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Transcriptional reporters for genes activated by cell wall stress through a non-catalytic mechanism involving Mpk1 and SBF

Ki-Young Kim and David E. Levin*

Department of Molecular and Cell Biology, Goldman School of Dental Medicine, Boston University, Boston, Massachusetts 02118

Abstract

The Mpk1 MAP kinase of the Cell Wall Integrity (CWI) signaling pathway induces transcription of the *FKS2* gene in response to cell wall stress through a non-catalytic mechanism that involves stable association of Mpk1 with the Swi4 transcription factor. This dimeric complex binds to a Swi4 recognition site in the *FKS2* promoter. The Swi6 transcription factor is also required to bind this ternary complex for transcription initiation to ensue. In this context, the Mlp1 pseudokinase serves a redundant function with Mpk1. We have identified three additional genes, *CHAI*, *YLR042c*, and *YKR013w* that are induced by cell wall stress through the same mechanism. We report on the behavior of several promoter-*lacZ* reporter plasmids designed to detect cell wall stress transcription through this pathway.

Keywords

cell wall integrity signaling; Mpk1; Swi4; Swi6; transcriptional reporters

Introduction

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (Klis, 1994; Cid *et al.*, 1995). The cell must remodel this rigid structure during vegetative growth, and during pheromone-induced morphogenesis. Wall remodeling is monitored and regulated by the cell wall integrity (CWI) signaling, which activates a MAP kinase cascade (reviewed in Levin, 2005). The MAP kinase cascade is a linear pathway comprised of Pkc1, a MEKK (Bck1), a pair of redundant MEKs (Mkk1/2) and a MAP kinase (Mpk1/Slt2). Mpk1 is a functional homolog of human ERK5 (Truman *et al.*, 2006), a MAPK that is activated in response to growth factors as well as physical and chemical stresses (Abe, *et al.*, 1996; Yan *et al.*, 2001).

CWI signaling is induced in response to a variety of cell wall stressors. First, signaling 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 signaling (Davenport *et al.*, 1995; Kamada *et al.*, 1995). Third, treatment with mating pheromone stimulates signaling at a time that is coincident with the onset of morphogenesis (Buehrer and Errede, 1997). Finally, CWI signaling is also stimulated by agents that interfere with cell wall biogenesis, such as the chitin antagonist Calcofluor white (Ketela *et al.*, 1999), Congo Red, caffeine, or Zymolyase (de Nobel *et al.*, 2000; Martin *et al.*, 2000).

*Correspondence and present address: delevin@bu.edu.

CWI signaling pathway induces activation of two known transcription factors. One of these is Rlm1 (Dodou and Treisman, 1997; Watanabe *et al.*, 1997), which is activated through phosphorylation by Mpk1 (Jung *et al.*, 2002). A second transcription factor that plays a role in CWI signaling is SBF (Madden *et al.*, 1997; Baetz *et al.*, 2001). SBF is a dimeric transcriptional regulator, comprised of Swi4 and Swi6, which is essential to normal regulation of G1-specific transcription (reviewed in Breeden, 2003). Swi4 is the sequence-specific DNA-binding subunit (CA/GCGAAA; Taylor *et al.*, 2000), but Swi6 is required for binding to cell cycle-regulated promoters (Andrews and Moore, 1992; Baetz and Andrews, 1999; Sidorova and Breeden, 1993). Swi6 allows Swi4 to bind DNA at cell cycle-regulated promoters by relieving an auto-inhibitory intramolecular association of the Swi4 C-terminus with its DNA-binding domain. Additionally, Swi6 is the transcriptional activation component of SBF (Sedgwick *et al.*, 1998).

That SBF has a second function related to CWI signaling was suggested with several findings. First, the cell lysis defect of an *mpk1* mutant is suppressed by overexpression of Swi4 (Madden *et al.*, 1997). Second, both *swi4Δ* and *swi6Δ* mutants are hypersensitive to Calcofluor White, supporting a role for SBF in cell wall biogenesis (Igual *et al.*, 1996). Third, Mpk1 associates with SBF *in vivo* (Madden *et al.*, 1997) and with Swi4 (but not Swi6) *in vitro* (Baetz *et al.*, 2001). Fourth, Swi6 is phosphorylated *in vivo* and *in vitro* by Mpk1 in response to cell wall stress (Madden *et al.*, 1997).

We found previously that CWI signaling drives expression of the *FKS2* gene through SBF in a manner that is independent of the role of this factor in G₁-specific transcription (Kim *et al.*, 2008; Truman *et al.*, 2009). Mpk1 and its pseudokinase paralog, Mlp1, which is also activated by the MEKs of the CWI signaling pathway, use a non-catalytic mechanism to activate transcription of *FKS2* that is dependent on SBF and on activating signal. Activated (phosphorylated) Mpk1, or Mlp1, form a complex with Swi4 that associates with an SBF-binding site in the *FKS2* promoter independently of Swi6 (Kim *et al.*, 2008). In this context, Mpk1 (or Mlp1) relieves the auto-inhibitory Swi4 interaction by binding to a MAPK docking site on Swi4 that neighbors the C-terminal Swi6-binding site (Truman *et al.*, 2009). Mutational ablation of the MAPK interaction site on Swi4 specifically blocks cell wall stress-induced *FKS2* transcription, without interfering with the cell cycle functions of Swi4. Although Swi6 is not required for Swi4 to bind to the *FKS2* promoter, it must still be recruited to the Mpk1-Swi4 complex to activate transcription.

Finally, we demonstrated recently that Mpk1 phosphorylation of Swi6 is a negative regulatory event that interferes with Swi6 entry to the nucleus (Kim *et al.*, in press). Nucleocytoplasmic shuttling of Swi6 in response to CWI signaling is a biphasic process. First, upon activation of Mpk1, Swi6 is recruited to the nucleus in a manner dependent on formation of the Mpk1-Swi4 complex, but independent of Mpk1 catalytic activity. Swi6 subsequently exits the nucleus in response to phosphorylation by Mpk1 on Ser238, which interferes with the function of an adjacent nuclear localization signal recognized by the Kap120 β-importin.

Here, we identify three additional genes, *CHAI* (*YCL064c*), *YKR013w*, and *YLR042c*, that are regulated through this cell wall stress transcriptional pathway. We also describe new *lacZ* reporter plasmids with promoter regions from these genes.

Materials and methods

Strains, Growth conditions, and Transformations

The *S. cerevisiae* strains used in this study are listed in Table 1 and are derived from EG123 (Siliciano and Tatchell, 1984). Yeast cultures were grown in YPD (1% Bacto yeast extract,

2% Bacto Peptone, 2% glucose) with or without 2% sorbitol for osmotic support, or SD (0.67% Yeast nitrogen base, 2% glucose) supplemented with the appropriate nutrients to select for plasmids and gene replacements. *Escherichia coli* DH5 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 to carbenicillin resistance by standard methods. Promoter-*lacZ* expression experiments were carried out in SD medium with the exception of the experiments in Figure 1, which included sorbitol (10%) to prevent cell lysis of mutant strains. Methods for β -galactosidase assays are described in Zhao *et al.* (1998). Cell wall stress was induced by increasing the growth temperature from 23°C to 39°C, or by treatment with Congo Red (50 μ g/ml; Sigma), calcofluor white (40 μ g/ml; Sigma), or Zymolyase 100T (2 units/ml; ICN Biomedicals).

Plasmids

The *FKS2* (−540 to −375)-*CYC1-lacZ* reporter plasmid (p2052), was described previously (Kim *et al.*, 2008). A reporter plasmid bearing *CHAI* residues −500 to −263 [*CHAI* (−500 to −263)-*CYC1-lacZ*] (plasmid p2876) was constructed by replacing the 134-bp *SmaI/XhoI* fragment of the *CYC1* promoter of pLGΔ-312 (Guarente and Mason, 1983) with a 237-bp PCR-amplified *CHAI* fragment, resulting in fusion of *CHAI* promoter sequences to the *CYC1* minimal promoter.

Four reporter plasmids bearing *YKR013w* promoter sequences were constructed. A full-length promoter-*lacZ* reporter was created [*YKR013w* (−1000 to −1)-*lacZ*] (plasmid p2959) by replacing the entirety of the *CYC1* promoter of pLGΔ-312 on a *SmaI/BamHI* fragment with a 1000-bp PCR-amplified *YKR013w* fragment, resulting in fusion of *YKR013w* promoter sequences to the start codon of *lacZ*. The second reporter bearing *YKR013w* residues −461 to −287 [*YKR013w* (−461 to −287)-*CYC1-lacZ*] (plasmid p2879) was constructed by replacing the 134-bp *SmaI/XhoI* fragment of the *CYC1* promoter of pLGΔ-312 with a 174-bp fragment of *YKR013w*, as described above for *CHAI*. Two additional reporters further subdivided this region of the *YKR013w* promoter to create plasmids *YKR013w* (−461 to −364)-*CYC1-lacZ* (plasmid p2960) and *YKR013w* (−371 to −287)-*CYC1-lacZ* (plasmid p2878).

Finally, a reporter plasmid bearing *YLR042c* residues −1000 to −1 [*YLR042c* (−1000 to −1)-*lacZ*] (plasmid p2880) was constructed by replacing the entirety of the *CYC1* promoter of pLGΔ-312 on a *SmaI/BamHI* fragment with a 1000-bp PCR-amplified *YLR042c* fragment. All PCR-amplified sequences were confirmed by DNA sequence analysis across the entire amplified region. Primer sequences available upon request.

Results

We wished to identify additional genes whose regulation in response to cell wall stress was similar to that of *FKS2*. Two previous studies were useful toward this goal. García *et al.* (2004 (2009) determined the global transcriptional responses to cell wall stress induced by Congo Red and Zymolyase. Among the many genes whose expression was induced by cell wall stress, only a small number did not require the Rlm1 transcription factor. These included *CHAI*, *FKS2*, *PHO89*, *YLR042c* (García *et al.* 2004), and *YKR013w* (García *et al.* 2009). Each of these genes, except *PHO89*, contains at least one consensus site for Swi4 binding (C[A/G]CGAAA). Therefore, we constructed *lacZ* reporter plasmids bearing 1000 nucleotides 5' to the translational start codon for each of these genes (except *FKS2*).

The *YKR013w* promoter contains three putative Swi4-binding sites at positions −377, −361, and −209. The full-length *YKR013w* promoter fusion to *lacZ* expressed a high level of basal activity, which complicated detection of induced transcription in response to cell wall stress

induced by elevated growth temperature (Figure 1). The high level of basal transcriptional activity is likely due to the periodic regulation of *YKR013w* through the cell cycle (Spellman *et al.*, 1998) through one or more of its Swi4-binding sites active during G1 transcription. Therefore, we created additional reporters that separated these sites from one another. A reporter that carries the two 5'-most Swi4-binding sites, nucleotides -461 to -287 fused to the basal *CYC1* promoter, behaved essentially like the full-length promoter, but displaying approximately half the basal activity with no detectable induction by cell wall stress (Figure 1). Therefore, we separated these sites from each other in two additional reporters. One of these contained the 5'-most Swi4 site (nucleotides -461 to -364); the other contained the middle Swi4 site (nucleotides -371 to -287). Both of these reporters displayed low basal expression, suggesting that both Swi4 sites are required for cell cycle regulation. Although the reporter bearing the Swi4 site at position -361 was responsive to cell wall stress, the reporter with the Swi4 site at position -377 was not, suggesting that there is an important functional distinction between these two Swi4 sites.

The *YLR042c* promoter contains a single putative Swi4-binding site at position -179. Similarly, the *CHAI* promoter contains a single putative Swi4-binding site at position -286. However, it also contains a heat shock responsive Hsf1-binding element at position -701 and an ER stress-responsive Hac1-binding element at position -200, which confounded transcriptional activation by cell wall stress in response to elevated temperature (not shown). To isolate the SBF-binding site from the other elements, we created a *CHAI-lacZ* reporter in which *CHAI* nucleotides -500 to -263 were fused to the basal *CYC1* promoter, as described previously for *FKS2* (Kim *et al.*, 2008). The *PHO89-lacZ* reporter was non-responsive to cell wall stress in our hands. Although the reporter we created possessed 1 kb of 5' untranslated sequence, we did not find a consensus Swi4-binding site at any point within 2 kb of the translational start site for this gene. We cannot explain the discrepancy between our results and the microarray data (García *et al.* 2004). Therefore, we did not pursue *PHO89* further.

We compared the new promoter-*lacZ* reporters to an *FKS2-lacZ* reporter (Kim *et al.*, 2008) for transcriptional induction in response to cell wall stress by increased growth temperature, or treatment with Congo Red, calcofluor white, or Zymolyase. All of these reporters were induced after 15 hours by all treatments, although there was variability among the reporters regarding relative induction by each stress (Figure 2). However, in general, elevated growth temperature was the strongest inducer for all of the reporters. In this regard, the *CHAI-lacZ* reporter showed the greatest relative increase (approximately 10-fold) at high temperature. The *YLR042c-lacZ* reporter was induced equivalently by heat stress, Congo Red, and calcofluor white, suggesting this as a good general reporter for cell wall stress.

Induction by all cell wall stresses except elevated temperature was entirely dependent upon Mpk1, but independent of Mpk1 catalytic activity, because the catalytically inactive *mpk1-K54R* mutant behaved essentially like wild-type for all stresses. Induction was only partially dependent on the Mlp1 pseudokinase (Figure 2), a catalytically inactive paralog of Mpk1 (Kim *et al.*, 2008). However, all induction was blocked in an *mpk1Δ mlp1Δ* double mutant, reflecting the partial overlap in function of Mpk1 and Mlp1 in this context. The greater dependence on Mpk1 over Mlp1 results from the fact that *MLP1* is transcriptionally induced by Mpk1 through the Rlm1 factor (Jung and Levin, 1999; Jung *et al.*, 2002). Therefore, in the absence of Mpk1, very little Mlp1 is present (not shown).

Transcriptional induction of all of these reporters by cell wall stress was strictly dependent on both *SWI4* and *SWI6* (Figure 2), consistent with the presumptive use of the SBF-binding sites in these reporters through the non-catalytic mechanism described for *FKS2* (Kim *et al.*, 2008). Taken together, we conclude that all of these genes are induced in response to cell

wall stress through a common mechanism that involves activation of Mpk1 and Mlp1 to engage Swi4 and Swi6.

To assess the kinetics of reporter activation, we conducted a time course for activity in response to temperature stress at 39°C. We had determined previously that *FKS2-lacZ* reporters were activated slowly, with β -galactosidase activity continuing to increase for at least 15 hours after induction (Kim *et al.*, 2008). This proved to be true for the new reporters as well (Figure 3). In all cases, reporter activity increased over time up to 15 hours of induction. This is in contrast to Rlm1-driven *lacZ* reporters, also activated by Mpk1, which typically reach a maximum level between two and four hours after thermal induction (Jung *et al.*, 2002).

Discussion

The transcriptional program activated in response to cell wall stress through the CWI pathway is largely directed by the Rlm1 transcription factor (Jung and Levin, 1999; García *et al.*, 2004; Roberts *et al.*, 2000). However, induction of the *FKS2* gene through the CWI pathway is known to be independent of Rlm1 (Zhao *et al.*, 1998; Jung and Levin, 1999). Induction of *FKS2* by wall stress requires the CWI MAPK cascade, but does not require the catalytic activity of the Mpk1 MAPK (Kim *et al.*, 2008). Instead, transcriptional activation of *FKS2* proceeds through a non-catalytic mechanism that involves the binding of activated (phosphorylated) Mpk1, or its pseudokinase paralog, Mlp1, to the Swi4 transcription factor. This dimer binds to the *FKS2* promoter, but requires the further binding of the Swi6 transcriptional activator for transcriptional initiation.

In this study, we show that *FKS2* is not the only gene induced through this non-catalytic mechanism. Three additional genes, *CHA1*, *YKR013w*, and *YLR042c*, which were identified previously as induced by cell wall stress independently of Rlm1 (García *et al.* 2004, 2009), behave in a similar manner to *FKS2*. *CHA1* encodes a serine-threonine ammonia lyase required for the catabolic deamination of these hydroxy amino acids (Catabolism of Hydroxy Amino acids; Petersen *et al.*, 1988). It is not clear why such an enzyme would be induced in response to cell wall stress. Both *YKR013w*, and *YLR042c* are uncharacterized, but *YLR042c* encodes a putative GPI-cell wall protein (Caro *et al.*, 1997), consistent with a role in cell wall biogenesis. However, deletion of any of these genes does not result in detectable sensitivity cell wall stress (data not shown). This may reflect functional redundancies, as in the case of *FKS1* and *FKS2*. Alternatively, cell wall stress sensitivity may require preventing the change in expression of numerous genes simultaneously.

We presented three new *lacZ* reporter plasmids that all displayed identical behavior to that of an *FKS2-lacZ* reporter with regard to the genetic requirements for activation by cell wall stresses. However, there appear to be quantitative differences for induction among these various reporters. For example, the *CHA1-lacZ* reporter displayed the greatest relative induction in response to heat stress. By contrast, the *YLR042c-lacZ* reporter appears to be the most generally responsive to all cell wall stresses tested.

Among the four genes identified, at least two of them possess complex promoters that are responsive to multiple stresses. The *FKS2* gene is especially complex, with a Crz1-binding calcineurin responsive element and a pair of Mig1-binding glucose depletion responsive elements (Zhao *et al.*, 1998). In addition to a Swi4-binding site, the *CHA1* gene possesses an Hsf1-binding heat shock responsive element and a Hac1-binding ER stress-responsive element. It is very likely that additional genes regulated by the non-catalytic Mpk1 pathway have gone undetected because of the complexity of their promoters and the consequent overlap of various stress signals.

One important outstanding question concerns the distinction between Swi4 recognition sites bound by Swi4-Mpk1 in response to cell wall stress from those recognized by Swi4-Swi6 for cell cycle regulation. The core seven nucleotide binding motif is identical in both cases (Kim et al., 2008), but surrounding sequences that have not yet been established dictate which dimeric factor will associate with a particular Swi4-recognition site. In this regard, it is interesting to note that the *YKR013w* promoter contains three potential Swi4-recognition sites and is regulated both by cell wall stress and periodically through the cell cycle. We found that a reporter containing the two 5'-most sites, which are separated from each other by only eight nucleotides and reside in opposite orientation, was capable of high basal (cell cycle) regulation. When we separated these two sites from each other, the 3' Swi4 site was capable of conferring cell wall stress regulation, and displayed low basal expression as compared with the reporter that contained both sites, suggesting that this reporter is not regulated through the cell cycle. By contrast, a reporter containing the 5' Swi4 site displayed low basal expression and was unresponsive to cell wall stress. This suggests that, at least in this context, cell wall stress regulation is conferred by only one of these sites, whereas cell cycle regulation requires both. Further dissection of the *YKR013w* promoter should provide insight into specific sequence requirements for Swi4-Mpk1 and Swi4-Swi6 recognition.

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Figure 1.

Dissection of the *YLR013w* promoter. Reporter plasmids *YKR013w* (– 1000 to –1)-*lacZ* (p2959), *YKR013w* (–461 to –287)-*CYC1-lacZ* (p2879), *YKR013w* (–461 to –364)-*CYC1-lacZ* (p2960), or *YKR013w* (–371 to –287)-*CYC1-lacZ* (p2878) were transformed into a wild-type yeast strain (1788). Transformants were cultivated in SD –Ura medium and grown at room temperature (23 C), or subjected to cell wall stress by shift to 39 C for 15 hours. -galactosidase activity was measured in crude extracts. Each value represents the mean and standard deviation from three independent transformants. Sp. Act., specific activity; U unit. A. Reporter plasmids p2959 and p2879. B. Reporter plasmids p2960 and p2878.

Figure 2.

Genetic requirements for cell wall stress induction of *FKS2*, *CHA1*, *YLR042c* and *YKR013w* reporter plasmids. Each reporter plasmid was transformed into the indicated strains. For expression of the catalytically inactive Mpk1-K54R mutant, plasmid pRS315 *mpk1-K54R-3xHA* (p2193; Kim et al., 2009) was co-transformed into the *mpk1Δ* mutant. Transformants were cultivated in selective medium supplemented with 2% sorbitol to prevent cell lysis. Samples were grown at room temperature (RT), or subjected to cell wall stress by shift to 39° C (thermal stress; TS), Congo red (CR; 50μg/ml), Calcofluor white (CFW; 40μg/ml), or Zymolyase (Zy; 2 U/ml) treatment for 15 hours. A. Plasmid *FKS2* (–540 to –375)-*CYC1-lacZ* (p2052). B. Plasmid *CHA1* (–500 to –263)-*CYC1-lacZ* (p2876). C. Plasmid *YLR042c* (–1000 to –1)-*lacZ* (p2880). D. Plasmid *YKR013w* (–371 to –287)-*CYC1-lacZ* (p2878). β-galactosidase activity was measured in crude extracts. Each value represents the mean and standard deviation from three independent transformants. Sp. Act., specific activity; U unit.

Figure 3.

Time course for induction of *lacZ* reporters by heat stress. The reporter plasmids in Figure 2, or empty vector (pLGΔ-178; Guarente and Mason, 1983) were transformed into a wild-type yeast strain (1788). Transformants were grown in SD –Ura medium at room temperature, or stressed at 39°C for the indicated times. β-galactosidase activity was measured in crude extracts. Each value represents the mean and standard deviation from three independent transformants. Sp. Act., specific activity; U unit.

Table 1*S. cerevisiae* strains

Strain	Relevant genotype	Reference or source
1788	<i>MATa/MAT α leu2-3,112 trp1-1 ura3-52 his4 can1^r</i>	I. Herskowitz
DL456	<i>MATa/MAT α mpk1Δ::TRP1/mpk1Δ::TRP1</i>	Lee <i>et al.</i> , 1993
DL2651	<i>MATa/MAT α mlp1Δ::LEU2/mlp1Δ::LEU2</i>	Truman <i>et al.</i> , 2009
DL3145	<i>MATa/MAT α swi4Δ::TRP1/swi4 Δ::TRP1</i>	Kim <i>et al.</i> , 2008
DL3148	<i>MATa/MAT α swi6 Δ::LEU2/swi6 Δ::LEU2</i>	Kim <i>et al.</i> , 2008
DL3327	<i>MATa/MAT α mpk1Δ::TRP1/mpk1Δ::TRP1 mlp1 Δ::ura3/mlp1Δ::ura3</i>	Kim <i>et al.</i> , in press