

Mechanism of Mpk1 Mitogen-Activated Protein Kinase Binding to the Swi4 Transcription Factor and Its Regulation by a Novel Caffeine-Induced Phosphorylation[∇]

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The Mpk1 mitogen-activated protein kinase (MAPK) of the cell wall integrity signaling pathway uses a noncatalytic mechanism to activate the SBF (Swi4/Swi6) transcription factor. Active Mpk1 forms a complex with Swi4, the DNA-binding subunit of SBF, conferring the ability to bind DNA. Because SBF activation is independent of Mpk1 catalytic activity but requires Mpk1 to be in an active conformation, we sought to understand how Mpk1 interacts with Swi4. Mutational analysis revealed that binding and activation of Swi4 by Mpk1 requires an intact D-motif-binding site, a docking surface common to MAPKs that resides distal to the phosphorylation loop but does not require the substrate-binding site, revealing a novel mechanism for MAPK target regulation. Additionally, we found that Mpk1 binds near the autoinhibitory C terminus of Swi4, suggesting an activation mechanism in which Mpk1 substitutes for Swi6 in promoting Swi4 DNA binding. Finally, we show that caffeine is an atypical activator of cell wall integrity signaling, because it induces phosphorylation of the Mpk1 C-terminal extension at Ser423 and Ser428. These phosphorylations were dependent on the DNA damage checkpoint kinases, Mec1/Tel1 and Rad53. Phosphorylation of Ser423 specifically blocked SBF activation by preventing Mpk1 association with Swi4, revealing a novel mechanism for regulating MAPK target specificity.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (12, 28). 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 pathway controlled by the Rho1p GTPase (reviewed in reference 31). 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), stimulating GS activity in a GTP-dependent manner. A second essential function of Rho1 is to bind and activate protein kinase C, which is encoded by *PKC1*. Loss of Pkc1 function, or of any of the components of the mitogen-activated protein kinase (MAPK) cascade under its control, results in a cell lysis defect that is attributable to a deficiency in cell wall construction. The MAPK cascade is a linear pathway comprised of a MEK kinase (Bck1), a pair of redundant MEKs (Mkk1/2), and a MAPK (Mpk1/Slt2). Mpk1 is a functional homolog of human extracellular signal-regulated kinase 5 (ERK5) (48), a MAPK that is activated in response to growth factors as well as physical and chemical stresses (1, 57).

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 to 39°C; 23), consistent with the finding that strains with null mutations in many of

the pathway components display cell lysis defects only when cultivated at high temperatures. Second, hypoosmotic shock induces a rapid but transient activation of signaling (13, 23). Third, treatment with mating pheromone stimulates signaling at a time that is coincident with the onset of morphogenesis (9). Finally, CWI signaling is also stimulated by agents that interfere with cell wall biogenesis, such as the chitin antagonist calcofluor white (25), Congo red, caffeine, or zymolyase (14, 35).

One consequence of CWI signaling is activation of the Rlm1 transcription factor (15, 55) through phosphorylation by Mpk1 (21). Rlm1 regulates the transcription of a wide array of cell wall metabolism genes (17, 22, 40). However, the *FKS2* gene, which encodes one of two alternative catalytic subunits of the GS, is an unusual transcriptional target of CWI signaling, because it is induced independently of Rlm1 (22, 60).

A second transcription factor that plays a role in CWI signaling is SBF (6, 33). SBF is a dimeric transcriptional regulator, comprised of Swi4 and Swi6, which is essential to normal regulation of G₁-specific transcription (reviewed in reference 8). Swi4 is the sequence-specific DNA-binding subunit (47), but Swi6 is required for binding to cell cycle-regulated promoters (4, 5, 43). Swi6 allows Swi4 to bind DNA by relieving an intramolecular association of the Swi4 C terminus with its DNA-binding domain. The fact that SBF has a second function related to CWI signaling was established with the finding that Mpk1 associates with SBF in vivo (33) and with Swi4 (but not Swi6) in vitro (6). Additionally, Swi6 is phosphorylated in vivo and in vitro by Mpk1 in response to cell wall stress (33).

We reported previously that CWI signaling drives expression of the *FKS2* gene through SBF (27). Mpk1 and its pseudokinase paralog, Mlp1, use a noncatalytic mechanism to activate

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype ^a	Source or reference
1788	<i>MATa/MATα</i> EG123 <i>leu2-3,112 trp1-1 ura3-52 his4 can1^r</i>	I. Herskowitz
DL100	<i>MATa</i> EG123 <i>leu2-3,112 trp1-1 ura3-52 his4 can1^r</i>	I. Herskowitz
DL456	<i>MATa/MATα</i> EG123 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i>	23
DL2649	<i>MATa</i> EG123 <i>mlp1Δ::LEU2</i>	This study
DL3145	<i>MATa/MATα</i> EG123 <i>swi4Δ::TRP1/swi4Δ::TRP1</i>	27
DL3195	<i>MATa/MATα</i> S288c <i>mpk1Δ::KanMX4/mpk1Δ::KanMX4</i>	27
DL3196	<i>MATa/MATα</i> S288c <i>mpk1Δ::KanMX4/mpk1Δ::KanMX4 mlp1Δ::KanMX4/mlp1Δ::KanMX4</i>	27
DL3929	<i>MATa</i> EG123 <i>mlp1Δ::LEU2 mpk1::MPK1-FLAG-HphMX4</i>	This study
DL3930	<i>MATa</i> EG123 <i>mlp1Δ::LEU2 mpk1::mpk1-S423A-FLAG-HphMX4</i>	This study
DL3931	<i>MATa</i> EG123 <i>mlp1Δ::LEU2 mpk1::mpk1-S428A-FLAG-HphMX4</i>	This study
DL3932	<i>MATa</i> EG123 <i>mlp1Δ::LEU2 mpk1::mpk1-S423A, S428A-FLAG-HphMX4</i>	This study
DL3950	<i>MATα</i> RDK2669 <i>sm1Δ::TRP1</i>	M. Smolka
DL3953	RDK2669 <i>sm1Δ::TRP1 rad53Δ::HIS3</i>	M. Smolka
DL3954	<i>MATa/MATα</i> RDK2669 <i>sm1Δ::TRP1/SML1 mec1Δ::HIS3/MEC1 tel1Δ::URA3/TEL1</i>	M. Smolka
DL3955	RDK2669 <i>sm1Δ::TRP1 mec1Δ::HIS3 tel1Δ::URA3</i> (p2704)	This study
DL3956	RDK2669 <i>sm1Δ::TRP1 mec1Δ::HIS3 tel1Δ::URA3</i> (p2824)	This study
DL3957	RDK2669 <i>sm1Δ::TRP1 mec1Δ::HIS3 tel1Δ::URA3</i> (p2825)	This study
PJ694a	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	S. Fields
PJ694α	<i>MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	S. Fields

^aStrain background EG123 is described by Siliciano and Tatchell (45).

transcription of *FKS2* that is dependent on SBF and on an activating signal to Mpk1. Activated (phosphorylated) Mpk1, or Mlp1, form a complex with Swi4 that associates with an SBF-binding site in the *FKS2* promoter independently of Swi6. However, Swi6 is recruited to this complex to activate transcription. Because transcriptional activation of *FKS2* through SBF is independent of Mpk1 catalytic activity but nevertheless requires Mpk1 to be in an active (phosphorylated) conformation, we were interested in how Mpk1 interacts with Swi4. MAPKs bind to their substrates and regulators through two distinct protein-protein interaction sites. A docking sequence (D motif) on the substrate or regulator engages in a high-affinity interaction with the D-motif-binding (DB) site of the MAPK, which is remote from the active site and is comprised of an acidic patch called the common docking site and a hydrophobic docking groove (2, 59). The bound protein wraps around the MAPK to make additional contacts with a second surface called the substrate-binding (SB) site, which is positioned near the catalytic center (59).

Here, we demonstrate that binding and activation of Swi4 by phosphorylated Mpk1 requires an intact DB site, but not the SB site, revealing a novel mechanism by which a MAPK activates a target. This finding also indicates that DB site interactions are regulated by the phosphorylation state of the MAPK. Additionally, Mpk1 binds near the C terminus of Swi4, adjacent to the Swi6-binding site, suggesting a Swi4 activation mechanism in which Mpk1 substitutes for Swi6 in promoting Swi4 DNA binding. Finally, we show that caffeine is an atypical activator of CWI signaling, because it induces phosphorylation of Mpk1 on two serine residues in its C-terminal extension. These modifications are dependent on the DNA damage checkpoint kinases Mec1/Tel1 and Rad53, and phosphorylation specifically at Ser423 blocks Mpk1 activation of SBF by preventing its association with Swi4.

MATERIALS AND METHODS

Strains, growth conditions, and transformations. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Yeast cultures were grown in YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) with or without 10% sorbitol or in SD (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate nutrients to select for plasmids and gene replacements. *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 to carbenicillin resistance by standard methods. Two-hybrid tests were conducted using yeast strains PJ694a and PJ694α (gift of Stanley Fields) transformed with Gal4 activation domain (AD) fusion plasmids of Swi4 or a Gal4 DNA-binding domain (DBD) fusion plasmid of Mpk1 (26), respectively. Transformed strains were crossed under selective conditions to generate diploids expressing the desired pair of fusions. Diploid strains were grown to mid-log phase for extract preparation. Promoter-*lacZ* expression experiments were carried out as described previously (27). β-Galactosidase assays for promoter-*lacZ* expression experiments and for two-hybrid analyses were conducted as described previously (60). Because *MPK1* mutants display an osmotically remedial cell lysis defect under conditions of cell wall stress (31), it was necessary to conduct gene expression experiments with these mutants in the presence of sorbitol for osmotic support, which diminished the stress responses somewhat.

Plasmid and strain construction. Plasmids used in this study are listed in Table 2. Plasmid p2704 contains the *MPK1* coding sequence and its own promoter (residues -1 to -453) cloned with a C-terminal FLAG epitope by SalI/NotI into centromeric plasmid pRS315 (44). This plasmid was used as the basis for construction of the various mutant alleles of *MPK1* by QuikChange site-directed mutagenesis (53). Plasmid p2713 contains the *SWI4* coding sequence and its promoter (residues -1 to -829), amplified by PCR from genomic yeast DNA (S288c), and cloned by SalI/NotI into centromeric plasmid pRS315. This plasmid was used for construction of mutant *SWI4* alleles (*swi4-I913A, I915A, and swi4-L966A, L968A*; plasmids p2714 and p2715, respectively) by QuikChange mutagenesis. The *swi4-I913A, I915A* allele was amplified by PCR from p2714 with a C-terminal six-histidine epitope as a SpeI/XhoI fragment and cloned into pUT36 (p2415) (27) for expression of the mutant form of Swi4-6HIS under the control of the *MET25* promoter (p2716).

Gal4 AD fusions to Swi4 for two-hybrid analysis were constructed in pGAD424 (Clontech Technologies; p1173) by PCR amplification of the coding region using primers that include a SalI site in the upstream primer and a BglII site in the downstream primer. The Gal4 DBD fusion to the catalytic domain of

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference or source
pRS315	<i>LEU2</i> -based centromeric plasmid	44
p1172	pGBT9; two-hybrid vector with Gal4-DBD	Clontech
p1173	pGAD424; two-hybrid vector with Gal4-AD	Clontech
p1366	<i>PRM5 (YIL117c)-lacZ</i> with <i>URA3</i> marker	21
p2052	<i>FKS2 (-540 to -375)-CYC1-lacZ</i> with <i>URA3</i> marker	27
p2066	<i>CLN2 (-600 to -400)-CYC1-lacZ</i> with <i>URA3</i> marker	27
p2266	pGBT9 <i>MPK1(1-328)</i>	26
p2313	YEp351 <i>MPK1-FLAG</i>	26
p2316	YEp351 <i>mpk1-T190A, Y192F-FLAG</i>	27
p2317	YEp351 <i>mpk1-D237A-FLAG</i>	27
p2352	pGAD424 <i>SWI4(1-1093)</i>	27
p2415	pUT36; <i>URA3</i> -based <i>MET25</i> promoter	27
p2418	pUT36 <i>SWI4-6HIS</i>	27
p2704	pRS315 <i>MPK1-FLAG</i>	This study
p2705	pRS315 <i>mpk1-H116A-FLAG</i>	This study
p2706	pRS315 <i>mpk1-R196A-FLAG</i>	This study
p2707	pRS315 <i>mpk1-K234A-FLAG</i>	This study
p2708	pRS315 <i>mpk1-D237A-FLAG</i>	This study
p2709	pRS315 <i>mpk1-K83A-FLAG</i>	This study
p2710	pRS315 <i>mpk1-Q166A-FLAG</i>	This study
p2711	pRS315 <i>mpk1-W321A-FLAG</i>	This study
p2712	pRS315 <i>mpk1-K54R-FLAG</i>	This study
p2713	pRS315 <i>SWI4</i>	This study
p2714	pRS315 <i>swi4-(I913A, I915A)</i>	This study
p2715	pRS315 <i>swi4-(L966A, L968A)</i>	This study
p2716	pUT36 <i>swi4-(I913A, I915A)-6HIS</i>	This study
p2717	pGAD424 <i>SWI4(1-671)</i>	This study
p2718	pGAD424 <i>SWI4(672-1093)</i>	This study
p2824	YEp351 <i>mpk1-S423A-FLAG</i>	This study
p2825	YEp351 <i>mpk1-S428A-FLAG</i>	This study
p2826	YEp351 <i>mpk1-S423A, S428A-FLAG</i>	This study

Mpk1 [pGBT9-Mpk1(1-328) (pGBT9-Mpk1 with amino acid residues 1 to 328); p2266] was a gift of M. Molina.

Phospho-site mutants of Mpk1 were constructed by QuikChange mutagenesis of *MPK1* in plasmid YEp351 (*MPK1-FLAG*) (p2313) to create *mpk1-S423A-FLAG* (p2824), *mpk1-S428A-FLAG* (p2825), and *mpk1-S423A, S428A-FLAG* (p2826). For construction of strains expressing integrated phospho-site mutants of Mpk1, *MPK1-FLAG* was amplified by PCR with BamHI/AscI overhangs and inserted into BamHI/AscI-cut vector pAG32 (contains the *Hph* gene, which confers resistance to hygromycin B) (18) (p2823), creating pAG32-*MPK1-FLAG* (p2819). *MPK1* was then mutated at either Ser423 or Ser428 to Ala by QuikChange mutagenesis, resulting in pAG32-*mpk1-S423-FLAG* (p2820), *mpk1-S428A-FLAG* (p2821), and *mpk1-S423A, S428A-FLAG* (p2822). The *MPK1-FLAG-Hph* cassettes from these alleles were then PCR amplified using primers for the 5' end of *MPK1* and the 3' end of the *Hph* cassette. The 5' primers contained overhangs to the promoter of *MPK1*, whereas the *Hph* cassette primer contained overhangs to sequence directly downstream of the *MPK1* gene. The resulting cassettes were purified and transformed into yeast strain DL2649 (*mlp1Δ*) on YPD containing 300 μg/ml hygromycin B. Colonies were checked by colony PCR for proper integration of the cassette. The resulting strains contained the *MPK1-FLAG* alleles replacing the native *MPK1* gene. All PCR-amplified sequences and site-directed mutations were confirmed by DNA sequence analysis across the entire amplified region or the complete gene, respectively. Primer sequences are available upon request.

The *mecl1Δ tel1Δ sml1Δ* strains (DL3955, DL3956, and DL3957) were isolated as segregants from the triply heterozygous diploid strain, DL3954 (MBS115; gift of M. Smolka), which had been transformed with plasmids that express mutant forms of Mpk1-FLAG. The strain construction was done in this way because *mecl1Δ tel1Δ sml1Δ* strains are genetically unstable and refractory to transformation.

Coimmunoprecipitation. Protein extraction and coimmunoprecipitation of Mpk1-FLAG from yeast strain DL456 (*mpk1Δ*) or DL3145 (*swi4Δ*) expressing Mpk1-FLAG with or without Swi4-6HIS (from 300 μg protein) were conducted

as described previously (23), using mouse monoclonal anti-FLAG (M2) affinity beads (Sigma) or protein A affinity beads (no-antibody controls; Sigma). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (on 7.5% gels) of samples representing 100 μg of initial protein was followed by immunoblot detection of either Mpk1-FLAG (M2 mouse monoclonal anti-FLAG antibody; Sigma) at a dilution of 1:10,000 or Swi4-6HIS (mouse monoclonal anti-tetra-His antibody; Qiagen) at a dilution of 1:3,000. Secondary antibodies (goat anti-mouse antibodies) were used at a dilution of 1:5,000. Input (lysate) controls represented 50 μg of initial protein.

ChIP. Chromatin immunoprecipitation (ChIP) experiments to detect association of Swi4-6HIS with the *CLN2* promoter or the *FKS2* promoter were carried out as described previously (27) with the following modifications. Swi4-6HIS was precipitated with Ni-nitrilotriacetic acid Superflow beads (30 μl; Qiagen). Primer sequences for amplifying the *FKS2* promoter were GGCTAAAGAAATTTGG GCGG (upstream) and CGTGCTGGGTAGACTTTTAG (downstream), amplifying a 346-bp region. Primers for the *CLN2* promoter were TATCCTCCG CACTTTTACCC (upstream) and CTACGCCAAATGTGCTCTTC (downstream), amplifying a 273-bp region.

Immunoblot detection of Swi6 and phosphorylated Mpk1. Activated Mpk1 was detected with rabbit polyclonal anti-phospho-p44/p42 MAPK (Thr²⁰²/Tyr²⁰⁴) antibodies (New England Biolabs) as described previously (50), with the following modifications. Mpk1-FLAG was immunoprecipitated from extracts (100 μg protein extract) prior to SDS-PAGE and immunoblot analysis using 25% of the sample. The anti-phospho-p44/p42 antibody was used at a dilution of 1:3,000, and the secondary antibody (donkey anti-rabbit antibody; GE Healthcare) was used at a dilution of 1:4,000. Serine-phosphorylated Mpk1 was also detected from immunoprecipitated Mpk1-FLAG (as described above) using mouse monoclonal antiphosphoserine antibodies (Sigma) at a dilution of 1:2,000 and the secondary antibody (goat anti-mouse antibody; Jackson Immunoresearch) at a dilution of 1:5,000. The antiphosphoserine antibody is reported by the manufacturer to detect specifically phosphoserine.

RESULTS

Mutations in the SB site and DB site of Mpk1 define separation-of-function alleles. Rlm1 is an Mpk1 target whose activity depends on phosphorylation by Mpk1 (21, 55). In contrast, Swi4 is an Mpk1 target whose activity in response to Mpk1 activation is independent of phosphorylation by Mpk1 (27). To understand how Mpk1 interacts with and activates Swi4, we created a set of mutations in Mpk1 that were predicted to ablate protein binding at either the DB site or the SB site. The mutations created were based on the residues important for the mammalian MAPK ERK2 association with its substrates and regulators (Elk1 and MKP; 59). Mutations in the Mpk1 DB site (K83A, Q166A, and W321A; Fig. 1), located on the backside of the protein relative to the catalytic pocket, were predicted to interfere with high-affinity docking interactions but to still allow activation of phosphorylation targets through interaction with the SB site. In contrast, mutations in the Mpk1 SB site (H116A, R196A, K234A, and D237A; Fig. 1) were predicted to block activation of targets that must interact with the catalytic pocket, while still allowing docking interactions at the DB site.

We used two transcriptional readouts that distinguish between Mpk1 activation of Rlm1 by phosphorylation (*PRM5-lacZ*; 21) and Mpk1 activation of Swi4 by its noncatalytic mechanism (*FKS2-lacZ*; 27). All of the mutations within the Mpk1 SB site completely blocked Rlm1-driven transcription in response to CWI signaling induced by either thermal stress (39°C) or caffeine treatment (Fig. 2A). In contrast, these mutant forms were able to activate Swi4-dependent transcription normally in response to thermal stress (Fig. 2B). On the other hand, all of the Mpk1 DB site mutants were completely defective for activation of Swi4-dependent transcription, but two of

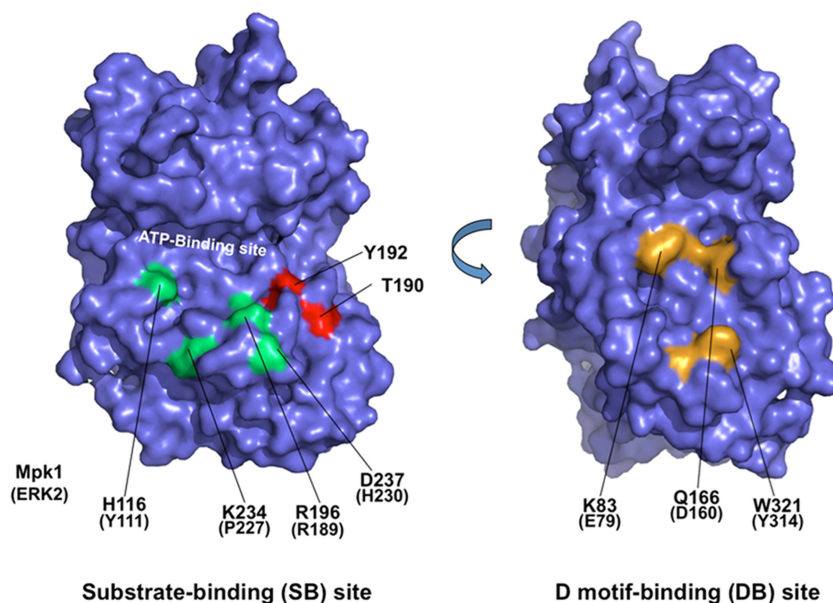


FIG. 1. Positions of substrate-binding site mutations and D-motif-binding site mutations within the predicted structure of Mpk1. The structure was modeled against known MAPK structures using the SWISS-MODEL program (<http://swissmodel.expasy.org/SWISS-MODEL.html>). The two images are rotated 180° with respect to each other. Mutated substrate-binding site residues are indicated in green, and mutated D-motif-binding site residues are indicated in yellow. Residues phosphorylated in the activation loop (T190 and Y192) are indicated in red.

these mutants (K83A and Q166A mutants) retained some Rlm1-dependent transcription. The *mpk1-W321A* mutant appears to be defective for both functions. None of the mutations impaired phosphorylation of Mpk1 by its activating kinases (Mkk1/2) in response to cell wall stress (Fig. 2C), indicating that the observed deficits in Mpk1 function do not result from an inability to interact with its activators. In fact, all of these mutants displayed an increased basal level of Mpk1 phosphorylation, likely reflecting cell wall stress associated with their functional deficits. Interestingly, caffeine failed to activate *FKS2-lacZ* transcription through wild-type Mpk1 (Fig. 2B) even though it stimulates phosphorylation of Mpk1 (Fig. 2C) (35, 49), its protein kinase activity in vitro against an artificial substrate (21), and Rlm1-driven transcription (Fig. 2A) (21). This failure to activate Swi4-dependent transcription is unusual among cell wall stressors. We shall return to this point.

MPK1 null mutants are sensitive to cell wall stress (31). For example, they lyse at elevated growth temperatures (37 to 39°C; 31) and in response to caffeine treatment (34). In contrast, an *rlm1Δ* mutant is caffeine sensitive, but not temperature sensitive (54), a finding that suggests separation of Mpk1 functions may be discernible through differential growth phenotypes. To determine whether the strains with separation-of-function mutations in *MPK1* described above display different growth phenotypes, we tested their sensitivity to heat stress and caffeine treatment. Figure 3A shows that all of the SB site mutants are caffeine sensitive, but not temperature sensitive for growth. This parallels the inability of these mutants to activate Rlm1-driven transcription and is consistent with *rlm1Δ* mutant phenotypes (54). However, an ATP-binding site mutant of Mpk1 (*mpk1-K54R*), which is devoid of detectable catalytic activity (33, 58), is both caffeine sensitive and temperature sensitive (Fig. 3). This suggests that although the Mpk1 SB

site mutants cannot activate Rlm1, they retain the ability to phosphorylate one or more additional targets. Conversely, all of the DB site mutants, which cannot activate Swi4, are temperature sensitive, but the two that retain some ability to activate Rlm1-driven transcription (with K83A and Q166A mutations) are not caffeine sensitive (Fig. 3). This lends further support to the notion that caffeine sensitivity is a consequence of diminished Rlm1 activity.

Mpk1 binds to Swi4 through its DB site. We next tested by coimmunoprecipitation the effects of mutations in the DB site and SB site on cell wall stress-activated Mpk1 binding to Swi4 (27). For these experiments, we chose Mpk1-R196A as a representative of the SB site mutants, and Mpk1-K83A as a representative of the DB site mutants. Epitope-tagged forms of Mpk1 (Mpk1-FLAG) were immunoprecipitated from extracts of cells that had been exposed to cell wall stress (Fig. 4). When mild heat shock was used to activate signaling to Mpk1, epitope-tagged Swi4 (Swi4-6HIS) coprecipitated with both wild-type Mpk1-FLAG and the SB site mutant (Mpk1-R196A-FLAG), but not the DB site mutant (Mpk1-K83A-FLAG), supporting the conclusion that Mpk1 engages Swi4 through its DB site. This is an interesting finding, because it suggests that the phosphorylation state of Mpk1 alters the conformation of the DB site, which resides on the opposite face of the protein from the phosphorylation loop, in such a way that it enables binding to Swi4.

Mpk1 binds to a D motif in the C-terminal region of Swi4. The results presented above reveal that Mpk1 binding to and activation of Swi4 require an intact DB site but are completely independent of the SB site. Therefore, we used this information to identify the Mpk1 interaction site on Swi4. The canonical D motif found in MAPK substrates and regulators possesses both basic and hydrophobic residues in the arrangement

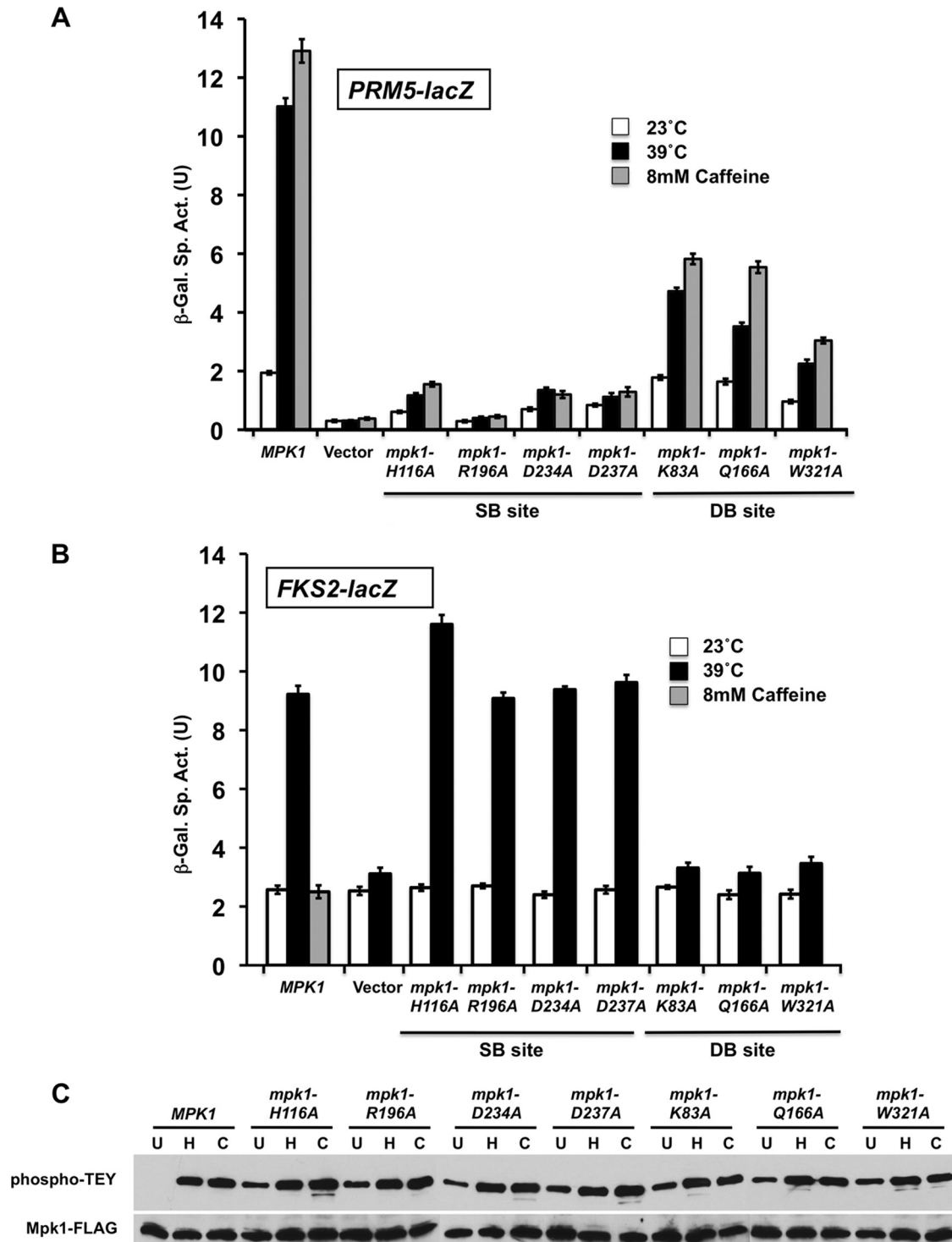


FIG. 2. Mutations in the substrate-binding site and the D-motif-binding site of Mpk1 cause differential effects on cell wall stress induction of *FKS2* and *PRM5* expression. (A) Cell wall stress-induced *PRM5* transcription. A *PRM5-lacZ* reporter plasmid (p1366) was cotransformed with centromeric plasmids expressing *MPK1-FLAG* (p2704), plasmids with the indicated point mutations in *MPK1*, or empty vector (pRS315) into an *mpk1Δ* mutant (yeast strain DL19195). Transformants were grown to saturation at 23°C in SD medium with 10% sorbitol lacking Ura and Leu. Cultures were diluted into 100 ml of medium so that subsequent incubation at 23°C, 39°C, or 23°C with 8 mM caffeine for 2 h resulted in mid-log-phase cultures (A_{600} of 1.0). β -Galactosidase activity was measured in crude extracts. The specific activity of β -galactosidase (in units) [β -Gal Sp. Act. (U)] is shown on the y axis. Each value represents the mean \pm standard deviation (error bar) from three independent transformants. (B) Cell wall stress-induced *FKS2* transcription. An *FKS2-lacZ* reporter plasmid (p2052) was cotransformed with centromeric plasmids expressing *MPK1* (p2704), plasmids with the indicated point mutations in *MPK1*, or empty vector (pRS315) into an *mpk1Δ mlp1Δ* mutant (DL19196). The *mpk1Δ mlp1Δ* mutant was used for this experiment because the Mlp1 pseudokinase contributes to *FKS2* expression. Transformants were grown as described above and diluted into 100 ml of medium so that subsequent incubation at 23°C, 39°C, or 23°C with 8 mM caffeine (*MPK1* only) for 15 h resulted in mid-log-phase cultures (A_{600} of 1.0 to 1.5). (C) Point mutations in Mpk1 are not compromised for phosphorylation by their activating protein kinases (Mkk1/2). Protein extracts from cells treated as in panel A (left untreated at 23°C [lanes U], heated to 39°C [lanes H], or treated with 8 mM caffeine [lanes C]) were subjected to immunoprecipitation of Mpk1-FLAG with anti-FLAG antibodies followed by SDS-PAGE. Dual phosphorylation of Mpk1 was detected with anti-phospho-p44/p42 MAPK antibodies; total Mpk1 was detected with anti-FLAG antibodies.

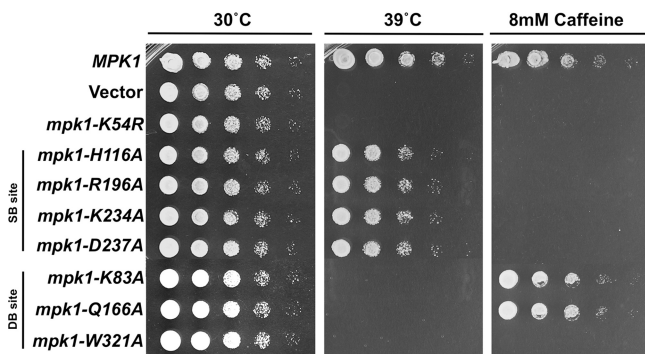


FIG. 3. Mutations in the substrate-binding site and the D-motif-binding site of Mpk1 cause differential phenotypes. An *mpk1Δ* strain (yeast strain DL456) was transformed with centromeric plasmids expressing the indicated alleles of *MPK1* or empty vector (pRS315). Transformants were grown overnight to saturation in selective medium (SD lacking Leu), and serial 10-fold dilutions were plated by pin plating from 96-well plates onto YPD alone or YPD plus 8 mM caffeine and incubated at the indicated temperature for 2 days.

(Arg/Lys)₁₋₂-X₂₋₆-Φ_A-x-Φ_B (where Φ indicates hydrophobic residues Leu, Ile, and Val) (2, 39, 42, 59). The basic residues interact with an acidic patch within the MAPK DB site called the common docking site (59), and the hydrophobic residues bind to a hydrophobic groove that is adjacent to the common docking site (11, 19).

We first narrowed the region of interest in Swi4 using a two-hybrid assay. We reported previously that Mpk1 displays a two-hybrid interaction with Swi4 under activating conditions (27). An N-terminal AD-Swi4 fusion [Gal4AD-Swi4(1-671)] failed to interact with a DBD-Mpk1 fusion [Gal4DBD-Mpk1(1-328)] (Fig. 5). However, a C-terminal Swi4 fusion [Gal4AD-Swi4(672-1093)] displayed a signal-dependent interaction with Mpk1, thereby limiting our search to the C-terminal half of

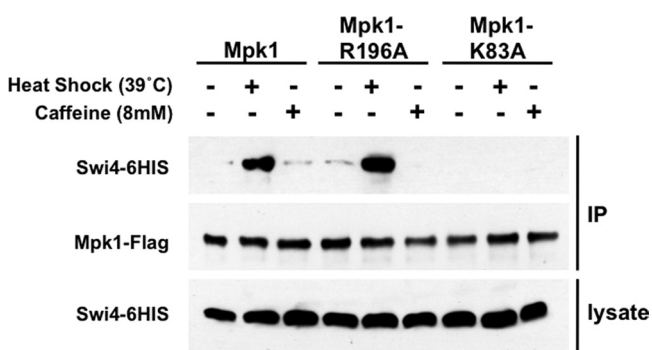


FIG. 4. Coimmunoprecipitation of Swi4 with mutant forms of Mpk1 in response to heat shock and caffeine treatment. Yeast strain DL456 (*mpk1Δ*) was cotransformed with plasmids bearing the indicated FLAG-tagged *MPK1* allele (wild-type *MPK1* [p2704], *mpk1-R196A* [p2706], and *mpk1-K83A* [p2709]) and HIS-tagged *SWI4* (p2418). Transformants were cultivated to mid-log phase in selective medium lacking methionine (to induce expression of Swi4-6HIS) and either maintained at 25°C or subjected to cell wall stress by changing the temperature to 39°C (+) or by adding 8 mM caffeine for 2 h (+). Cell extracts (lysate) and immunoprecipitates (IP) with anti-FLAG M2 affinity gel were subjected to SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibodies to detect Mpk1 or with anti-HIS antibodies to detect Swi4.

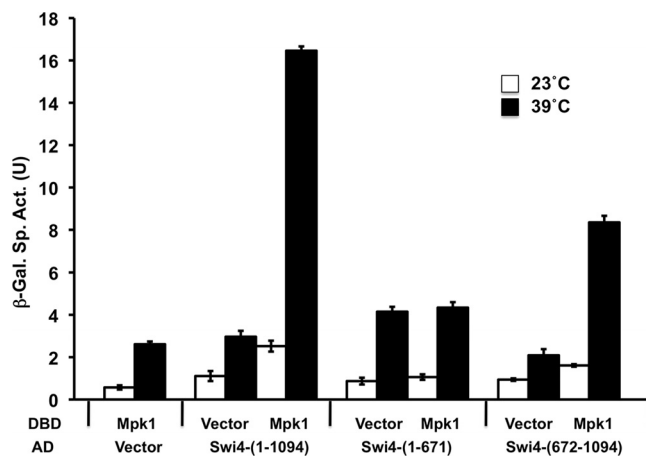


FIG. 5. Two-hybrid association of Mpk1 with the C-terminal region of Swi4. A Gal4 DNA-binding domain (DBD) fusion to the catalytic domain of Mpk1 (residues 1 to 328; p2266) was tested for interaction with Gal4 activation domain (AD) fusions to full-length Swi4 (residues 1 to 1093; p2352), the N-terminal region (residues 1 to 671; p2717), and the C-terminal region (residues 672 to 1093; p2718) in yeast two-hybrid strain SFY526. Transformants were cultivated in selective medium for 15 h at either 23°C or 39°C. Vector controls (Gal4DBD vector [p1172] and Gal4AD vector [p1173]) are included for each fusion. β -Galactosidase (β -Gal) activity was measured in crude extracts. The specific activity of β -galactosidase (in units) [β -Gal Sp. Act. (U)] is shown on the y axis. Each value represents the mean and standard deviation (error bar) from three independent transformants.

Swi4. Within that region of Swi4 are two sequences that resemble the canonical D motif, 962-KSQALKL-1068 and 909-KKRLLIT-915. We mutated the two hydrophobic residues (underlined) in each of these potential Swi4 D motifs to Ala (Swi4-LALA or Swi4 IAIA, respectively). The *swi4-LALA* mutant was able to activate *FKS2-lacZ* transcription normally in response to heat stress (Fig. 6A). However, the *swi4-IAIA* mutant was severely compromised for *FKS2-lacZ* transcription, suggesting that its ability to dock with Mpk1 was impaired. Additionally, an epitope-tagged form of the Swi4-IAIA mutant protein (Swi4-IAIA-6HIS) failed to coimmunoprecipitate with heat shock-activated Mpk1-FLAG (Fig. 6B), supporting this conclusion.

In contrast, the Swi4-IAIA mutant was not compromised for its interaction with Swi6, which is required for the cell cycle functions of SBF, because it displayed nearly normal transcription of the cell cycle-regulated *CLN2-lacZ* reporter (Fig. 6C) and it bound in vivo to Swi6 (Fig. 6D). We have shown previously by ChIP that wild-type Swi4 binds to the *FKS2* promoter in an Mpk1-dependent but Swi6-independent manner in response to cell wall stress (27). On the other hand, Swi4 binds to cell cycle promoters only when in complex with Swi6, which relieves autoinhibition of DNA binding caused by the Swi4 C terminus (4, 5, 43). Therefore, as a final demonstration that the Swi4-IAIA mutant is specifically defective for its Mpk1-associated function, we tested the ability of this mutant to occupy the *CLN2* and *FKS2* promoters. Figure 6E shows that the Swi4-IAIA mutant is defective for *FKS2* promoter binding but is competent to bind the *CLN2* promoter. These findings support a model in which Mpk1 binds to the D motif near the C terminus of Swi4, relieving autoinhibition of DNA binding in a

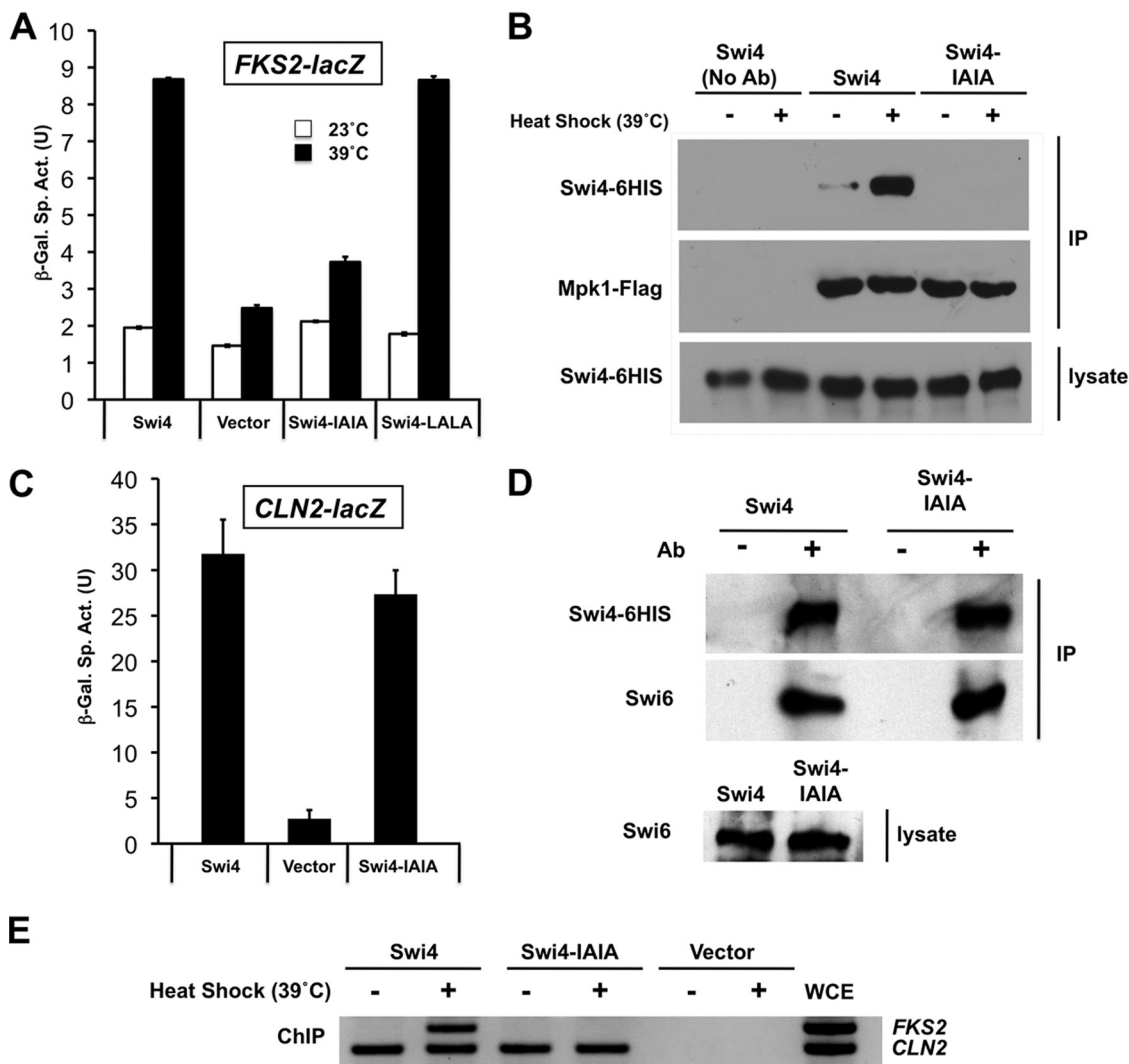


FIG. 6. Behavior of a strain with a mutation in the Mpk1-binding site of Swi4. (A) Cell wall stress-induced *FKS2* transcription is impaired in the *swi4-LALA* mutant. An *FKS2-lacZ* reporter plasmid (p2052) was cotransformed with centromeric plasmids expressing *SWI4* (p2713), plasmids with the indicated point mutations in *SWI4* (I913A/I915A [Swi4-IAIA] [p2714] or L966A/L968A [Swi4-LALA] [p2715]), or empty vector (pRS315) into a *swi4Δ* mutant (yeast strain DL3145). Transformants were grown as described in the legend to Fig. 2A and subjected to cell wall stress at 39°C or maintained at 23°C. β-Galactosidase activity was measured in crude extracts. The specific activity of β-galactosidase (in units) [β-Gal Sp. Act. (U)] is shown on the y axis. Each value represents the mean and standard deviation (error bar) from three independent transformants. (B) The Swi4-IAIA mutant protein fails to coimmunoprecipitate with activated Mpk1. A *swi4Δ* mutant (DL3145) was cotransformed with plasmids bearing FLAG-tagged *MPK1* (p2704) and a HIS-tagged *SWI4* allele (wild-type *SWI4* [p2418] or *swi4-LALA* [p2716]). Transformants were cultivated to mid-log phase in selective medium lacking methionine (to induce expression of Swi4-6HIS) and either maintained at 25°C or subjected to cell wall stress by heat shock (changing the temperature to 39°C) for 2 h (+). Cell extracts (lysate) and immunoprecipitates (IP) with anti-FLAG M2 affinity gel were subjected to SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibodies to detect Mpk1 or anti-HIS antibodies to detect Swi4. No Ab, no antibody. (C) *CLN2* transcription is not impaired in the *swi4-IAIA* mutant. A *CLN2-lacZ* reporter plasmid (p2066) was cotransformed with centromeric plasmids expressing *SWI4* (p2713), the *swi4-LALA* mutant (p2714), or empty vector (pRS315) into a *swi4Δ* mutant (DL3145). Transformants were grown as described above but were maintained at 23°C. (D) The Swi4-IAIA mutant is not impaired for Swi4 binding. A *swi4Δ* mutant (DL3145) was transformed with a plasmid bearing a HIS-tagged *SWI4* allele (wild-type *SWI4* [p2418] or *swi4-LALA* [p2716]). Transformants were cultivated to mid-log phase at 30°C in selective medium lacking methionine (to induce expression of Swi4-6HIS). Cell extracts (lysate) and immunoprecipitates (IP) with Ni-nitrilotriacetic acid beads were subjected to SDS-PAGE and analyzed by immunoblotting with anti-HIS antibodies to detect Swi4 or anti-Swi6 antibodies to detect coprecipitated endogenous Swi6. Ab, antibody. (E) The Swi4-IAIA mutant is defective for *FKS2*, but not *CLN2*, promoter occupancy. Transformants from panel D and a vector control (p2415) were cultivated in YPD medium or subjected to heat shock at 39°C for 1 h prior to ChIP analysis using primers designed to detect the endogenous *FKS2* or *CLN2* promoter. PCRs of whole-cell extract (WCE) from the unstressed wild-type Swi4 sample is also shown.

manner similar to that of Swi6, which binds to a site in Swi4 immediately C terminal to the D motif.

Mpk1 activated by caffeine treatment fails to bind Swi4 and undergoes additional phosphorylation on serine. Noting that

activation of Mpk1 by caffeine treatment failed to induce *FKS2* transcription, we asked whether this treatment impaired the ability of Mpk1 to bind Swi4. Intriguingly, when caffeine was used to activate Mpk1, this stress failed to induce Mpk1 bind-

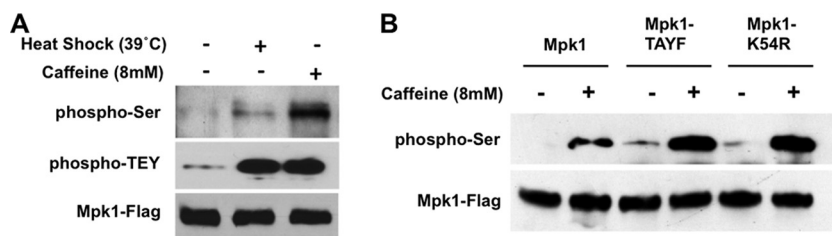


FIG. 7. Mpk1 undergoes serine phosphorylation in response to caffeine treatment. (A) Caffeine induces serine phosphorylation of Mpk1. Yeast strain DL456 (*mpk1Δ*), transformed with a plasmid expressing FLAG-tagged Mpk1 (p2313), was either maintained at 25°C or subjected to cell wall stress (heat shock) by changing the temperature to 39°C (+) or by adding 8 mM caffeine (+) for 2 h. Cell extracts were subjected to immunoprecipitation of Mpk1-FLAG, followed by immunodetection of total Mpk1 with anti-FLAG antibodies, activated Mpk1 with phospho-p42/p44 antibodies (phospho-TEY), and serine phosphorylation of Mpk1 with antiphosphoserine antibodies. (B) Caffeine-induced serine phosphorylation of Mpk1 is independent of Mpk1 activation state or catalytic activity. Yeast strain DL456 (*mpk1Δ*), transformed with a plasmid expressing FLAG-tagged Mpk1 (p2313), nonactivatable Mpk1-TAYF (p2316), or catalytically inactive Mpk1-K54R (p2317), were subjected to caffeine stress (+) and processed as described above for detection of Mpk1 phosphorylated on serine and total Mpk1.

ing to Swi4 (Fig. 4). This likely explains the failure of caffeine to induce Swi4-dependent transcription and indicates that Mpk1 can be directed selectively to one target over another depending on the activating signal. Nevertheless, caffeine-activated Mpk1 is capable of phosphorylating Swi6 in vivo (K.-Y. Kim, unpublished results).

To begin to explore the difference between Mpk1 activated by caffeine or by heat stress, we sought to detect additional phosphorylation events on Mpk1 using a phosphoserine-specific antibody. The rationale for using this antibody was that the activating dual phosphorylations of Mpk1 are on threonyl and tyrosyl residues within the activation loop (TEY), which should not interfere with detection of phosphoserine. Immunoprecipitated Mpk1-FLAG activated either by heat shock or caffeine was subjected to immunodetection with phospho-p42/p44 antibody to measure Mpk1 activation or with a phosphoserine-specific antibody to detect additional modifications (Fig. 7A). Although both the caffeine and heat shock treatments resulted in similar levels of activated Mpk1, caffeine-activated Mpk1 displayed strong serine phosphorylation, which was only weakly detected in Mpk1 from unstressed cells and heat-shocked cells. This new modification is unlikely to be caused by autophosphorylation of Mpk1, because there are no Ser-Pro MAPK target sites within Mpk1. This conclusion was confirmed by the finding that caffeine treatment induced serine phosphorylation on mutant forms of Mpk1 (Fig. 7B) that are either blocked for catalysis (*mpk1-K54R*) or for phosphorylation by the upstream MEKs (*mpk1-TAYF*). Interestingly, serine phosphorylation appeared to be slightly enhanced in the inactive forms of Mpk1, suggesting the possibility of negative-feedback regulation of this phosphorylation by Mpk1 activity. Along similar lines, Mpk1 is known to phosphorylate its other regulators, Msg5 and Mkk2 (16, 20).

Caffeine induces phosphorylation of Mpk1 on Ser423 and Ser428. A previous report of phospho-proteomic sites in yeast (3) revealed that Mpk1 is phosphorylated on Ser423 and Ser428 in response to treatment with the mutagen, methyl methanesulfonate. Because these are the only known phosphosites on Mpk1 other than those in the activation loop and because caffeine treatment has been reported to induce frameshift mutations in *E. coli* (38), we mutated these residues individually and in combination to determine whether they are the sites phosphorylated in response to caffeine treatment.

Figure 8A shows that caffeine induced serine phosphorylation of Mpk1 at both residues Ser423 and Ser428. The phosphoserine-specific antibody did not detect the Mpk1-S423A, S428A double mutant form even upon overexposure of the film (not shown), suggesting that these are the only serine phosphorylations that occur in Mpk1. A reduced level of serine phosphorylation was detected in both the Mpk1-S423A and Mpk1-S428A mutant forms. Because both single mutants were detected by the phosphoserine antibody, phosphorylation at Ser423 and Ser428 can occur independently of each other. It should also be noted that neither one of the single mutants nor the double mutant affected the ability of Mpk1 to acquire its traditional activating phosphorylations at Thr190 or Tyr192. This is important because the Mpk1-Swi4 interaction requires these phosphorylations.

To assess the impact of caffeine-induced serine phosphorylation of Mpk1 on the behavior of this MAPK, we tested the *mpk1-S423A* and *mpk1-S428A* mutants for their ability to induce *FKS2-lacZ* transcription in response to heat stress and caffeine treatment. In contrast to the behavior of wild-type Mpk1, caffeine strongly induced *FKS2-lacZ* transcription in the *mpk1-S423A* mutant (Fig. 8B), suggesting that phosphorylation of Ser423 normally inhibits a function of Mpk1 associated with *FKS2-lacZ* transcription. The *mpk1-S428A* mutant was not appreciably affected, leaving unanswered the importance of phosphorylation of this residue. Neither mutation had an appreciable effect on the ability of heat stress to induce *FKS2-lacZ* transcription.

As noted above, although caffeine treatment induces activation of Mpk1, the MAPK activated in this manner does not bind to Swi4. Because the *S423A* mutation conferred upon Mpk1 the capacity to drive *FKS2-lacZ* transcription in response to caffeine treatment, we examined the ability of the Mpk1-S423A mutant form to bind Swi4 under this condition. Figure 8C shows that the Mpk1-S423A mutant acquired the ability to associate with Swi4 in response to caffeine. Thus, caffeine treatment induces a novel Ser phosphorylation on Mpk1, which imposes a shift in the target specificity of this MAPK.

Caffeine-induced phosphorylation of Mpk1 on Ser423 is dependent on Rad53. Phosphorylation of Mpk1 on Ser423 and Ser428 is provoked by DNA damage (3). In this regard, it is interesting to note the context of these phosphorylation sites. Specifically, Ser428 resides at a Ser Gln site, a target motif for

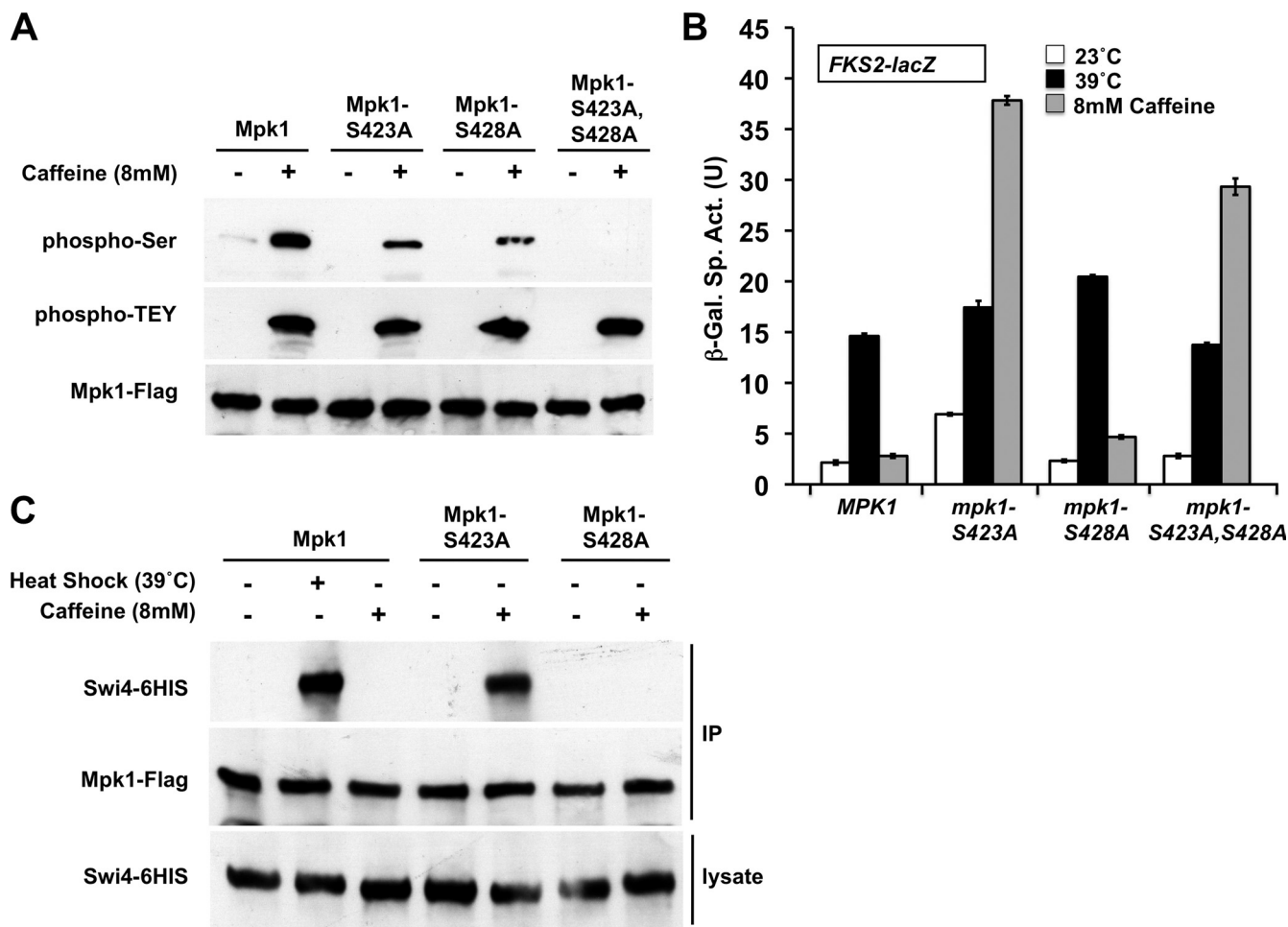


FIG. 8. Behavior of Mpk1 mutants that block serine phosphorylation induced by caffeine. (A) Ser423 and Ser428 of Mpk1 are phosphorylated in response to caffeine. Yeast strain DL456 (*mpk1Δ*), transformed with plasmids expressing FLAG-tagged Mpk1 (p2313) or Ser-to-Ala mutant forms (S423A [p2824], S428A [p2825], or S423A S428A [p2826]) were subjected to caffeine stress (+), and extracts were processed for detection of serine-phosphorylated Mpk1 (phospho-Ser), activated Mpk1 (phospho-TEY), and total Mpk1 (Mpk1-Flag) as described in the legend to Fig. 7. (B) Blocking phosphorylation of Mpk1 at Ser423 allows caffeine-induced cell wall stress to drive *FKS2* transcription. An *FKS2-lacZ* reporter plasmid (p2052) was transformed into yeast strains with the indicated *MPK1* alleles integrated into the genome (*MPK1* [DL3929], *mpk1-S423A* [DL3930], *mpk1-S428A* [DL3931], and *mpk1-S423A S428A* [DL3932]). Transformants were grown as described in the legend to Fig. 2A and subjected to thermal or caffeine stress or maintained at 23°C. β -Galactosidase activity was measured in crude extracts. β -Galactosidase specific activity (in units) [β -Gal Sp. Act. (U)] is shown on the y axis. Each value represents the mean and standard deviation (error bar) from three independent transformants. (C) Blocking phosphorylation of Mpk1 on Ser423 allows caffeine-activated Mpk1 to bind Swi4. Yeast strain DL456 (*mpk1Δ*), cotransformed with plasmids expressing FLAG-tagged Mpk1 (p2313) or Ser-to-Ala mutant forms (Mpk1-S423A [p2824] or Mpk1-S428A [p2825]) and Swi4-6HIS (p2418) were subjected to heat shock or caffeine stress (+), and extracts were processed for coimmunoprecipitation of Swi4 with Mpk1 as described in the legend to Fig. 6B.

the partially redundant Mec1/Tel1 DNA damage checkpoint kinases (46). Additionally, Ser423 resides at a Ser Phe site, which fits the S/T- ψ consensus motif (where ψ is a hydrophobic residue, such as F, I, V, or L) for the Rad53 DNA damage checkpoint kinase, whose activity is under the control of Mec1/Tel1 (46). Therefore, we asked whether the novel caffeine-induced Mpk1 phosphorylations were dependent on Mec1/Tel1 or Rad53. Because these genes are essential, the *mec1Δ/tel1Δ* and *rad53Δ* mutants were tested in a background that suppresses their lethality (*sml1Δ*) (46, 61). Serine phosphorylation of Mpk1-S428A (but not wild-type Mpk1) was abolished in the *rad53Δ* mutant (Fig. 9A), strongly suggesting that Ser423 is a direct target of Rad53. Phosphorylation of both Ser423 and Ser428 was abolished in the *mec1Δ/tel1Δ* mutant. This, to-

gether with the *rad53Δ* result, strongly suggests that Ser428 is a direct target of Mec1/Tel1 and Ser423 is an indirect target of these kinases through Rad53. We reasoned that a *rad53Δ* mutant should confer upon Mpk1 the capacity to drive *FKS2-lacZ* transcription in response to caffeine treatment in the same manner as the Mpk1-S423A mutant. Figure 9B shows that this prediction was borne out, revealing that caffeine-induced modification of Mpk1 target specificity is regulated by the Rad53 kinase.

DISCUSSION

MAPK substrates and regulators bind to the kinase through a bipartite recognition mechanism (11, 59). A kinase interaction site (D motif), which is located on the backside of the

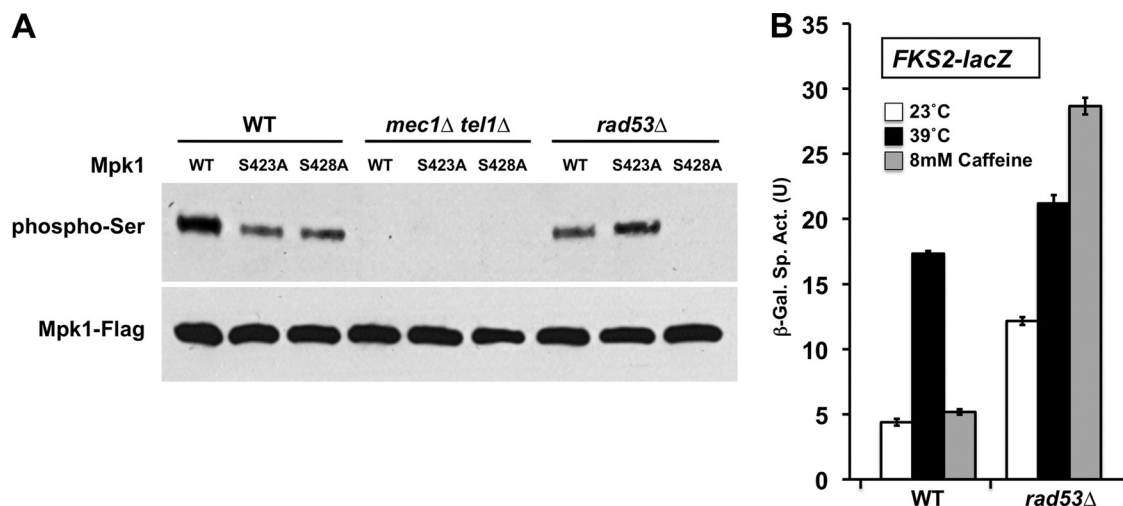


FIG. 9. Dependence of Mpk1 serine phosphorylation on Mec1/Tel1 and Rad53 and its role in *FKS2* transcription. (A) Caffeine-induced phosphorylation of Mpk1 Ser423 is blocked in a *rad53Δ* mutant, and phosphorylation of both Ser423 and Ser428 are blocked in a Mec1/Tel1 mutant. Yeast strains with the *sml1Δ* gene (wild type [WT] [strain DL3950]), *mec1Δ tel1Δ sml1Δ* gene (strains DL3955 to DL3957), and *rad53Δ sml1Δ* gene (strain DL3953) that had been transformed with plasmid expressing Mpk1-FLAG (p2704), Mpk1-S423A-FLAG (p2824), or Mpk1-S428A-FLAG (p2825) were subjected to caffeine stress. The cell extracts were processed for detection of serine-phosphorylated Mpk1 (phospho-Ser) and total Mpk1 (Mpk1-Flag) as described in the legend to Fig. 7. (B) Blocking Ser423 phosphorylation in a *rad53Δ* mutant allows caffeine-induced cell wall stress to drive *FKS2* transcription. An *FKS2-lacZ* reporter plasmid (p2052) was transformed into yeast strains with the *sml1Δ* gene (WT; DL3950) and *rad53Δ sml1Δ* gene (DL3953). Transformants were grown as described in the legend to Fig. 2A and subjected to thermal or caffeine stress or maintained at 23°C. β-Galactosidase activity was measured in crude extracts. β-Galactosidase specific activity (in units) [β-Gal Sp. Act. (U)] is shown on the y axis. Each value represents the mean and standard deviation (error bar) from three independent transformants.

protein kinase relative to the catalytic pocket (Fig. 1), engages in high-affinity associations with the DB site. The substrate-binding region, located near the kinase active site, binds to other structural elements in the interacting protein that are C terminal to the D motif and is required for phosphorylation of substrates (59). In this study, we sought to understand how the CWI pathway MAPK, Mpk1, interacts with the Swi4 transcription factor. Dually phosphorylated Mpk1 activates Swi4-driven transcription through a noncatalytic mechanism that involves the formation of an Mpk1-Swi4 complex on the promoter of the *FKS2* gene (27). The ability of Mpk1 to activate Swi4 without phosphorylating it raised an interesting mechanistic question. Because formation of the Swi4-Mpk1 complex is strictly dependent upon activation of Mpk1 by its cognate MEKs (Mkk1/2), we wondered whether Mpk1 interacts with Swi4 as it would a phosphorylation substrate. To address this question, we constructed a set of mutant forms of Mpk1 that were predicted to ablate binding of proteins at either the DB site or the SB site.

Interaction of Swi4 with the DB site of Mpk1. Our study revealed that Mpk1 binds to and activates Swi4 in a manner that requires an intact DB site but is independent of the SB site. This result was somewhat surprising, because of the dependence of this interaction on phosphorylation of Mpk1 at its activation loop, suggesting that the affinity of Swi4 for the Mpk1 DB site is altered by the phosphorylation state of Mpk1. Comparison of the structures of phosphorylated and unphosphorylated ERK2 (10), a mammalian MAPK to which Mpk1 is closely related, reveals that the greatest conformational changes are in the phosphorylation loop and little change is evident within the DB site or the regions that surround it. On

the other hand, binding of D-motif peptides derived from ERK2 regulators to the DB site of unphosphorylated ERK2 induces large conformational changes in the activation loop and elsewhere (62), revealing that there is communication between the two sites. Specifically, binding of peptides from the HePTP tyrosine phosphatase or the MEK2 kinase induce the dual phosphorylation sites of ERK2, which are buried in the unphosphorylated form, to become solvent exposed as in active ERK2. In fact, the peptide-induced conformational changes result in an ERK2 structure that is overall more similar to the phosphorylated form than to the unphosphorylated form (62). This observation suggests that regulators and substrates may bind preferentially to the DB site of activated MAPKs, although this has not been explored directly in ERK2 or other mammalian MAPKs. On the other hand, the unphosphorylated yeast MAPK Kss1 binds to the transcriptional repressors Dig1 and Dig2 through its DB site, blocking transcriptional activation of filamentous growth genes (30).

We are aware of only one prior precedent for a protein binding specifically or preferentially to the DB site of an active form of a MAPK. The Ptp2 protein phosphatase, which inactivates Hog1, binds only to the phosphorylated form of this MAPK (56). This interaction was shown recently to be predominantly through the Hog1 DB site (37).

We identified the D motif (kinase interaction site) in Swi4, which resides near its C terminus but N terminal to its Swi6 interaction site. The C terminus of Swi4 is an autoinhibitory domain that prevents its binding to DNA, but association of Swi6 to the Swi4 C terminus relieves autoinhibition and allows the heterodimer to bind SCB sites in cell cycle-regulated genes (4, 5, 43). In contrast, under conditions of cell wall stress,

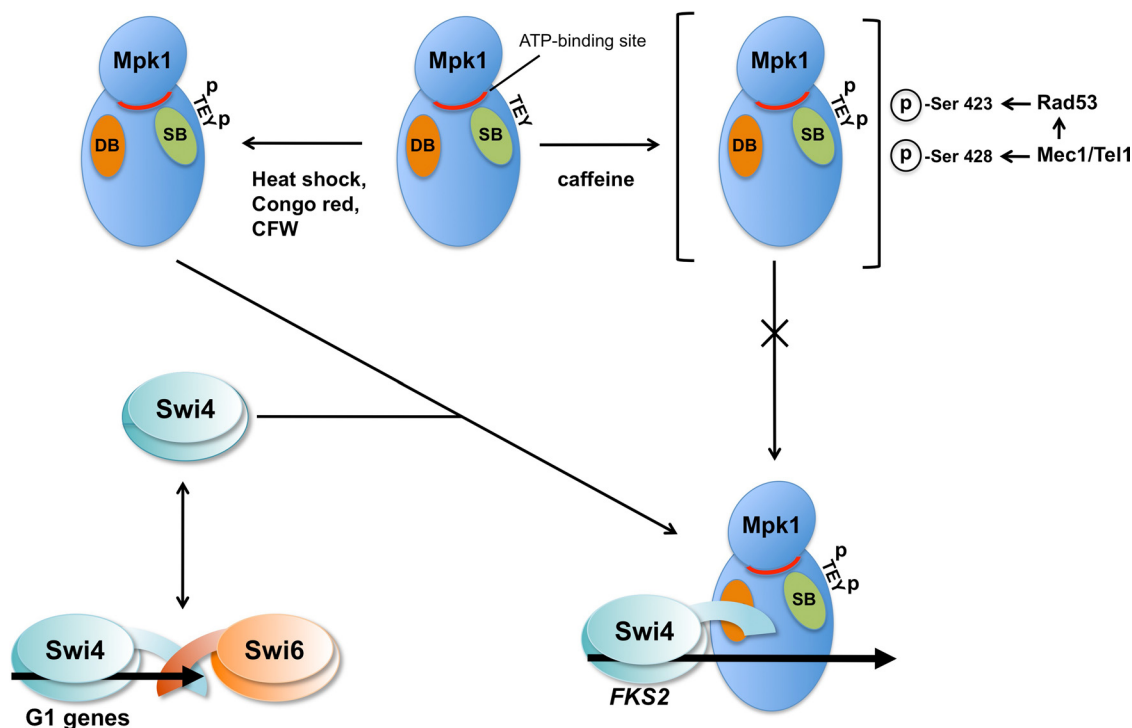


FIG. 10. Model for interaction of Mpk1 with Swi4. Mpk1 activated by cell wall stressors other than caffeine is capable of activating Swi4-dependent transcription. Swi4 binds Mpk1 through the D motif near the C terminus of Swi4, which allows Swi4 to bind the *FKS2* promoter. This interaction is proposed to relieve the autoinhibitory interaction of the Swi4 C terminus that prevents DNA binding. In this regard, Mpk1 serves the same function as Swi6 in allowing Swi4 to bind DNA. Swi6 is recruited to the Mpk1-Swi4 complex and binds to Swi4 through their respective C termini (not shown), in a manner similar to their interaction on G_1 -induced cell cycle genes (e.g., *CLN2*). Mpk1 activated by caffeine is not capable of Swi4-driven transcription. Under these conditions, activated Mpk1 is incapable of interacting with Swi4. Additional phosphorylations on Mpk1 Ser423 and Ser428 are provoked by caffeine treatment through the DNA damage checkpoint kinases Mec1/Tel1 and Rad53. Phosphorylation of Ser423, which is dependent on Rad53, specifically prevents Mpk1 binding to Swi4. DB, D-motif-binding site; SB, substrate-binding site; p, phosphate group; CFW, calcofluor white.

Mpk1 binds to Swi4 and allows this complex to bind an SCB in the *FKS2* promoter (27). Our finding that Mpk1 interacts with Swi4 at a D motif that neighbors its Swi6-binding site suggests that Mpk1 serves to relieve autoinhibition of Swi4 DNA binding in a manner that is similar to that of Swi6 (Fig. 10).

In contrast to the requirements for Swi4 activation, the Rlm1 transcription factor, which is activated through phosphorylation by Mpk1 (21, 55), was inactive in SB site mutants of *MPK1*. Although Rlm1 also binds to Mpk1 through a D motif (21), we found that ablation of the Mpk1 DB site did not completely block Rlm1 activation. Consequently, the DB site mutants and SB site mutants of Mpk1 displayed different phenotypic defects that reflected differential activation of Rlm1 and Swi4.

Caffeine-activated Mpk1 does not bind Swi4 because it undergoes a Rad53-dependent phosphorylation at Ser423. Caffeine has been used for many years to induce cell wall stress (21, 34, 35), yet the mechanism by which it acts is not understood (29). We found that caffeine is an unusual cell wall stress agent, because although it induces activation of Mpk1 and consequent transcriptional activation through Rlm1 (21), it failed to induce Swi4-driven transcription. This failure was accompanied by a failure of caffeine-activated Mpk1 to form a complex with Swi4.

We found that caffeine treatment provokes novel phosphor-

ylations on Ser423 and Ser428 of Mpk1 independently of the standard dual phosphorylation associated with MAPK activation. We found additionally that phosphorylation at Ser423 was responsible for blocking association of active Mpk1 with Swi4, specifically preventing Mpk1 from driving transcription of the *FKS2* gene. This phosphorylation was dependent on the DNA damage checkpoint kinase, Rad53, and its activating kinases, Tel1 and Mec1. In contrast, phosphorylation of Ser428 was dependent only on Tel1/Mec1. Taken together with the sequence motifs at these phosphorylation sites, our findings suggest that Ser423 is a direct target of Rad53 and Ser428 is a direct target of Mec1/Tel1 (Fig. 10). These results reveal a novel mechanism by which signal-specific targeting of a MAPK can be achieved. Ser423 resides outside of the catalytic domain in the long, C-terminal extension of Mpk1, which is shared only with its mammalian ortholog, ERK5. We do not yet know how this modification interferes with the binding of Mpk1 to Swi4. Although both Ser423 and Ser428 become phosphorylated under caffeine stress, Ser428 has no apparent impact on the Mpk1-Swi4 interaction or on consequent induction of *FKS2*. Perhaps phosphorylation of Ser428 by Mec1/Tel1 is important for regulating the interaction of Mpk1 with other targets.

The primary target of caffeine in *Saccharomyces cerevisiae* appears to be the TORC1 complex (29, 32). This conclusion derives both from the identification of TORC1 components

(Tor1, Tco89, and Kog1) in a global screen of heterozygous deletion mutants for enhanced caffeine sensitivity (32) and from a global gene expression analysis in which the transcriptional response to caffeine was found to overlap 25% with that of rapamycin, an inhibitor of TORC1 (29). However, several observations reveal the existence of additional caffeine targets. First, caffeine induces a nonuniform growth arrest (29), whereas rapamycin induces arrest specifically at G₁ phase as a result of inhibition of protein synthesis initiation (7). Second, the sensitivity of strains with mutations in the CWI pathway to caffeine is the consequence of cell lysis, as judged by microscopic observation of nonrefractile ghosts (D. E. Levin, unpublished results) and suppression of the growth defect by inclusion of sorbitol in the growth medium for osmotic support (29; Levin, unpublished). In contrast, caffeine sensitivity of a *tor1Δ* mutant is not suppressed by sorbitol (29). Finally, the ATM and ATR DNA damage checkpoint kinases are inhibited by caffeine in *Saccharomyces cerevisiae*, fission yeast, and mammalian cells (24, 36, 41, 51, 52). In this regard, it is difficult to explain why caffeine induces Tel1/Mec1-dependent phosphorylations on Mpk1, considering that Mec1 and Tel1 are the yeast homologs of ATR and ATM, respectively (46). Clearly, additional connections between the action of caffeine on these kinases and CWI signaling await discovery.

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