain of the Wsc1 cell wall stress sensor

Heather A. Vay, Bevin Philipt and David E. Levin

Correspondence

David E. Levin

levin@jhmi.edu

Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health, Baltimore, 615 N Wolfe St, MD 21205, USA

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Wsc1 is a member of a family of highly O-glycosylated cell surface proteins that reside in the plasma membrane of *Saccharomyces cerevisiae* and function as sensors of cell wall stress. These proteins activate the cell wall integrity signalling pathway by stimulating the small G-protein Rho1, protein kinase C (Pkc1) and a MAP kinase cascade. The cytoplasmic domains of Wsc1 family members interact with the Rom2 guanine nucleotide exchange factor to stimulate GTP-binding of Rho1. Here, a mutational analysis of the cytoplasmic domain of Wsc1 is presented. The data identify two regions of the Wsc1 cytoplasmic tail that are conserved with other family members as important for Rom2 interaction. These regions are separated by an inhibitory region, which includes a cluster of seryl residues that appear to be phosphorylated. Mutational analysis of these residues supports a model in which Wsc1 interaction with Rom2 is negatively regulated by phosphorylation.

INTRODUCTION

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (Cid *et al.*, 1995; Klis, 1994). The cell must remodel this rigid structure during vegetative growth and during pheromone-induced morphogenesis. Wall remodelling is monitored and regulated by the cell integrity signalling pathway controlled by the Rho1 GTPase. Two essential functions have been identified for Rho1. First, it serves as an integral regulatory subunit of the 1,3- β -glucan synthase complex (GS) that stimulates GS activity in a GTP-dependent manner (Drgonova *et al.*, 1996; Qadota *et al.*, 1996). A pair of closely related genes, *FKS1* and *FKS2*, encode alternative catalytic subunits of the GS complex (Douglas *et al.*, 1994; Inoue *et al.*, 1995; Mazur *et al.*, 1995; Ram *et al.*, 1995) that are the presumed targets of Rho1 activity.

A second essential function of Rho1 is to bind and activate protein kinase C (Kamada *et al.*, 1996; Nonaka *et al.*, 1995), which is encoded by *PKC1* (Levin *et al.*, 1990). Loss of *PKC1* function, or of any of the components of the MAP kinase cascade under its control, results in a cell lysis defect that is attributable to a deficiency in cell wall construction (Levin & Bartlett-Heubusch, 1992; Levin *et al.*, 1994; Paravicini *et al.*, 1992). The MAP kinase cascade is a linear pathway comprising a MEKK (Bck1; Costigan *et al.*, 1992; Lee & Levin, 1992), a pair of redundant MEKs (Mkk1/2; Irie *et al.*, 1993) and a MAPK (Mpk1/Slt2; Lee *et al.*, 1993; Torres

et al., 1991). One of the consequences of signalling through the MAP kinase cascade is the activation of the SRF-like transcription factor, Rlm1 (Watanabe *et al.*, 1997; Jung *et al.*, 2002). Signalling through Rlm1 regulates the expression of at least 25 genes, most of which have been implicated in cell wall biogenesis (Jung & Levin, 1999).

Cell wall integrity signalling is induced in response to several environmental stimuli. First, signalling is activated persistently in response to growth at elevated temperatures (e.g. 37–39 °C; Kamada *et al.*, 1995), consistent with the finding that null mutants in many of the pathway components display cell lysis defects only when cultivated at high temperature. Second, hypo-osmotic shock induces a rapid, but transient activation of signalling (Davenport *et al.*, 1995; Kamada *et al.*, 1995). Third, treatment with mating pheromone stimulates signalling at a time that is coincident with the onset of morphogenesis (Buehrer & Errede, 1997; Errede *et al.*, 1995). Indeed, mutants defective in cell integrity signalling undergo cell lysis during pheromone-induced morphogenesis. Finally, agents that cause cell wall stress, such as caffeine and the chitin antagonist Calcofluor White, also activate signalling (Ketela *et al.*, 1999; Martin *et al.*, 2000).

The mechanisms by which cell wall stress is transmitted to Rho1 is an area of active investigation. Several regulators of Rho1 activity have been identified. Rom1 and Rom2 comprise a redundant pair of guanine nucleotide exchange factors (GEFs) for Rho1 (Ozaki *et al.*, 1996). Bem2 and Sac7 are GTPase-activating proteins (GAPs) for Rho1 (Kim *et al.*, 1994; Peterson *et al.*, 1994; Schmidt *et al.*, 1997). Finally, a family of cell surface sensors for the activation of

†Present address: Wyeth Pharmaceuticals, Cambridge, MA 02140, USA.

Abbreviation: λ PPase, λ protein phosphatase.

cell integrity signalling has been described. These include Wsc1, Wsc2, Wsc3, Mid2 and Mtl1 (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998; Ketela *et al.*, 1999; Rajavel *et al.*, 1999). Among these, Wsc1 and Mid2 are the major sensors dedicated to signalling wall stress during vegetative growth and pheromone-induced morphogenesis (Rajavel *et al.*, 1999; Ketela *et al.*, 1999). Indeed, a *wsc1Δ mid2Δ* double mutant displays a severe cell lysis defect, indicating that these genes have an overlapping function during vegetative growth. The cytoplasmic domains of both Wsc1 and Mid2 interact with the N-terminal domain of the Rom2 (and presumably Rom1) guanine nucleotide exchange factor to stimulate GTP loading of Rho1 (Philip & Levin, 2001).

All members of the Wsc1/Mid2 family are transmembrane proteins that reside in the plasma membrane (Ketela *et al.*, 1999; Lodder *et al.*, 1999; Rajavel *et al.*, 1999; Verna *et al.*, 1997). Their overall structures are similar in that they possess small cytoplasmic domains, each has a single transmembrane region and their extracellular domains are rich in Ser/Thr residues. These Ser/Thr-rich regions are highly O-mannosylated, probably resulting in extension and stiffening of the polypeptide. Therefore, the extracellular domains have been proposed to act as rigid probes of the extracellular matrix (Rajavel *et al.*, 1999). Despite the broad similarity among the proteins, there is very limited sequence identity among their cytoplasmic domains. Here we present a mutational analysis of the cytoplasmic domain of Wsc1.

METHODS

Strains, growth conditions and transformations. Yeast strains used in this study were derived from EG123 (Kamada *et al.*, 1995). Wild-type haploid (1783) or diploid (1788) yeast cultures were grown in YEPD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose) or YEPD supplemented with 10% sorbitol to prevent lysis of strain DL2282 (*wsc1Δ::LEU2 mid2Δ::URA3*; Rajavel *et al.*, 1999). Synthetic minimal (SD) medium (Rose *et al.*, 1990) supplemented with the appropriate nutrients was used to select for plasmid maintenance. Yeast transformations were carried out by the lithium acetate method (Ito *et al.*, 1983). *wsc1* mutant plasmids were tested for function in yeast strain DL2282, for Wsc1^{HA} protein expression and phosphorylation in the haploid parental strain 1783 and for Mpk1 activation in the diploid parental strain 1788. *Escherichia coli* DH5α was used to propagate all plasmids. *E. coli* cells were cultured in Luria broth medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl) and transformed by standard methods.

WSC1 plasmids

All plasmids used in this study are shown in Table 1.

Expression plasmids. To construct the C-terminal truncation mutants of WSC1, sequences (including 600 bp 5' to the translational start site) were amplified by PCR through the final amino acid desired in the truncation using YEp352-WSC1^{HA} (p1657) as template and inserted blunt-ended into the *Sma*I site of episomal plasmid YEp352 [3xHA] (p845; Rajavel *et al.*, 1999). The resultant clones fused the 3xHA epitope, in-frame with an additional glycine residue, to the various C termini of Wsc1. These mutant alleles were subcloned as *Kpn*I–*Pst*I fragments into centromeric plasmid pRS314 (Sikorski & Hieter, 1989).

For construction of point mutants, pRS314-WSC1^{HA} (p1672) was used as template for site-directed mutagenesis by the PCR overlap extension method (Ho *et al.*, 1989). The T7 and T3 primers were used in primary PCR reactions with forward and reverse mutagenic primers, respectively. The products of these reactions were mixed in secondary PCR reactions using only the T7 and T3 primers. The full-length products derived from these reactions were subcloned either as *Kpn*I–*Sac*I fragments into pRS314 (K301A, Y303A and Q304A) or as *Apa*I–*Pst*I fragments into pRS314-WSC1^{HA} (L369A, V371A, V372A, N373A, P374A, D375A and D378A). YEp352-*wsc1*-S328/329/331A-T337/341A^{HA} (p1847) was constructed stepwise by first mutating the two Thr residues in pRS314-WSC1^{HA}, followed by mutagenesis of the three Ser residues and finally subcloning the pentuple mutant as an *Mfe*I–*Sall* fragment into YEp352-WSC1^{HA}. YEp352-*wsc1*-S319/320A^{HA} (p1868), YEp352-*wsc1*-S322/323A^{HA} (p1869) and YEp352-*wsc1*-S319/320/322/323A^{HA} (p1850) were constructed by simultaneous mutation of two or all four Ser residues, followed by subcloning the double and quadruple mutants as a *Mfe*I–*Sall* fragment into YEp352-WSC1^{HA}. pRS314-*wsc1*-(1-346)-S319/320/322/323A^{HA} (p1866) was constructed by subcloning the truncated C terminus of *wsc1*-(1-346)^{HA} (from p1513) into pRS314-*wsc1*-S319/320/322/323A^{HA} by an *Apa*I–*Pst*I fragment (the *Apa*I site spans residues 326/327). The complete DNA sequences of all inserts were determined. Sequence analysis was performed by the JHU Biosynthesis and Sequencing Facility. PCR was performed using *Pfu* polymerase (Stratagene). Primers are available upon request.

Two-hybrid plasmids and assays. Sequences encoding the C-terminal tails (residues 291–378) of *wsc1* point mutants were amplified by PCR using primers that placed a *Bam*HI site before the N-terminal residue and a *Pst*I site after the C-terminal residue. These fragments were cloned into the *Bam*HI and *Pst*I sites of pGBT9 (Clontech Laboratories) so as to fuse the *wsc1* sequences in-frame with the Gal4-DNA-binding domain. All fusions were confirmed by DNA sequence analysis. pGBT9 clones of mutant *wsc1* tails were cotransformed with pGAD424-*rom2*-(1-660) (p1667) into yeast strain SFY526 (Clontech Laboratories) and transformants were tested as described in Philip & Levin (2001) for two-hybrid interactions.

Immunodetection and phosphatase treatment of Wsc1^{HA}.

Extracts of yeast strain EG123 expressing forms of Wsc1^{HA} from YEp352 were made and tested by immunoblotting with mouse mAb 12CA5, as described by Philip & Levin (2001) with the following modifications to the lysis buffer: phosphatase inhibitors (30 mM sodium pyrophosphate and 0.2 mM sodium vanadate) and 0.5 mM EGTA were added and 0.5% NP40 was substituted for Triton X-100. The phosphorylation state of Wsc1^{HA} forms was determined by treatment with λ protein phosphatase (New England Biolabs). Extracts (15–25 µg protein) were treated with 400 U λ protein phosphatase for 2 h at 30 °C in λ phosphatase buffer (50 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij35 and 2 mM MnCl₂) with or without phosphatase inhibitors (45 mM KF, 23 mM sodium pyrophosphate and 1.5 mM sodium vanadate). Treated extracts were subjected to SDS-PAGE on 4–15% polyacrylamide gradient Ready Gels (Bio-Rad).

Immunodetection of Mpk1^{HA} and activated Mpk1 after heat shock.

Yeast cells grown to an OD₆₀₀ of 0.5–1.0 at 23 °C in YEPD were exposed to a mild heat shock (39 °C) by 1:1 dilution with fresh medium prewarmed to 55 °C and maintained at 39 °C for the indicated times. The cell response was terminated by further dilution (1:1) with ice-cold stop mix (Kamada *et al.*, 1995). Extracts were made and Mpk1^{HA} was detected by immunoblot after samples (5 µg protein) were subjected to SDS-PAGE using mouse mAb 12CA5 (BaBCo) and horseradish peroxidase-linked secondary antibody (Amersham) as described by Kamada *et al.* (1995). Activated Mpk1 was detected with rabbit polyclonal anti-phospho-p44/p42 MAPK (Thr²⁰²/Tyr²⁰⁴) antibody (New England Biolabs) essentially as described

Table 1. Plasmids

Plasmid number	Plasmid name	Source/reference
Centromeric plasmids		
118	pRS314	Sikorski & Hieter (1989)
1672	pRS314- <i>WSC1</i> ^{HA}	This study
1512	pRS314- <i>wsc1</i> -(1-367) ^{HA}	This study
1513	pRS314- <i>wsc1</i> -(1-346) ^{HA}	This study
1518	pRS314- <i>wsc1</i> -(1-326) ^{HA}	This study
1514	pRS314- <i>wsc1</i> -(1-316) ^{HA}	This study
1511	pRS314- <i>wsc1</i> -(1-300) ^{HA}	This study
1786	pRS314- <i>wsc1</i> -K301A ^{HA}	This study
1785	pRS314- <i>wsc1</i> -Y303A ^{HA}	This study
1787	pRS314- <i>wsc1</i> -Q304A ^{HA}	This study
1881	pRS314- <i>wsc1</i> -L369A ^{HA}	This study
1889	pRS314- <i>wsc1</i> -V371A ^{HA}	This study
1882	pRS314- <i>wsc1</i> -V372A ^{HA}	This study
1883	pRS314- <i>wsc1</i> -N373A ^{HA}	This study
1884	pRS314- <i>wsc1</i> -P374A ^{HA}	This study
1885	pRS314- <i>wsc1</i> -D375A ^{HA}	This study
1880	pRS314- <i>wsc1</i> -D378A ^{HA}	This study
1854	pRS314- <i>wsc1</i> -S319/320/322/323A ^{HA}	This study
1866	pRS314- <i>wsc1</i> -(1-346)-S319/320/322/323A ^{HA}	This study
Episomal plasmids		
672	YEp352-MPK1 ^{HA}	Kamada <i>et al.</i> (1995)
845	YEp352 [3xHA]	Rajavel <i>et al.</i> (1999)
1657	YEp352- <i>WSC1</i> ^{HA}	Rajavel <i>et al.</i> (1999)
1449	YEp352- <i>wsc1</i> -(1-367) ^{HA}	This study
1459	YEp352- <i>wsc1</i> -(1-346) ^{HA}	This study
1516	YEp352- <i>wsc1</i> -(1-326) ^{HA}	This study
1460	YEp352- <i>wsc1</i> -(1-316) ^{HA}	This study
1847	YEp352- <i>wsc1</i> -S328/329/331A-T337/341A ^{HA}	This study
1850	YEp352- <i>wsc1</i> -S319/320/322/323A ^{HA}	This study
1868	YEp352- <i>wsc1</i> -S319/320A ^{HA}	This study
1869	YEp352- <i>wsc1</i> -S322/323A ^{HA}	This study
Two-hybrid plasmids		
1172	pGBT9	Clontech
1324	pGBT9- <i>wsc1</i> -(291-378)	Philip & Levin (2001)
1818	pGBT9- <i>wsc1</i> -(291-378)-K301A	This study
1790	pGBT9- <i>wsc1</i> -(291-378)-Y303A	This study
1819	pGBT9- <i>wsc1</i> -(291-378)-Q304A	This study
1893	pGBT9- <i>wsc1</i> -(291-378)-L369A	This study
1901	pGBT9- <i>wsc1</i> -(291-378)-V371A	This study
1894	pGBT9- <i>wsc1</i> -(291-378)-V372A	This study
1895	pGBT9- <i>wsc1</i> -(291-378)-N373A	This study
1896	pGBT9- <i>wsc1</i> -(291-378)-P374A	This study
2018	pGBT9- <i>wsc1</i> -(291-378)-D375A	This study
1891	pGBT9- <i>wsc1</i> -(291-378)-D378A	This study
1667	pGAD424- <i>rom2</i> -(1-660)	Philip & Levin (2001)

by Martin *et al.* (2000) except that 20 µg protein was fractionated and primary antibody was used at a 1 : 1000 dilution.

RESULTS AND DISCUSSION

As a first step in the functional analysis of the cytoplasmic domain of Wsc1, we constructed a set of C-terminal

truncation mutants of this sensor (Fig. 1a) in which the HA epitope was fused to the new C termini. C-terminal fusions have been shown previously not to impair Wsc1 function (Lodder *et al.*, 1999; Rajavel *et al.*, 1999). We tested the ability of these truncated forms to complement the cell lysis defect of a *wsc1Δ mid2Δ* double mutant when expressed from a centromeric plasmid. The *wsc1Δ mid2Δ* mutant,

Fig. 1. (a) C-terminal truncations of Wsc1. (b) Complementation of the cell lysis defect of a *wsc1Δ mid2Δ* mutant by some of the Wsc1 C-terminal truncations. Yeast strain DL2282 (*wsc1Δ mid2Δ*) was transformed with centromeric plasmid pRS314 (vector) or pRS314 bearing the indicated allele of *WSC1*^{HA} (p1672, p1511, p1512, p1513, p1514 or p1518). Transformants were streaked onto YEPD and allowed to grow at 30 °C for 2 days.

which lyses at all growth temperatures in the absence of osmotic support (Rajavel *et al.*, 1999), provides a more sensitive test of Wsc1 function than does a *wsc1Δ* mutant, which only displays a cell lysis defect at high temperatures (e.g. 37–39 °C). Deletion of the C-terminal 11 amino acid residues in *wsc1(1-367)* was sufficient to eliminate complementation at 30 °C (Fig. 1b), suggesting that residues at the extreme C terminus of this protein are important to its function. Similarly, deletion of the C-terminal 32 residues in *wsc1(1-346)* eliminated complementation. Interestingly, removal of an additional 20 or 30 residues [in *wsc1(1-326)* or *wsc1(1-316)*, respectively] partially restored complementation function, suggesting the presence of sequences between residues 317 and 345 that inhibit Wsc1 function. Finally, removal of nearly the entire cytoplasmic domain in *wsc1(1-300)* eliminated complementation, indicating that residues between 301 and 316 are necessary and sufficient for partial function of the cytoplasmic domain. All of the truncated

Fig. 2. (a) Conserved residues within the cytoplasmic tail of Wsc1, 2 and 3. (b) Complementation of a *wsc1Δ mid2Δ* mutant by point mutants in the KxYQ motif of Wsc1. Yeast strain DL2282 (*wsc1Δ mid2Δ*) was transformed with centromeric plasmid pRS314 (vector) or pRS314 bearing the indicated allele of *WSC1*^{HA} (p1672, p1785, p1786 or p1787). Transformants were streaked onto YEPD and allowed to grow at 30 °C for 2 days. (c) Interaction of point mutants in the Wsc1 KxYQ motif with the N-terminal domain of Rom2. Plasmids expressing fusions between the Gal4-DNA-binding domain (in pGBT9) and the cytoplasmic domain of the indicated Wsc1 forms (p1324, p1790, p1818 or p1819) were cotransformed with pGAD424-rom2-(1-660) (p1667; expressing a Gal4-activation domain fusion to Rom2) into yeast strain SFY526. Transformants were patched onto YEPD for 24 h and tested for two-hybrid interaction. Dark patches indicate interaction.

forms of Wsc1 were expressed at wild-type levels (data not shown).

Although the cytoplasmic domain of Wsc1 has diverged appreciably from those of Wsc2 and Wsc3, and is only about half the length of the other two, there are two conserved regions of sequence among all three (Fig. 2a). One of these, identified by Lodder *et al.* (1999), is the KxYQ box, which resides at residues 301–304 in Wsc1. The other region of Wsc1 that is conserved with the other sensors comprises the 10 residues at its extreme C terminus (residues 369–378; Fig. 2a). These conserved sequences correspond to the two regions of the cytoplasmic domain assessed to be important for Wsc1 function by our truncation analysis. Therefore, we mutated each of the conserved residues within both regions individually to alanine residues to determine their contribution to function. Fig. 2(b) shows that among the conserved residues in the KxYQ box, only the *Y303A* mutation failed to complement the growth defect of a *wsc1Δ*

mid2Δ mutant at 30 °C (or at 37 °C, data not shown). Consistent with this result, only the Y303A mutant failed to interact with Rom2 by two-hybrid analysis (Fig. 2c), suggesting that Tyr303 is important for Rom2 association.

Fig. 3. (a, b) Complementation of a *wsc1Δ mid2Δ* mutant by point mutants in the extreme C terminus of Wsc1. Yeast strain DL2282 (*wsc1Δ mid2Δ*) was transformed with centromeric plasmid pRS314 (vector) or pRS314 bearing the indicated allele of *WSC1*^{HA} (p1672, p1880, p1881, p1882, p1883, p1884, p1885 or p1889). Transformants were streaked onto YEPD and allowed to grow at 30 (a) or 37 °C (b) for 2 days. (c) Interaction of point mutants in the extreme C terminus Wsc1 with the N-terminal domain of Rom2. Plasmids expressing fusions between the Gal4-DNA-binding domain (in pGBT9) and the cytoplasmic domain of the indicated Wsc1 forms (p1324, p1891, p1893, p1894, p1895, p1896, p1901 or p2018) were cotransformed with pGAD424-*rom2*-(1-660) (p1667; expressing a Gal4-activation domain fusion to Rom2) into yeast strain SFY526. Transformants were patched onto YEPD for 24 h and tested for two-hybrid interaction. Dark patches indicate interaction. (d) Point mutants at the extreme C terminus of Wsc1 are more severely compromised than truncation mutants that remove regions of the C terminus. Yeast strain DL2282 was transformed with pRS314 or pRS314 bearing the indicated allele of *WSC1*^{HA}. Transformants were treated as in (a).

We next mutated the seven conserved residues at the C terminus of Wsc1 individually to alanyl residues. Among these mutants, only the L369A and N373A alleles failed to complement the *wsc1Δ mid2Δ* mutant at 30 °C (Fig. 3a). Two additional mutants, V371A and D375A, displayed some impairment at 37 °C (Fig. 3b). Here again, the two-hybrid interactions of these mutant forms with Rom2 were consistent with their ability to complement the *wsc1Δ mid2Δ* mutant (Fig. 3c). All four mutants at the extreme C terminus that displayed some deficiency in complementation were deficient in Rom2 interaction, suggesting that this region of Wsc1 also interacts with Rom2. Interestingly, the *wsc1*-L369A and *wsc1*-N373A alleles were less effective at complementation than either the *wsc1*(1-316) or *wsc1*(1-326) truncation mutants (Fig. 3d), supporting the notion that negative regulatory sequences reside between residues 317 and 345. As with the truncation mutants, epitope-tagged versions of all the point mutants in *WSC1* were expressed at wild-type levels (data not shown).

Lodder *et al.* (1999) demonstrated that Wsc1 is phosphorylated within the cytoplasmic domain. Phosphorylation of Wsc1 can be detected as a band shift with increased mobility after treatment with λ protein phosphatase (λ PPase; Fig. 4a and Lodder *et al.*, 1999). Treatment of Wsc1(1-367) or Wsc1(1-346) with λ PPase resulted in a band shift (Fig. 4b). However, λ PPase treatment of Wsc1(1-326) or Wsc1(1-316) failed to produce a band shift, indicating that the regions bounded by residues 326 and 346 are important for phosphorylation. This was also a region determined by deletion analysis to be important for negative regulation of Wsc1 (Fig. 1b). There are five potential phosphorylation sites

Fig. 4. Wsc1 is phosphorylated on its cytoplasmic domain. (a) Protein extract of yeast strain 1783 expressing Wsc1^{HA} from YEp352 (p1657) was treated with λ PPase in the presence or absence of phosphatase inhibitors. Samples were subjected to SDS-PAGE separation followed by immunoblot detection of Wsc1^{HA} using the 12CA5 antibody. (b) Extracts of yeast strain 1783 expressing C-terminal truncations of Wsc1^{HA} from YEp352 (p1449, p1459, p1460, p1516 or p1657) were treated with λ PPase and the indicated form of Wsc1 detected as in (a).

(three seryl and two threonyl residues) within this region (Fig. 5a). To determine if any of these are real phosphorylation sites, we mutated all five seryl and threonyl residues collectively to alanyl residues (designated ST5A). To our surprise, the Wsc1-ST5A mutant form behaved like wild-type with respect to the λ PPase-induced band shift (Fig. 5b), indicating that the phosphorylation site(s) do not reside between residues 326 and 346 and must be further N-terminal to 326. There may be a protein kinase docking site within the region between residues 326 and 346, which would explain the importance of this region for phosphorylation.

We next mutated the four seryl residues that reside between 316 and 326 (Fig. 5a) collectively to alanyl residues (designated S4A). In this case, the Wsc1-S4A mutant protein failed to undergo a λ PPase-induced band shift (Fig. 5b), migrating at the same position as λ PPase-treated wild-type Wsc1. We separated these mutations into adjacent pairs (S319/320A and S322/323A). Both of these mutant proteins behaved as unphosphorylated forms (Fig. 5c), suggesting that all four seryl residues may be phosphorylated in an interdependent manner. Alternatively, some of these seryl residues may be important for substrate recognition by the protein kinase to phosphorylate the others.

To determine the contribution of the phosphorylated seryl residues to Wsc1 function, we introduced the S4A mutation into the *wsc1(1-346)* truncation allele. Fig. 5(d) shows that eliminating these phosphorylation sites partially suppressed the growth defect associated with *wsc1(1-346)*, supporting the conclusion that phosphorylation of Wsc1 serves a negative regulatory role. Phosphorylation site mutants of Wsc1 did not display enhanced two-hybrid interaction with Rom2 (data not shown). However, because only the cytoplasmic domain of Wsc1 was expressed in the two-hybrid clone to allow nuclear localization, it may not be phosphorylated in this setting. Finally, we tested the effect of the S4A mutation on Mpk1 activity by following dual phosphorylation of the MAP kinase (Martin *et al.*, 2000). We did not detect constitutive activity of Mpk1 in cells expressing the *WSC1-S4A* allele under non-inducing conditions (Fig. 5e). However, a shift from 23 to 39 °C reproducibly induced a more rapid activation of Mpk1 than observed in wild-type cells (Fig. 5e), suggesting that dephosphorylation of Wsc1 potentiates signal transduction. Full activation of Mpk1 in response to mild heat shock normally requires 30 min (Kamada *et al.*, 1995; Fig. 5e). By contrast, Mpk1 was fully activated within 10 min after temperature upshift in the *WSC1-S4A* mutant.

These results, taken together, suggest a model for the regulated interaction of Wsc1 with Rom2. We propose that two regions of the Wsc1 cytoplasmic tail interact with Rom2 (Fig. 6). One of these includes tyrosine 303, which is proximal to the plasma membrane. Because a truncation that removes most of the cytoplasmic tail [*wsc1(1-316)*] was partially functional, we conclude that this region is sufficient for Rom2 stimulation in the absence of other cytoplasmic domain sequences. The other Rom2-interacting

Fig. 5. (a) Sequence of the Wsc1 cytoplasmic tail. Seryl and threonyl residues between 326 and 346 (boxed) were mutated in a cluster to alanyl residues (yielding *wsc1-ST5A*). Seryl residues 319, 320, 322 and 323, which were mutated in a cluster in *wsc1-S4A*, are the presumptive phosphorylation sites. Truncation sites are also shown by vertical lines. (b, c) Extracts of yeast strain 1783 expressing the indicated point mutants of Wsc1^{HA} from YE352 (p1847, p1850, p1868, p1869 or p1657) were treated with λ PPase and the indicated form of Wsc1 detected as in Fig. 4. (d) The S4A mutation partially suppressed the defect of the *wsc1(1-346)* truncation mutant. Yeast strain DL2282 (*wsc1 Δ mid2 Δ*) was transformed with centromeric plasmid pRS314 (vector) or pRS314 bearing the indicated allele of *WSC1*^{HA} (p1513, p1672 or p1866). Transformants were streaked onto YE2D and allowed to grow at 28 °C for 2 days. (e) The S4A mutation potentiates Mpk1 activation in response to mild heat shock. Wild-type yeast strain 1788 transformed with plasmids bearing *MPK1*^{HA} (p672) and either *WSC1* (p1672) or *WSC1-S4A* (p1854) were grown to mid-exponential phase at 23 °C in YE2D and shifted to 39 °C for the indicated times. Extracts were tested by immunoblotting for dual phosphorylation of Mpk1 and Mpk1^{HA} (upper panel) and for Mpk1^{HA} protein levels (lower panel).

Fig. 6. Model for the regulated interaction of the Wsc1 cytoplasmic tail with Rom2. Phosphorylation of seryl residues 319/320/322/323 interferes with the interaction of the two indicated regions of Wsc1 with Rom2.

region is at the extreme C terminus of Wsc1, defined by residues 369–375. Disruption of either of these interactions in the context of the full-length protein prevents Rom2 interaction. The presence of a negative regulatory region between residues 316 and 345 also influences function. We identified a cluster of seryl residues within this region of Wsc1 (S319, 320, 322 and 323) that appear to be phosphorylated. Seryl-to-alanyl mutation of this cluster of residues partially suppressed the growth defect of a Wsc1 mutant missing the C-terminal interaction region. Moreover, a WSC1 mutant missing these phosphorylation sites potentiated activation of Mpk1 by mild heat shock. Therefore, we propose that phosphorylation of the Wsc1 cytoplasmic tail interferes with Rom2 interaction (Fig. 6)

and that dephosphorylation provides a means of regulating this interaction in response to cell wall stress. However, we have not been able to detect stress-induced dephosphorylation of Wsc1. Perhaps only a small fraction of the Wsc1 is dephosphorylated in response to wall stress. It will be interesting to identify the protein kinase and phosphatase that are responsible for the regulation of Wsc1.

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REFERENCES

- Buehrer, B. & Errede, B. (1997). Coordination of the mating and cell integrity mitogen-activated protein kinase pathways in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17**, 6517–6525.
- Cid, V. J., Duran, A., Rey, F., Snyder, M. P., Nombela, C. & Sanchez, M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiol Rev* **59**, 345–386.
- Costigan, C., Gehrung, S. & Snyder, M. (1992). A synthetic lethal screen identifies *SLK1*, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. *Mol Cell Biol* **12**, 1162–1178.
- Davenport, K. R., Sohaskey, M., Kamada, Y., Levin, D. E. & Gustin, M. C. (1995). A second osmosensing signal transduction pathway in yeast. *J Biol Chem* **270**, 30157–30161.
- Douglas, C. M., Foor, F., Marrinan, J. A. & 11 other authors (1994). The *Saccharomyces cerevisiae* *FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3- β -D-glucan synthase. *Proc Natl Acad Sci U S A* **91**, 12907–12911.
- Drgonova, J., Drgon, T., Tanaka, K., Kollar, R. Chen G.-C., Ford, R. A., Chan, C. S. M., Takai, Y. & Cabib, E. (1996). Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* **272**, 277–279.
- Errede, B., Cade, R. M., Yashar, B. M., Kamada, Y., Levin, D. E., Irie, K. & Matsomoto, K. (1995). Dynamics and organization of MAP kinase signal pathways. *Mol Repr Dev* **42**, 477–485.
- Gray, J. V., Ogas, J. P., Kamada, Y., Stone, M., Levin, D. E. & Herskowitz, I. (1997). A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. *EMBO J* **16**, 4924–4937.
- Ho, S.-N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- Inoue, S. B., Takewaki, N., Takasuka, T. & 7 other authors (1995). Characterization and gene cloning of 1,3- β -D-glucan synthase from *Saccharomyces cerevisiae*. *Eur J Biochem* **231**, 845–854.
- Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K. & Oshima, Y. (1993). *MKK1* and *MKK2*, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. *Mol Cell Biol* **13**, 3076–3083.
- Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 163–168.
- Jacoby, J. J., Nilius, S. M. & Heinisch, J. J. (1998). A screen for upstream components of the yeast protein kinase C signal transduction pathway identifies the product of the *SLG1* gene. *Mol Genet* **258**, 148–155.

- Jung, U. S. & Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signaling pathway. *Mol Microbiol* **34**, 1049–1057.
- Jung, U. S., Sobering, A. K., Romeo, M. J. & Levin, D. E. (2002). Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall MAP kinase. *Mol Microbiol* **46**, 781–789.
- Kamada, Y., Jung, U. S., Piotrowski, J. & Levin, D. E. (1995). The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev* **9**, 1559–1571.
- Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohya, Y. & Levin, D. E. (1996). Activation of yeast protein kinase C by Rho1 GTPase. *J Biol Chem* **271**, 9193–9195.
- Ketela, T., Green, R. & Bussey, H. (1999). *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. *J Bacteriol* **181**, 3330–3340.
- Kim, Y. J., Francisco, L., Chen, G. C., Marcotte, E. & Chan, C. S. M. (1994). Control of cellular morphogenesis by the Ipl2/Bem2 GTP-activating protein: possible role of protein phosphorylation. *J Cell Biol* **127**, 1381–1394.
- Klis, F. M. (1994). Review: cell wall assembly in yeast. *Yeast* **10**, 851–869.
- Lee, K. S. & Levin, D. E. (1992). Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. *Mol Cell Biol* **12**, 172–182.
- Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K. & Levin, D. E. (1993). A yeast mitogen-activated protein kinase homolog (Mpk1) mediates signaling by protein kinase C. *Mol Cell Biol* **13**, 3067–3075.
- Levin, D. E. & Bartlett-Heubusch, E. (1992). Mutants in the *S. cerevisiae* PKC1 gene display a cell cycle-specific osmotic stability defect. *J Cell Biol* **116**, 1221–1229.
- Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M. & Thorner, J. (1990). A candidate protein kinase C gene *PKC1*, is required for the *S. cerevisiae* cell cycle. *Cell* **62**, 213–224.
- Levin, D. E., Bowers, B., Chen, C., Kamada, Y. & Watanabe, M. (1994). Dissecting the protein kinase C/MAP kinase signaling pathway of *Saccharomyces cerevisiae*. *Cell Mol Biol Res* **40**, 229–239.
- Lodder, A. L., Lee, T. K. & Ballester, R. (1999). Characterization of the Wsc1 protein, a putative receptor in the stress response of *Saccharomyces cerevisiae*. *Genetics* **152**, 1487–1499.
- Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C. & Molina, M. (2000). Regulatory mechanisms for modulation of signaling through the cell integrity Slr2-mediated pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **275**, 1511–1519.
- Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J. A., Nielsen, J. B. & Foor, F. (1995). Differential expression and function of two homologous subunits of yeast 1,3- β -D-glucan synthase. *Mol Cell Biol* **15**, 5671–5681.
- Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. & Takai, Y. (1995). A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J* **14**, 5931–5938.
- Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kamayama, T., Nonaka, H., Hirano, H., Matsuura, Y. & Takai, Y. (1996). Rom1p and Rom2p are small GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP-binding protein in *Saccharomyces cerevisiae*. *EMBO J* **15**, 2196–2207.
- Paravicini, G., Cooper, M., Friedli, L., Smith, D. J., Carpentier, J.-L., Klig, L. S. & Payton, M. A. (1992). The osmotic integrity of the yeast cell requires a functional PKC1 gene product. *Mol Cell Biol* **12**, 4896–4905.
- Peterson, J., Zheng, Y., Bender, L., Myers, A., Cerione, R. & Bender, A. (1994). Interactions between the bud emergence proteins Bem1 and Bem2 and the Rho-type GTPases in yeast. *J Cell Biol* **127**, 1395–1406.
- Philip, B. & Levin, D. E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. *Mol Cell Biol* **21**, 271–280.
- Qadota, H., Python, C. P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E. & Ohya, Y. (1996). Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3- β -glucan synthase. *Science* **272**, 279–281.
- Rajavel, M., Philip, B., Buehrer, B. M., Errede, B. & Levin, D. E. (1999). Mid2 is a putative sensor for cell integrity signaling in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19**, 3969–3976.
- Ram, A. F. J., Brekelmans, S. S. C., Oehlen, L. J. W. M. & Klis, F. M. (1995). Identification of two cell cycle regulated genes affecting the β -1,3-glucan content of cell wall in *Saccharomyces cerevisiae*. *FEBS Lett* **358**, 165–170.
- Rose, M. D., Winston, F. & Hieter, P. (1990). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schmidt, A., Bickle, M., Beck, T. & Hall, M. (1997). The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell* **88**, 531–542.
- Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.
- Torres, L., Martin, H., Garcia-Saez, M. I., Arroyo, J., Molina, M., Sanchez, M. & Nombela, C. (1991). A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae* *lyt2* mutants. *Mol Microbiol* **5**, 2845–2854.
- Verna, J., Lodder, A., Lee, K., Vagts, A. & Ballester, R. (1997). A family of genes required for the maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **94**, 13804–13809.
- Watanabe, Y., Takaesu, G., Hagiwara, M., Irie, K. & Matsumoto, K. (1997). Characterization of a serum response factor-like protein in *Saccharomyces cerevisiae*, Rlm1, which has transcriptional activity regulated by the Mpk1 (Slr2) mitogen-activated protein kinase pathway. *Mol Cell Biol* **17**, 2615–2623.