

Retrophosphorylation of Mkk1 and Mkk2 MAPKKs by the Slt2 MAPK in the Yeast Cell Integrity Pathway*

Received for publication, July 30, 2007 Published, JBC Papers in Press, August 20, 2007, DOI 10.1074/jbc.M706270200

María Jiménez-Sánchez¹, Víctor J. Cid², and María Molina

From the Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain

In *Saccharomyces cerevisiae*, a variety of stresses and aggressions to the cell wall stimulate the activation of the cell wall integrity MAPK pathway, which triggers the expression of a series of genes important for the maintenance of cell wall homeostasis. This MAPK module lies downstream of the Rho1 small GTPase and protein kinase C Pkc1 and consists of MAPKKK Bck1, MAPKKs Mkk1 and Mkk2, and the Slt2 MAPK. In agreement with previous reports suggesting that Mkk1 and Mkk2 were functionally redundant, we show here that both Mkk1 and Mkk2 alone or even chimerical proteins constructed by interchanging their catalytic and regulatory domains are able to efficiently maintain signal transduction through the pathway. Both Mkk1 and Mkk2 are phosphorylated *in vivo* concomitant to activation of the cell integrity pathway. Interestingly, hyperphosphorylation of the MEKs required not only the upstream components of the pathway, but also a catalytically competent Slt2 MAPK downstream. Active Slt2 purified from yeast extracts was able to phosphorylate Mkk1 and Mkk2 *in vitro*. We have mapped Ser⁵⁰ as a direct phosphorylation target for Slt2 in Mkk2. However, substitution of all (Ser/Thr)-Pro canonical MAPK target sites with alanine did not totally abrogate Slt2-dependent Mkk2 phosphorylation. Mutation or deletion of a conserved MAPK-docking site at the N-terminal extension of Mkk2 precluded its interaction with Slt2 and negatively affected retrophosphorylation. Our data show that the cell wall integrity MAPKKs are targets for their downstream MAPK, suggesting the existence of complex feedback regulatory mechanisms at this level.

Mitogen-activated protein kinase (MAPK)³ cascades are ubiquitous signaling pathways that respond to multiple extracellular stimuli in all eukaryotic cells. Signal is transmitted

through this pathway by the sequential phosphorylation of a module composed of three protein kinases. MAPKs are activated by phosphorylation in both threonine and tyrosine residues within a conserved TXY motif. This unusual mechanism is catalyzed by the dual-specificity kinases, MAPKKs, which in turn are activated by a MAPKKK (1–3). Upon activation, MAPKs phosphorylate conserved motifs consisting on a serine or threonine followed by a proline in substrates that will be mainly transcriptional factors but also cytoplasmic proteins such as protein kinases or phosphatases, cytoskeletal components, regulators of mRNA translation, etc. (4).

The existence of two sequentially activated kinases upstream the MAPK allows amplification of the signal by increasing the amount of protein kinase in every step of the MAPK module. For example, in the pheromone response pathway in *Saccharomyces cerevisiae*, this amplification occurs in every step (5, 6), whereas in the ERK1/2 pathway in mammalian cells, the amplification exists only between the MAPKKK (Raf) and the MAPKK (MEK), but not in the MEK-ERK step (7). Furthermore, the cascade arrangement should allow signal transduction specificity to diverse inputs by avoiding cross-talk among pathways (8). This can be achieved by interaction of the integrants of a particular MAPK module with scaffolding proteins. This is the case of the *S. cerevisiae* mating pathway scaffold Ste5, which determines specificity of the MAPKKK Ste11 and MAPKK Ste7 toward the Fus3 MAPKs instead of their alternative downstream MAPK Kss1 (9–11). In some cases, it is the MAPKK of the pathway that plays the scaffolding function, as reported for Pbs2 in the *S. cerevisiae* high osmolarity glycerol response pathway (12). Direct interaction of both yeast Ste7 and mammalian MAPKKs with their corresponding MAPK targets involves a “docking domain” or “D-motif” that is usually located at the N-terminal non-catalytic extension of the protein (13–15). This motif cooperates with the scaffold to define the specificity of signaling (16). Finally, the modular configuration might as well serve as a mechanism to retain inactive MAPK in the cytoplasm, as pointed out by evidence that activation of the mammalian ERK pathway results in dissociation of ERK1 from the complex formed with MEK1 and its translocation to the nucleus to execute the response (17).

Although not seen in all MAPK pathways, an additional advantage of assembling several kinases in a signaling module would be the possible operation of feedback regulatory mechanisms among their components. This has been reported to happen in the mammalian Raf/MEK/ERK pathway. Once activated, ERK retrophosphorylates MEK1 in two residues, Thr²⁹² and Thr³⁸⁶. The biological function of this retrophosphoryla-

* This work was supported in part by Grant BIO2004-02019 from the Comisión Interministerial de Ciencia y Tecnología (CYCYT). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Recipient of a predoctoral Formación de Profesorado Universitario fellowship from the Spanish Ministerio de Educación y Ciencia.

² To whom correspondence should be addressed: Dept. de Microbiología II, Facultad de Farmacia UCM, Pza de Ramón y Cajal s/n., 28040 Madrid, Spain. Tel.: 34-91-394-1888; Fax: 34-91-394-1745; E-mail: vicjid@farm.ucm.es.

³ The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PAK, p21-activated kinase; CWI, cell wall integrity; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase.

tion is controversial, as some researchers did not find any variation on the catalytic activity of MEK1 in respect to its unphosphorylated form (18, 19), whereas others suggested a negative role for this retrophosphorylation mechanism (20). In general, little is known about regulation of MAPK pathways at the MAPKK level. However, mounting evidence suggests that MAPKKs are subject to fine regulation. For instance, some phosphorylation events on MAPKKs by other kinases different than MAPKKKs have been reported: MEK1 is also phosphorylated on Ser²⁹⁸ by p21-activated kinase (PAK) (21), leading to an enhanced MEK1-ERK complex association (22). Phosphorylation of MEK1 by ERK on Thr²⁹² upon cell adhesion stimulation inhibits the phosphorylation by PAK on Ser²⁹⁸. Therefore, competitive phosphorylation of MEK1 by ERK and PAK regulate adhesion-dependent complex formation and activation (23).

Three of the *S. cerevisiae* MAPK pathways are orthologous to mammalian ERK modules: the mating pheromone pathway, the invasive growth pathway, and the cell wall integrity (CWI) pathway (see Ref. 24 for a recent review). The MAPKK Ste7 is shared by the mating and the invasive growth pathways, respectively, phosphorylating either the Fus3 or Kss1 MAPKs (25–27). In addition to the phosphorylation of Ste7 by the MAPKKK Ste11, pheromone induction leads to hyperphosphorylation of Ste7 mediated by Fus3 and Kss1 (25, 28). Seven residues in Ste7 have been identified as targets of Fus3 and Kss1 phosphorylation (29). The higher activity of Ste7 in cells lacking Fus3 and Kss1 initially suggested a negative feedback regulatory mechanism (25, 28), but further analyses did not reveal an inhibition of its kinase activity, rather favoring the hypothesis that retrophosphorylation of Ste7 might be a mechanism for determining signaling specificity toward the mating or invasive growth pathways (29). MAPK docking domains (D-domains) were first described in Ste7 MAPKK (30) and they were subsequently found to be present in multiple MAPK substrates, suggesting that they constitute a general mechanism for substrate recognition by MAPKs. Although the D-domain of Ste7 is known to partially contribute to signal transduction (14), its involvement on retrophosphorylation of Ste7 by Fus3 and Kss1 has not been studied.

The CWI pathway in the yeast *S. cerevisiae* is essential to respond to cell wall damage and a variety of stresses, besides of being activated in a cell cycle-dependent manner during vegetative growth. Signaling through this pathway relies on stimulus-dependent activation of the yeast homolog of the protein kinase C (Pkc1) via its interaction with GTP-loaded Rho1 small GTPase. Pkc1 activates a MAPK module that consists of the MAPKKK Bck1, two MAPKKs, Mkk1 and Mkk2, and the MAPK Slt2, also known as Mpk1. In consequence, a transcriptional response involving the activation of the transcriptional factor Rlm1, the most prominent phosphorylation target of Slt2, leads to the expression of a number of genes involved in the maintenance of cell wall integrity (reviewed in Ref. 31). The polarisome protein Spa2 has been proposed to serve as a scaffold-like protein that recruits the MAPKKs and the MAPK of this pathway to sites of polarized growth (32). Although the yeast Slt2 MAPK pathway has

been subjected to intense analyses, very little is known about its regulation at the level of its two redundant MAPKKs.

To learn more about the role of Mkk1 and Mkk2, we have studied the contribution of each MAPKK in signaling through the CWI pathway, confirming a high degree of redundancy. We report here that Slt2 retrophosphorylates both MAPKKs and that a docking domain in their N-terminal extension is important for recognition of Slt2. Our data hint that feedback regulatory mechanisms might be exerted by the MAPK Slt2 on its upstream kinases.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—The *S. cerevisiae* strains used are listed in Table 1. *Escherichia coli* DH5 α F'(K12 Δ (lacZYA-argF)U169 deoR supE44 thi-1 recA1 endA1 hsdR17 gyrA96 relA1 (ϕ 80lacZ Δ M15)F') was used for routine molecular biology techniques. YPD (1% yeast extract, 2% peptone, and 2% glucose) broth or agar was the general non-selective medium used for yeast cell growth. Synthetic minimal medium (SD) contained 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% glucose and lacking appropriate amino acids and nucleic acid bases to maintain selection for plasmids. In SR, glucose was replaced with 2% raffinose. Galactose induction in liquid medium was performed by growing cells in SR to mid-exponential phase and then adding galactose to 2% for 6–8 h. Growth assays on plates were performed, typically, by growing transformants overnight in SD lacking uracil or YPD adjusting the culture to an $A_{600\text{ nm}} = 0.5$, and then spotting samples of the cell suspension and three serial 5-fold dilutions onto the surface of YPD plates followed by incubation at 24 or 37 °C for 2–3 days. When indicated, plates were supplemented with sodium orthovanadate, caffeine, or Congo red, previously dissolved in sterile water and added to the medium to the desired concentration.

Plasmid and Strain Construction—Transformation of *E. coli* and yeast, and other basic molecular biology methods, were carried out using standard procedures (33).

Deletion of *MKK1* and *MKK2* genes was performed by substitution of the *MKK1* and *MKK2* open reading frame with the *kanMX4* or *SpHIS5* genes amplified from the pFA6a-kanMX4 and pFA6a-SpHIS5, respectively (34), with primers S1MKK1 and S2MKK1 for *MKK1* and S1MKK2 and S2MKK2 for *MKK2*, that bore short flanking homology sequences in 5' homologous to the target chromosomal loci to direct recombination. Oligonucleotides used in this work are listed in Table 2.

Plasmids pRS306-Mkk1-6myc and pRS306-Mkk2-6myc were generated by amplification by PCR from the Mkk1 and Mkk2 coding sequence using primers MKK1Up and MKK1Lo for *MKK1* and MKK2Up and MKK2Lo for *MKK2*, and cloned into the BamHI site in a yeast vector, pRS306-myc6, which had been previously generated by inserting a *myc*₆ epitope into the polylinker in the integrative *URA3* vector, pRS306 (35). Integration of *myc*-tagged versions in the *MKK1* or *MKK2* loci was achieved by transforming the pRS306-based constructs linearized with HpaI. The plasmid carrying the pRS306-MKK1(1–222)-MKK2(216–506)-6myc chimera was constructed by overlapping PCR using primers MKK1Up and NB for amplifying the region corresponding to the N-terminal extension of

Slr2 Retrophosphorylates Mkk1 and Mkk2 MAPKKs

TABLE 1

Yeast strains used in this work

Strains	Source
YPH499	<i>MATa ade2-101 trp1-63 leu2-1 ura3-52 his3-Δ200 lys2-801</i> P. Hieter
YMJ1	YPH499 isogenic, <i>mkk1Δ::kanMX4</i> This work
YMJ2	YPH499 isogenic, <i>mkk2Δ::SpHIS5</i> This work
YMJ3	YPH499 isogenic, <i>mkk1Δ::kanMX4 mkk2Δ::SpHIS5</i> This work
YMJ14	YPH499 isogenic, <i>mkk2Δ::SpHIS5</i> This work
YMJ13	<i>MKK1(1-666)-MKK2(643-1518)::6myc::URA3</i> YPH499 isogenic, <i>mkk1Δ::kanMX4</i> This work
YMJ9	<i>MKK2(1-645)MKK1(669-1524)::6myc::URA3</i> YPH499 isogenic, <i>mkk2Δ::SpHIS5 MKK1::6myc::URA3</i> This work
YMJ10	YPH499 isogenic, <i>mkk1Δ::kanMX4 MKK2::6myc::URA3</i> This work
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> EUROSCARF
Y02487	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mkk1Δ::kanMX4</i> EUROSCARF
Y12112	BY4742 isogenic, <i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mkk2Δ::kanMX4</i> EUROSCARF
YMJ29	BY4742 isogenic, <i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mkk2Δ::kanMX4 mkk1Δ::SpHIS5</i> This work
YMJ21	BY4741 isogenic, <i>MKK1::6myc::LEU2</i> This work
YMJ22	BY4741 isogenic, <i>MKK2::6myc::LEU2</i> This work
<i>bck1Δ</i>	BY4741 isogenic, <i>bck1Δ::kanMX4</i> EUROSCARF
<i>slt2Δ</i>	BY4741 isogenic, <i>slt2Δ::kanMX4</i> EUROSCARF
<i>rlm1Δ</i>	BY4741 isogenic, <i>rlm1Δ::kanMX4</i> EUROSCARF
TD28	<i>MATa ura3-52 inos1-151canR</i> Ref. 38
TD28 Mkk1myc	TD28 isogenic, <i>MKK1::6myc::URA3</i> This work
TD28 Mkk2myc	TD28 isogenic <i>MKK1::6myc::URA3</i> This work
TD28 Slr2F54 c	TD28 isogenic <i>slt2-F54</i> Ref. 38
TD28 Slr2F54 Mkk1myc	TD28 isogenic <i>slt2-F54 MKK1::6myc::URA3</i> This work
TD28 Slr2F54 Mkk2myc	TD28 isogenic <i>slt2-F54 MKK2::6myc::URA3</i> This work
1783	<i>MATa ura3-52 his4 trp1-1 leu2-3112 canR</i> Ref. 55
DL454	1783 isogenic, <i>slt2Δ::TRP1</i> Ref. 55
Y190	<i>MATa, ura3-52, his3-Δ200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, URA3::GAL1_UAS-GAL1_TATA-lacZ, LYS2::GAL_UAS-HIS3_TATA-HIS3</i> Clontech

MKK1 and primers CB and MKK2Lo for the region coding the C-terminal kinase domain of Mkk2, and cloned into the BamHI site in the pRS306-6myc. For the pRS306-MKK2(1-215)-MKK1(223-508)-6myc construction, primers MKK2Up and NA were used for amplifying the region that corresponds to the N-terminal extension of Mkk2, and CA and MKK1Lo for the sequence coding the kinase domains of Mkk1. Cloning was made by using the BamHI site in the same vector. Integration was performed by transforming these constructs opened with *CellIII* for the chimera bearing the N-terminal domain of Mkk1, and with *BstEII* for the one bearing the N-terminal domain of Mkk2.

The *LEU2*-based plasmids pRS305-MKK1-6myc and pRS305-MKK2-6myc were made by excising *MKK1* and *MKK2* from the pRS306Mkk1-6myc and pRS306Mkk2-6myc described above, using the BamHI site, and cloning them into the same site in the yeast integrative vector pRS305-6myc (35). These constructions were integrated in the *MKK1* and *MKK2* loci after linearization with *CellIII* and *Eco81I*, respectively.

Plasmids pRS416-MKK1-6myc and pRS416-MKK2-6myc were generated by amplification of the *MKK1* and *MKK2* promoters and coding sequences fused to the *myc*₆ epitope from the yeast genomic DNA of strains that had integrated *MKK1*-6myc and *MKK2*-6myc from the pRS305-MKK1-6myc or pRS305-MKK2-6myc vectors, respectively. The primers used were *MKK1*ProHindUp (upper) for *MKK1* and *MKK2*ProHindUp (upper) for *MKK2*; and *MycHindLo* (lower) for both. These amplicons were inserted in the HindIII site of the yeast centromeric vector pRS416.

pEG(KG) plasmids bearing *SLT2* and *SLT2-F54* have been described previously (35). pGEX(KG) plasmids bearing *MKK1* and *MKK2* were obtained by cloning from the pEG(KG)-*MKK1* and pEG(KG)-*MKK2* plasmids described previously (36).

MKK1 was excised and inserted in the pGEX plasmid using the XbaI-SalI sites, and *MKK2* using the SmaI-HindIII sites.

To obtain the different *mkk1* and *mkk2* mutant versions, site-directed mutagenesis was carried out using a DpnI-based strategy (37) with Turbo *PfuI* DNA polymerase (Stratagene). pRS416-MKK1-K250R-6myc and pGEX-MKK1-K250R were mutagenized using primers MKK1KRUp and MKK1KRLo, and pRS416MKK2-K243R-6myc and pGEX-MKK2-K243R with MKK2KRUp and MKK2KRLo. The primers used for mutagenesis of the (Ser/Thr)-Pro sites of *MKK2* were: T17AK2Up, T17AK2Lo, T153AK2Up, T153AK2Lo, S42A-S50AK2Up, S42A-S50AK2Lo, S42AK2Up, S42AK2Lo, S50AK2Up, S50AK2Lo, S437AK2Up, S437AK2Lo, S471K2Up, and S471AK2Lo. To obtain the pRS416MKK2(Δ23), the pRS416MKK2-6myc plasmid was modified introducing a BamHI site before the ATG start codon of *MKK2* by site-directed mutagenesis using primers BamHI-1MKK2Up and BamHI-1MKK2Lo. To eliminate a second BamHI site in the polylinker of pRS416 the construct excised XbaI/HindIII and re-ligated in the opposite orientation. The *MKK2*(Δ23) fragment was amplified using primers 1-23NMKK2Up and MKK2Lo, and inserted in the BamHI site of the plasmid described before to replace the original *MKK2*. pRS416MKK2-L20AxL22A-6myc plasmid was constructed by site-directed mutagenesis using primers LxLK2Up and LxLK2Lo.

Two-hybrid pGAD424 plasmid bearing *SLT2* and pGBT9 bearing *MKK2* have been described previously (36). To construct the pGBT9MKK2(Δ23) plasmid, the fragment *mkk2*(Δ23) from the pRS416-MKK2(Δ23) was cloned in the BamHI site of the pGBT9 plasmid, and the phase was corrected by site-directed mutagenesis using primers pGBT9-23K2XXUp and pGBT9-23K2XXLo. pGBT9-MKK2-L20AxL22A was made by mutagenesis of *MKK2* with primers LxLK2Up and LxLK2Lo.

TABLE 2

Oligonucleotides used in this work

Oligonucleotides	
S1MKK1	5'-CCAAATTAACTCTATCCTTCCATTGCACAATTTGCCAGTCGTACGCTGCAGGTCGAC-3'
S2MKK1	5'-TTTTAGTTAGGCTATCCTGGGTATGAATCTTGATGGAGGATCGATGAATTCGAGCTCG-3'
S1MKK2	5'-GTTATCATATCTACAAAATACCAATTATATACACAGGATACGTACGCTGCAGGTCGAC-3'
S2MKK2	5'-ACTATATACGTATATGTTAAGAAATTTTCGGAGTGTCTAGATCGATGAATTCGAGCTCG-3'
MKK1UP	5'-GGGGATCCATGGCTTCACTGTTTCAGACC-3'
MKK1Lo	5'-GGGGATCCATGCATATCTTTCCAGCACTTCCTCA-3'
MKK2UP	5'-GGGGATCCATGGCTTCAATGTTTCAGACCA-3'
MKK2Lo	5'-GGGGATCCATGCATTATCCCATCCTTTTCTTTTC-3'
NB	5'-CTTCTCCTAGAAATTCCTAGTGTCTCGATCCTATCTTTTAAATTTGC-3'
CB	5'-GCAAATTTAAAAGATAGGATCGAGACACTAGGAATTCAGGAGAAG-3'
NA	5'-CTTCTCCTAGAAATGCCTAATGTAGTAATCTCATCCTTGAGATG-3'
CA	5'-CATCTCAAGGATGAGATTACTACATTAGGCATCTCAGGAGAAG-3'
MKK1ProHindUp	5'-CCAAGCTTATTATTAGTTTTTCTCACC-3'
MKK2ProHindUp	5'-CCAAGCTTAGCTTGAGAATTGCTCTAC-3'
MycHindLo	5'-CCAAGCTTAGGTAAATACGACTCACTATAG-3'
MKK1KRUp	5'-GGATCAAAAATATTCGCTTTAAGAGTGATAAACACATTAATACAGATCC-3'
MKK1KRLo	5'-GGTCTGTATTAAATGTGTTTATCACTCTTAAAGCGAATATTTTGTATCC-3'
MKK2KRUp	5'-GGAAAAAGGTTTTCGCTTGAGGACAATCAACACTATGAATACGTG-3'
MKK2KRLo	5'-CAGTATTCATAGTGTGATTGTCTCAACGCAAAACCTTTTTC-3'
T17AK2Up	5'-CCAATAGGAGTCAACAAAAGGCTCCAAAATTAACGCTTCCAG-3'
T17AK2Lo	5'-CTGGAAGCGTTAATTTTGGAGCCTTTTGGTGACTCCTAT TGG-3'
T153AK2Up	5'-CAAGAAAGATCTCGACGCACAGAGGCGAGGATTC-3'
T153AK2Lo	5'-GAATCCTCGCCTTCTGGTGCGTCGAGATCTTTCTTG-3'
S42A-S50AK2Up	5'-GGGCAACATCTCAACCGGGCACCGTACTCGTCAGTGAACGAAGCCCCATACTCCAACAATAGC-3'
S42A-S50AK2Lo	5'-GCTATTGTGGAGTATGGGGCTTCGTTCACTGACGAGTACGGTGCCCGTTGAGATGTTGCC-3'
S42AK2Up	5'-GGGCAACATCTCAACCGGGCACCGTACTCGTCAGTGAACG-3'
S42AK2Lo	5'-CGTTCACGTACGAGTACGGTGCCCGTTGAGATGTTGCC-3'
S50AK2Up	5'-GTACTCGTCAGTGAACGAAGCCCGTACTCCAACAATAGC-3'
S50AK2Lo	5'-GCTATTGTGGAGTACGGGGCTTCGTTCACTGACGAGTAC-3'
S437AK2Up	5'-GACGATGATCCTGACGTTTGTCTCCCAAGTTGAAAGATGAGCC-3'
S437AK2Lo	5'-GGCTCATCTTTCAACTGGGGAGCAAACGTCAGGATCATCGTC-3'
S471AK2Up	5'-GATGCCAGAGAGAGGCGCTGCTCCAGGCAAAATGTTAAAGC-3'
S471AK2Lo	5'-GCTTTAAACATTTGCTTGGGAGCAGGCTTCTCTGCGCATC-3'
BamHIMKK2Up	5'-GGTCTGAACATTGAAGCCATGGATCCTGTGTATATAATTGG-3'
BamHIMKK2Lo	5'-CCAATTATATACACAGGATCCATGGCTTCAATGTTTCAGACC-3'
1-23NK2Up	5'-GGGGATCCATGGTAAATTTAGTTTCAAAATGTG.3
pGBT9-23K2XXUp	5'-CCGGAATTCCTGGGGATCCCAATGGTAAATTTAGTTTCAAAATGC-3'
pGBT9-23K2XXLo	5'-GCATTTTGAACATAATTTACCATTTGGGATCCCCGGGAATTCGGG-3'
LxLK2Up	5'-CACCAAAAGACTCCAAAAGCAACGGCTCCAGTAAATTTAGTTC-3'
LxLK2Lo	5'-GACTAAATTTACTGGAGCCGTGCTTTTGGAGTCTTTTGGTG-3'

Protein Detection by Immunoblot—Standard procedures were used for yeast growth, cell harvesting, and cell breakage, as well as for preparation of protein-containing cell-free extracts, fractionation by SDS-polyacrylamide gel electrophoresis, and transfer to nitrocellulose membranes. Monoclonal anti-Myc (9E10 BAbCO) was used to detect proteins fused to the Myc epitope and anti-V-ATPase 60-kDa subunit (13D11, Molecular Probes) was used as a loading control. Yeast MAPK Slt2 was detected with polyclonal anti-Slt2 antibodies described before (38). Rabbit anti-phospho-p44/p42 MAPK (anti-Thr(P)²⁰²-Tyr(P)²⁰⁴) antibodies (New England Biolabs) were used to detect dually phosphorylated forms of Slt2. Bound primary antibodies were revealed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies, as appropriate, and a chemiluminescence detection system (ECLTM; Amersham Biosciences).

Immunoprecipitation and Alkaline Phosphatase Treatment—Cells expressing Mkk1-6myc or Mkk2-6myc were treated with 5 mM sodium orthovanadate for 2 h and lysed as described above. Immunoprecipitation was performed by incubating 500 μ g of protein extracts with 1 μ l of anti-Myc antibody for 1 h and subsequently 2 h with G-protein-Sepharose beads. After washing 3 times with lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 50 mM β -glycerol phosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate) and 3 times with alkaline phosphatase buffer (50

mM Tris-HCl, 1 mM MgCl₂), beads were preincubated for 10 min at 30 °C. Samples were treated or not with 10 mM sodium orthovanadate and 20 units of calf intestine alkaline phosphatase. The reaction was incubated for 30 min at 30 °C and stopped by the addition of SDS-sample loading buffer.

In Vitro Kinase Assays—GST, GST-Mkk1, GST-Mkk2, GST-Mkk1-K243R, GST-Mkk2-K250R, and GST-Mkk2-S42A-S50A-K250R were expressed in *E. coli* transformed with pGEX-derivative plasmids and affinity purified on glutathione-Sepharose beads as previously described (35). *In vitro* kinase activity of GST, GST-Slt2, and GST-Slt2K54F was assayed by incorporation of radiolabeled phosphate from [γ -³²P]ATP (Amersham Biosciences) as previously described (35).

Two-hybrid Assays—pGAD424- and pGBT9-based vectors expressing the appropriate *GAL4* fusions to *SLT2* or *MKK2*, respectively, were transformed in the Y190 yeast two-hybrid strain and assayed for interaction as described (36). For quantitative β -galactosidase assays, transformants were grown in selective medium to exponential phase and assayed by the *o*-nitrophenyl- β -D-galactopyranoside method (39). Values are the average of data obtained from different clones from the same transformation.

RESULTS

Mkk1 and Mkk2 Are Fully Redundant and Their Kinase Domains Are Interchangeable—Mkk1 and Mkk2 were first described as the two redundant MAPKKs of the cell integrity

Slt2 Retrophosphorylates Mkk1 and Mkk2 MAPKKs

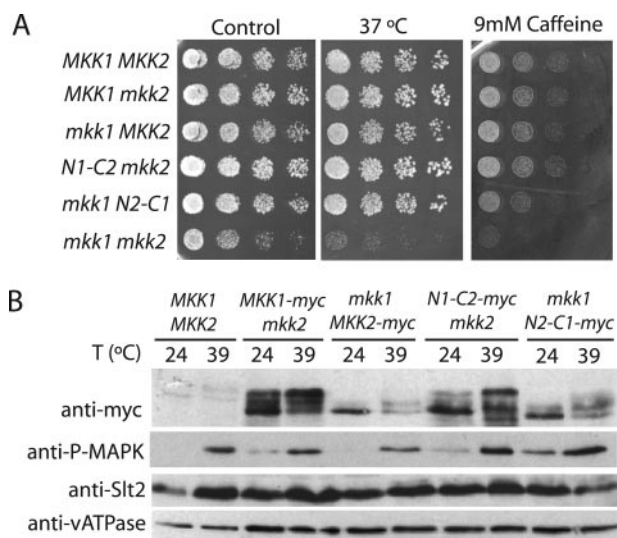


FIGURE 1. Phenotypic analyses of isogenic *mkk1* and *mkk2* strains and functional study of Mkk1-Mkk2 chimeras. A, growth of wild type yeast YPH499 strain or isogenic YMJ1, YMJ2, YMJ3, YMJ14, and YMJ13 strains bearing *mkk1*Δ and/or *mkk2*Δ or integrated MKK1(1–222)-MKK2(216–506) (N1-C2) or MKK2(1–215)-MKK1(223–508) (N2-C1), as indicated in standard medium or in conditions restrictive for CWI mutants. Cells were grown in YPD medium to an $A_{600\text{ nm}}$ of 0.5 and 5-fold dilution series were spotted onto YPD agar medium at 24 °C in the absence or presence of 9 mM caffeine or at 37 °C, as indicated. B, electrophoretic analyses of Mkk1-myc, Mkk2-myc, and the Myc-tagged chimerical proteins, and levels of phosphorylation of Slt2 in the strains used in A. Cells were grown to mid-log phase and maintained at 24 °C or heat-shocked at 39 °C for 2 h, as indicated. Protein extracts from these cultures were separated by SDS-PAGE and analyzed by Western blotting with anti-Myc, anti-phospho-p42/p44, anti-Slt2, or anti-vATPase as loading control.

pathway in *S. cerevisiae* (40). However, later studies attributed a more important contribution of Mkk1 to the transmission of the signal through the pathway (41). These previous reports were performed on congenic strains, so an influence of the genetic background on previous phenotypic studies could not be discarded. To address this question we first constructed isogenic *mkk1*Δ and *mkk2*Δ and double *mkk1*Δ *mkk2*Δ mutants in two different genetic backgrounds (strains BY4741 and YPH499). This set of mutants was phenotypically analyzed by checking their sensitivity to high incubation temperatures, cell wall-interfering agents as Congo red and calcofluor white, as well as to caffeine, zymolyase, and sodium orthovanadate, all known stimuli of the cell integrity pathway (31). Although the degree of sensitivity varied depending on the strain, single *mkk1* and *mkk2* and their isogenic wild type control were equally resistant to these stimuli, whereas sensitivity of the double *mkk1*Δ *mkk2*Δ was consistent with a total loss of function of the cell integrity pathway (data corresponding to the YPH499 background are shown in Fig. 1A).

Mkk1 and Mkk2 have highly similar C-terminal kinase domains (80.42% identity), but a more divergent N-terminal region (26.04% identity). This N-terminal extension has been proposed to play a regulatory role, as it is known to mediate interaction with other proteins, such as Slt2 (36) or the polarisome component Spa2 (42). In case there should be functional differences between both MAPKKs, it is logical to think that the N-terminal region, rather than the conserved kinase domains, would be responsible for such differences. To test interchange-

ability of Mkk1 and Mkk2 regulatory and catalytic domains, we constructed chimerical proteins bearing the N-terminal extension of one MAPKK and the C-terminal kinase domains of the other. These constructions consisted of Mkk1(1–222)-Mkk2(216–506) and Mkk2(1–215)-Mkk1(223–508). Myc-tagged fusions of both chimerical proteins were integrated in their corresponding chromosomal *loci* in a strain deleted for the other MAPKK. Thus, these recombinant clones had as the only MAPKK of the CWI pathway the corresponding chimerical protein. Sensitivity to different stimuli of the pathway was assayed on these strains (Fig. 1A, fourth and fifth rows). Both chimeras were able to maintain the function of the pathway, demonstrating that Mkk1 and Mkk2 are redundant and have interchangeable domains.

To confirm that Mkk1, Mkk2, or any of the chimeras were able to efficiently transduce the signal we analyzed the activation of the CWI pathway by immunoblotting yeast lysates with antibodies that recognize the dually phosphorylated form of Slt2 (41). For this purpose, we used strains that had integrated in their corresponding chromosomal *loci* a Myc-tagged version of Mkk1, Mkk2, or either chimera as their only functional CWI MAPKK. As shown in Fig. 1B, phosphorylation of Slt2 under stimulation conditions occurred in all recombinant strains.

Mkk1 and Mkk2 Are Persistently Phosphorylated upon Activation of the Cell Integrity Pathway—Yeast strains expressing Myc-tagged Mkk1 and Mkk2 from their chromosomal *loci* were treated with a variety of stimuli of the pathway. A change in the mobility of Mkk1 and Mkk2 was patent when the pathway was activated by temperature (Fig. 1B) or by other means (Fig. 2A). The amount of protein that is shifted as well as the number and intensity of shifted bands correlated with the level of phosphorylation of the Slt2 MAPK (Fig. 2A). Therefore, this mobility shift is a consequence of the activation of the pathway, independent on the nature of the stimulus, but likely dependent on its strength. Interestingly, the mobility pattern upon stimulation of Mkk1 and Mkk2 was different. Mkk1 ran in the gel as two distinct bands in basal conditions and shifted to a predominant form of lower mobility when the pathway was active, whereas Mkk2 displayed a unique band in basal conditions that shifted to a band of lower mobility or, in samples subjected to strong stimuli, such as orthovanadate or caffeine, to a minor band of even lowest mobility, always fainter than the equivalent form of Mkk1. A similar analysis of the Mkk1(1–222)-Mkk2(216–506) and Mkk2(1–215)-Mkk1(223–508) chimerical proteins as compared with integrated Myc-tagged wild type Mkk1 and Mkk2 revealed that the mobility pattern of the chimeras was dictated by their N-terminal extension (Fig. 1B), suggesting that this region was essential for the differential post-translational modifications on these proteins. This was intriguing because the target residues for the MAPKKK Bck1 lie within the C-terminal catalytic domains (40).

Next we wished to test whether the change in the mobility of Mkk1 and Mkk2 was because of phosphorylation. To moderately enhance the expression levels of the MAPKKs, we constructed centromeric vectors that expressed myc-tagged Mkks from their own promoter. The functionality of these constructs was checked by their capability of complementing the double *mkk1 mkk2* phenotype (data not shown). We immunoprecipi-

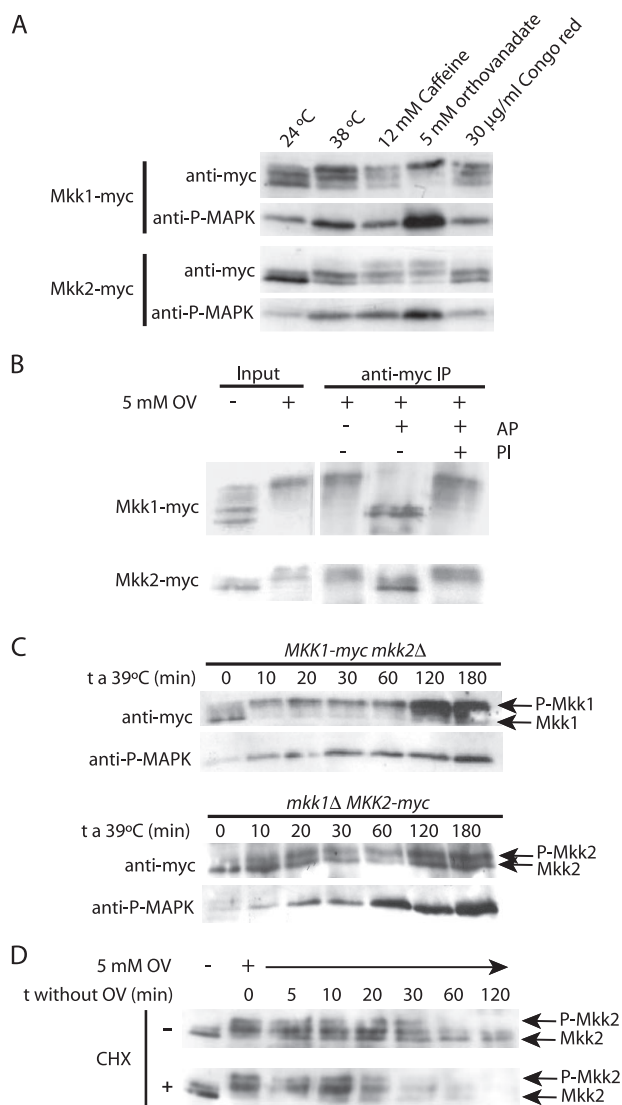


FIGURE 2. Stimuli of the CWI pathway lead to stable hyperphosphorylation of Mkk1 and Mkk2. *A*, Mkk1 and Mkk2 display a mobility shift in the presence of different stimuli of the pathway. The BY4741 strain or isogenic YMJ21 and YMJ22 strains, that had, respectively, integrated Mkk1-6myc and Mkk2-6myc, were grown to mid-log phase at 24 °C and then shifted to 38 °C or treated with 12 mM caffeine, 5 mM sodium orthovanadate, or 30 µg/ml Congo Red for 1 h. Mobility of Mkk1-6myc and Mkk2-6myc was analyzed by immunoblotting with anti-Myc antibodies, and the level of phosphorylation of Slt2 was detected with anti-phospho-p42/p44 antibodies. *B*, electrophoretic mobility shifts under CWI pathway stimulation conditions are due to phosphorylation. Transformants of the BY4741 strain carrying the pRS416Mkk1-6myc or pRS416Mkk2-6myc plasmids were treated with 5 mM sodium orthovanadate (OV) for 1 h to induce activation of the CWI pathway. Lysates from these cells were immunoprecipitated and treated with (+) or without (–) alkaline phosphatase (AP) from calf intestine in the absence (–) or presence (+) of phosphatase inhibitors (PI). The Mkk1-6myc and Mkk2-6myc forms were detected by immunoblotting with anti-Myc antibodies. Inputs refer to 6% of the total protein extracts that were subjected to immunoprecipitation. *C*, permanent phosphorylation of Mkk1 and Mkk2 under sustained stimulation of the CWI pathway. YPH499 integrants expressing from their chromosomal loci Myc-tagged copies of Mkk1 and Mkk2 (YMJ9 and YMJ10 strains) were incubated in YPD medium at 24 °C (control) or at 39 °C for the times indicated. Lysates from these cells were processed for immunoblot as in *A*. *D*, stability of phosphorylated and non-phosphorylated Mkk2 bands in conditions of inhibition of protein synthesis. The BY4741 strain transformed with pRS416Mkk2-6myc was incubated in the absence or presence of stimulus (sodium orthovanadate, OV) for 1 h. At that time, cells were washed and incubated into fresh OV-free medium (time 0) in the absence (upper panel) or presence (lower panel) of the protein synthesis inhibitor cycloheximide (CHX, 0.2 mg/ml). Cells recovering from such previous stimulation were collected at the times indicated and lysates were processed and immunoblotted with anti-Myc antibodies as above.

tated lysates from cells from these transformants grown in the presence of sodium orthovanadate to stimulate the pathway. Treatment of such immunoprecipitates with alkaline phosphatase *in vitro* in the absence of phosphatase inhibitors eliminated the bands of lower mobility (Fig. 2*B*). Thus, the major mobility shift that occurs under stimulating conditions is due to phosphorylation. To study the kinetics of this phosphorylation relative to the onset of the stimulus, we studied the electrophoretic pattern of Mkk1-myc and Mkk2-myc as well as the phosphorylation status of the Slt2 MAPK at several time points after shifting the culture to high temperature. MAPKK phosphorylation occurred before 10 min, in parallel to the initial increase of MAPK phosphorylation (Fig. 2*C*). As shown, longer incubation times in these activation conditions led to an enhanced amount of Mkk proteins without further altering their initial phosphorylation status. Persistence of the phosphorylated Mkk2 bands after exposure to orthovanadate for 1 h and subsequent removal of the stimulus by incubation in fresh medium was also evaluated both in the absence and presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 2*D*, phosphorylation of Mkk2 diminished gradually to finally disappear about 1 h after removal of the stimulus, a behavior that runs parallel to the loss of Slt2 phosphorylation (data not shown). Similar data were obtained for Mkk1 when analyzed in identical experimental conditions (data not shown). In the presence of cycloheximide, the phosphorylated Mkk2 bands disappeared as in the absence of this compound but, in contrast, the non-phosphorylated band of highest mobility did not re-appear. This suggests that the return to basal conditions relies on *de novo* synthesis of Mkk2 rather than on dephosphorylation of the promoted bands.

Phosphorylation of Mkk1 and Mkk2 Is Dependent on an Active Slt2 MAPK—A straightforward explanation for the mobility shift of Mkk1 and Mkk2 in activation conditions would lie on their phosphorylation by Bck1 in the two conserved MAPKKK phosphorylation residues (40). Accordingly, this phosphorylation was eliminated when Mkk1-myc or Mkk2-myc were expressed in a *bck1* strain (Fig. 3*A*). However, this view is in disagreement with our observation that the mobility of the chimerical Mkk proteins described above depended on their N-terminal regions, whereas the two residues phosphorylated by Bck1 are predicted to reside in their kinase C-terminal domain.

MAPKKs, like other protein kinases, display a lysine residue in their catalytic pocket responsible for their kinase activity. In the case of Mkk1 and Mkk2, such lysine maps in residues 250 and 243, respectively. To study the influence of their own kinase activity on their phosphorylation, we mutated this lysine in both MAPKKs to alanine. As expected, the inactive versions of both protein kinases were unable to complement the double *mkk1 mkk2* mutant (data not shown). Expression of any of these mutated versions in the absence of the other MAPKK of the cell integrity pathway abrogated the activation of Slt2 and they only displayed a small shift that could be attributable to Bck1-dependent phosphorylation (Fig. 3*B*). However, when the other MAPKK was present, the mobility shift of the tagged MAPKK was restored, concomitant to the activation of Slt2. All together, these data discard that the MAPKKs can autophos-

Slt2 Retrophosphorylates Mkk1 and Mkk2 MAPKKs

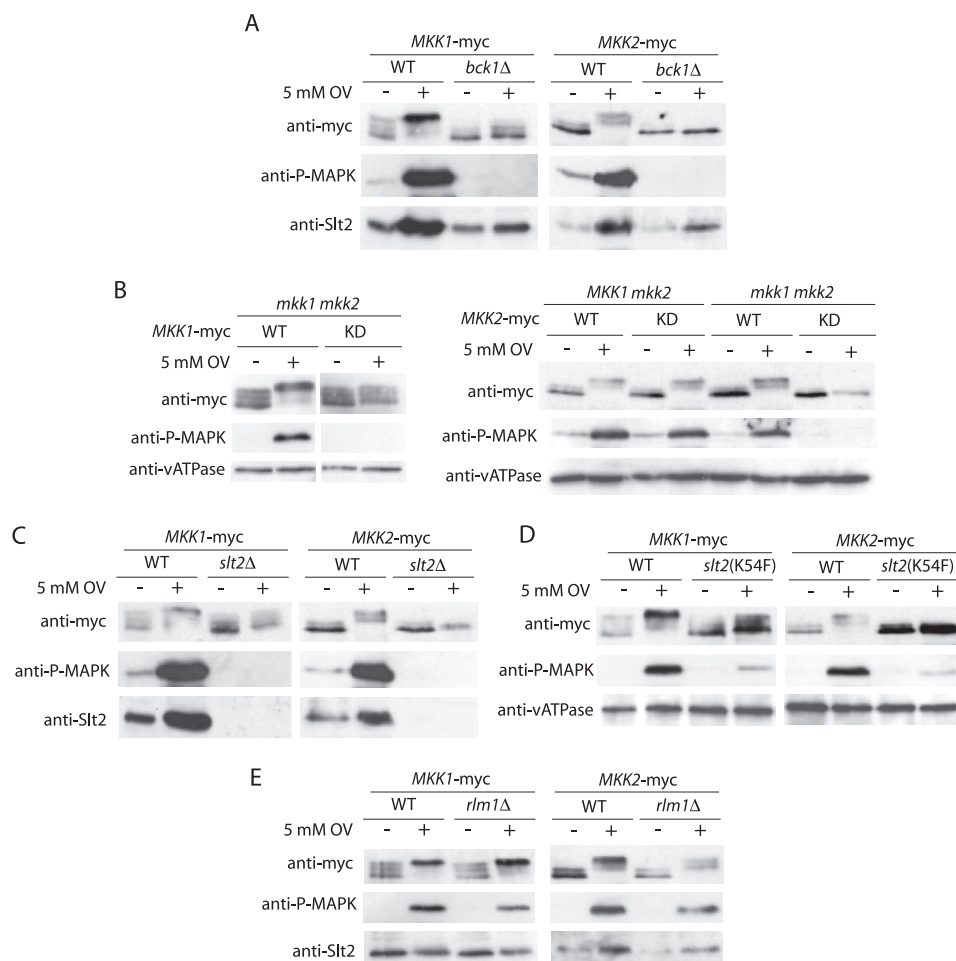


FIGURE 3. Analysis of Mkk1 and Mkk2 phosphorylation in different mutants of the CWI pathway. Plasmids pRS416Mkk1-6myc and pRS416Mkk2-6myc were transformed in the BY4741 strain or isogenic *bck1* (A), *slt2* (C), and *rlm1* (D) mutants, and in the wild type TD28 background or the isogenic catalytically inactive *slt2*-K54F mutant (E). The same plasmids, or their mutated versions that expressed K250R and K243R kinase-dead (KD) versions of Mkk1 and Mkk2, respectively, were transformed in the Y12112 *mkk2* strain or the isogenic *mkk1 mkk2* double deletant, as indicated. B, cultures of these transformants were grown to mid-log phase at 24 °C in SD and treated (+) or not (–) with 5 mM sodium orthovanadate for 1 h to stimulate the CWI pathway. Proteins were analyzed by immunoblotting with anti-Myc, anti-phospho-p42/p44, and either anti-Slt2 or anti-vATPase antibodies as loading control, as indicated.

phosphorylate when activated by Bck1 and suggest that the shift of Mkk1 and Mkk2 depends on the activity of downstream components of the pathway.

In agreement with this hypothesis, the lack of the MAPK Slt2 eliminated the phosphorylation of both MAPKKs when the pathway was activated (Fig. 3C). Moreover, when we studied phosphorylation of the MAPKKs in the presence of an inactive Slt2 by mutation of the Lys⁵⁴ of its catalytic center to phenylalanine (38), the shift in the MAPKKs did not occur either (Fig. 3D). Therefore, hyperphosphorylation of Mkk1 and Mkk2 requires the presence of a catalytically competent Slt2.

These results suggested a novel retrophosphorylation dependent on the presence and activity of the MAPK Slt2. To test whether this effect was direct or depended on a Slt2-driven transcriptional response, we studied phosphorylation of Mkk1 and Mkk2 in cells lacking Rlm1, the transcription factor that chiefly mediates the transcriptional response upon cell wall damage (43, 44). Both Mkk1 and Mkk2 were phosphorylated in an *rlm1*Δ mutant strain (Fig. 3E), so we can affirm that retro-

phosphorylation is a consequence of activity of Slt2 but independent of the transcriptional response triggered by this MAPK.

Slt2 Phosphorylates Mkk1 and Mkk2 in Vitro—Phosphorylation of Mkk1 and Mkk2 could be exerted directly by Slt2 or could be a consequence of the activation of other kinases by this MAPK. To test the first possibility, we performed *in vitro* kinase assays by using as the active enzyme the MAPK Slt2 fused to GST affinity purified from yeast extracts obtained in activation conditions. As substrates, we used GST-Mkk1 and GST-Mkk2, produced in *E. coli*. Both kinase-dead enzyme (GST-Slt2-K54F) and substrate (GST-Mkk1-K250R or GST-Mkk2-K243R) were included as controls. As shown in Fig. 4, active GST-Slt2 phosphorylates *in vitro* in both wild type and catalytically inactive forms of GST-Mkk1 and GST-Mkk2, whereas kinase-dead GST-Slt2 was unable to phosphorylate any of the MAPKKs. Therefore, Mkk1 and Mkk2 are substrates *in vitro* for Slt2. Occasionally, enzyme-free (GST) controls exhibited a faint signal, likely due to GST-Mkk autophosphorylation, because they were never observed in reactions containing kinase-dead MAPKKs.

Ser⁵⁰ in Mkk2 Is a Phosphorylation Target for Slt2 Both in Vivo and in Vitro—MAPKs are Ser/Thr kinases that phosphorylate consen-

sus sites where the Ser or Thr residue is followed by a Pro. The phosphorylation pattern of chimerical Mkk proteins with inter-exchanged catalytic domains (Fig. 1B) suggested that Slt2 retrophosphorylation targets should map at the N-terminal non-catalytic region of Mkk1 and Mkk2. Mkk1 has 6 Ser/Thr(Pro) sites in its non-kinase N terminus, whereas Mkk2 has only 4. As we have shown before that both protein kinases have redundant functions, we chose to work with Mkk2 for simplicity on our search for Slt2 phosphorylation sites. By site-directed mutagenesis, the Ser and Thr residues of the four Ser/Thr-Pro sites in the N-terminal domain of Mkk2 (Thr¹⁷, Ser⁴², Ser⁵⁰, and Thr¹⁵³) were mutated to Ala. The quadruple mutant protein had a phosphorylation pattern different from the wild type Mkk2 protein. It lacked the band of lowest mobility, so the shift was restricted to the intermediate band (Fig. 5A). In an attempt to eliminate all Slt2-dependent phosphorylation we changed to alanine the remaining two Ser/Thr-Pro residues that lie in the Mkk2 C-terminal region, namely Ser⁴³⁷ and Ser⁴⁷¹. Surprisingly, the sextuple mutant behaved like the quadruple, suggest-

ing that either Slt2 is capable of phosphorylating Ser and Thr residues that do not adjust to the consensus of MAPK targets, or that an unknown Slt2-dependent kinase is mediating phosphorylation.

To learn if all four sites in the N-terminal region of Mkk2 were needed for the partial elimination of the shift, we combined paired mutations, T17A together with T153A, and S42A with S50A. The first combination lead to an Mkk2 that was phosphorylated like the wild type, discarding these residues as

targets, whereas the S42A,S50A mutant displayed the same pattern as that of the quadruple mutant (Fig. 5B). Finally, single mutations revealed that Ser⁵⁰ is primarily responsible for the shift from the intermediate to the highest phosphorylated form, whereas Ser⁴² is not determinant for this phosphorylation event (Fig. 5C).

To confirm these data *in vitro*, we performed kinase assays as above by using as a substrate both kinase-dead GST-Mkk2(K243R) and a triple S42A,S50A,K243R mutant. In agreement with the above data, GST-Mkk2(S42A,S50A,K243R) was considerably less efficient as a substrate for active GST-Slt2 than GST-Mkk2(K243R) (Fig. 5D). However, *in vitro* phosphorylation was not totally eliminated by these mutations, so we cannot discard that Slt2 might phosphorylate *in vitro* positions other than the ones targeted here.

Retrophosphorylation of Ser⁵⁰ Negatively Regulates Mkk2 Function—Next we investigated whether phosphorylation of Ser⁵⁰ in Mkk2 contributed to functional aspects of the protein by phenotypic studies of the mutant protein. The Mkk2(S50A) mutant, like the rest of the mutants generated, was able to efficiently transduce the signal toward Slt2 as judged by immunoblotting with anti-P-MAPK antibodies (Fig. 5). We did not observe differences in the localization of a Mkk2(S50A)-GFP fusion as compared with the wild type in activation conditions (data not shown). Furthermore, substitution of Ser⁵⁰ by glutamic acid, a putative phosphomimetic mutation, did not affect either the stability of the protein in experiments using cycloheximide or the interaction between Mkk2 and Slt2 by using the two-hybrid system as previously reported (36) (data not shown). However, as shown in Fig. 6, Mkk2(S42A,S50A)-myc rescued *mkk1 mkk2* phenotypes of sensitivity to Congo red, orthovanadate, and caffeine more efficiently than Mkk2-myc alone. Interestingly, this suggests that the Slt2-mediated phosphorylation of Ser⁵⁰ negatively regulates Mkk2 function, but without affecting Mkk2 localization, stability, or its ability to interact with the MAPK.

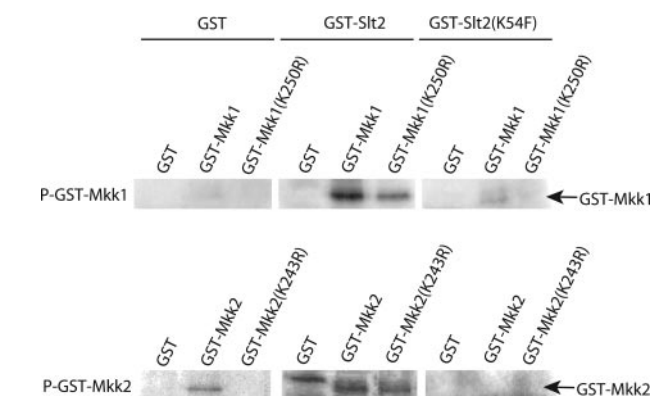


FIGURE 4. *In vitro* phosphorylation of recombinant GST-Mkk1 and GST-Mkk2 by GST-Slt2. Substrates for the *in vitro* kinase assay were expressed from *E. coli* transformed with pGEX-KG, pGEX-MKK1, pGEX-MKK1(K250R), pGEX-MKK2, and pGEX-MKK2(K243R), grown to mid-log phase and induced with isopropyl 1-thio- β -D-galactopyranoside to a final concentration of 0.4 mM for 3 h. Affinity purification was performed by using a glutathione-Sepharose matrix. As enzyme, the DL454 (*slt2* Δ) strain was transformed with the pEG(KG), pEG(KG)-SLT2, and pEG(KG)-SLT2K54F, grown in SR medium, and protein expression was induced by adding galactose to a final concentration of 2% for 6 h. Cells were shifted to 39 °C for 2 h to activate the CWI pathway and GST or GST-fused Slt2 versions were purified from the resulting lysates by affinity purification with glutathione-Sepharose. The reaction was performed at 30 °C for 30 min in the presence of [γ -³²P]ATP, solved in SDS-PAGE, and developed by autoradiography.

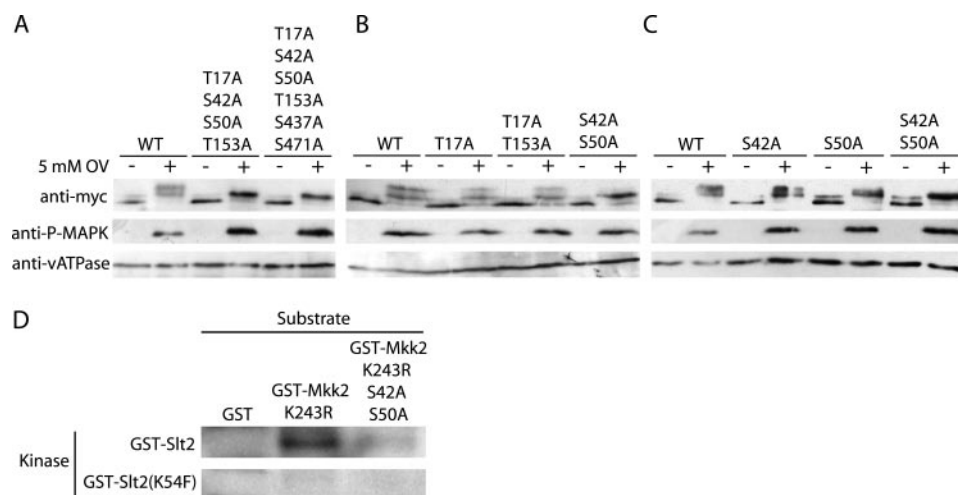


FIGURE 5. Phosphorylation of Mkk2 mutants in (Ser/Thr)Pro motifs. A–C, the pRS416MKK2-6myc plasmid bearing wild type MKK2 or mutations that generate the indicated amino acid changes were transformed in the YMJ29 *mkk1* Δ *mkk2* Δ strain, and grown to mid-log phase in SD medium at 24 °C. Protein extracts were prepared from cultures incubated in the presence (+) or absence (–) of 5 mM sodium orthovanadate for 1 h. Immunoblot analyses were performed using anti-Myc, anti-phospho-p42/p44, and anti-vATPase antibodies. D, effect of the S42A,S50A mutation in the phosphorylation of GST-Mkk2 by Slt2 *in vitro*. GST, GST-Mkk2(K243R), and GST-Mkk2(K243R,S42A,S50A) were purified from *E. coli* transformed with the appropriated plasmids and GST-SLT2 and GST-SLT2(K54F) were obtained from transformants of the DL454 yeast strain shifted to 39 °C for 2 h. Sample preparation and kinase assay were as described in the legend to Fig. 4. OV, sodium orthovanadate.

phorylation of Ser⁵⁰ negatively regulates Mkk2 function, but without affecting Mkk2 localization, stability, or its ability to interact with the MAPK.

Retrophosphorylation of Ser⁵⁰ in Mkk2 Depends on an N-terminal MAPK-docking Domain—Formation of the MAPKK-MAPK complex has been described to be mediated by an N-terminal MAPK-docking domain in the MAPKK (13). This domain obeys to the consensus (K/R)_{2–3}X_{1–6}(L/I)X(L/I) in mammalian MAPK pathways (45, 46) as well as in the yeast mating pheromone pathway (30). Although Mkk1 and Mkk2 are known to interact with Slt2 via their N-terminal region (36, 47), a canonical D-domain has not been mapped in these MAPKKs. We aligned the N-terminal region of Mkk1 and Mkk2 from *S. cerevisiae* as well as their orthologs in other fungi in

Slt2 Retrophosphorylates Mkk1 and Mkk2 MAPKKs

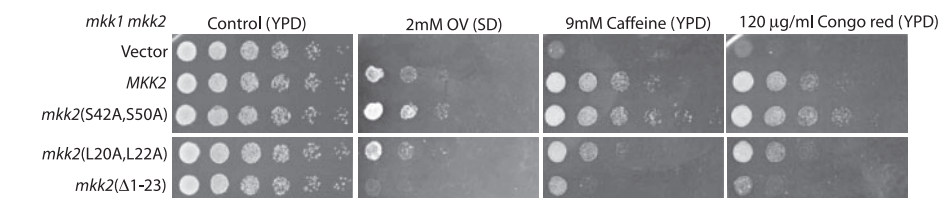


FIGURE 6. Phenotypic analysis of phosphorylation-deficient and putative MAPK-docking domain Mkk2 mutants. The YMJ29 *mkk1Δ mkk2Δ* double mutant strain was transformed with the pRS416 empty vector or pRS416-based constructs carrying wild type Mkk2-6myc or the Mkk2 mutant versions as indicated. Cells were grown in SD-Ura to an $A_{600\text{ nm}}$ of 0.5, serially 5-fold diluted, and spotted onto YPD agar medium at 24 °C in the absence or presence of 9 mM caffeine or 120 mg/ml Congo red, or in SD-Ura with 2 mM sodium orthovanadate (OV).

A

Species	Protein	Sequence	Position
<i>S. cerevisiae</i>	Mkk1	1 MASLFRP--PESAKCNPNPRLKLPPLLRNQ	29
<i>S. paradoxus</i>	Mkk1	1 MASLFRP--PESAKGNPNPRLKLPPLLRNQ	29
<i>S. castellii</i>	Mkk1	1 MASLFRP--PEQTKRNHVQPKLRPLPLFNEN	29
<i>S. cerevisiae</i>	Mkk2	1 MASMFRP--PESNRSHQKTPKLTLPVNLVQN	29
<i>S. paradoxus</i>	Mkk2	1 MASMFRP--PESNRSHQNTPKLTLPVNLVQN	29
<i>S. castellii</i>	Mkk2	1 MAFLFRP--PESMKRVNSPKLSLPTTLIRN	29
<i>C. glabrata</i>	Mkk	1 MASLFRP--PESTRQNARSRLKRLPLPLSNVS	29
<i>K. lactis</i>	Mkk	1 MASMFRP--PESRRNSKPKLSLPPQLVSN	29
<i>A. gossypii</i>	Mkk	1 MAFMFKP--PEQRKRNEKSPRLSLPSTLIGS	29
<i>N. grisea</i>	Mkk	4 PAPLLRPAIPGARSAGARTPRGLAIPSPN	54
<i>N. crassa</i>	Mkk	5 PAPLLRPAIPGARPGGRGPPRLGLAIPSPS	35
<i>Podospora</i>	Mkk	9 SVPLLRPALPGGNRSGRAPRLGLAIPSPN	59
<i>A. fumigatus</i>	Mkk	4 PVPLLRPPVPNGRNGPRPKLTGLIPSPN	35
<i>U. maydis</i>	Mkk	1 MASLIPPKP--RK--PGMPKLTIPTRPTSS	27
<i>C. albicans</i>	Mkk	4 VPPLFKVPNTKRTSNKVLNLSIPPTSNNK	34
<i>Blumeria</i>	Mkk	1----MPGQSTPRARPS-----PQLKLSPTPKSS	24
<i>Homo sapiens</i>	MEK1	1-----MPKKKPTPIQLNPAPDGS	18

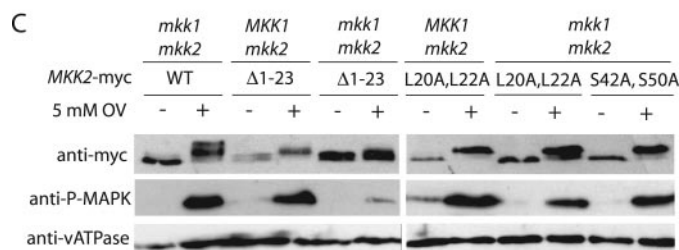
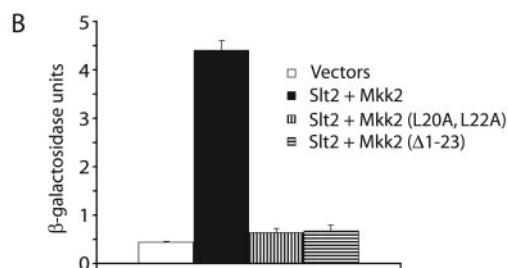


FIGURE 7. Fungal CWI MAPKKs have an unconventional but functional N-terminal MAPK-docking domain. A, multiple alignment of the first 29 amino acids of *S. cerevisiae* Mkk1 and Mkk2 with the equivalent regions of several Mkk orthologs from different fungi (as indicated) and human MEK1. Alignment was performed by CLUSTALW (ebi.ac.uk/clustalw). Dark shaded residues mark those that are identical in *S. cerevisiae* Mkk1 and Mkk2, and conserved in other organisms. Light shaded residues mark conservative changes with respect to *S. cerevisiae* Mkk1 and Mkk2. The (L/I)X(L/I) motif common to all D-domains is marked by arrows. B, mutation of Leu²⁰ and Leu²² in Mkk2 or elimination of the conserved 23 N-terminal residues impedes Mkk2-Slt2 interaction. Quantitative two-hybrid assays were performed on Y190 strain co-transformants expressing GAL4(AD)-Slt2 and GAL4(BD)-Mkk2 wild type or mutant fusions as indicated. Results in the graph are expressed in β-galactosidase Miller units. C, phosphorylation of Mkk2 docking-domain mutants as compared with the S42A,S50A phosphorylation-defective mutant. The pRS416MKK2-6myc plasmid bearing wild type Mkk2 or the mutations indicated was transformed either in the YMJ29 double *mkk1 mkk2* or single isogenic Y12112 *mkk2* mutant strains as indicated. Cultures and lysates were processed for immunoblot as described in the legend to Fig. 5A. OV, sodium orthovanadate.

search for conserved regions. Although the non-kinase N-terminal region of *S. cerevisiae* Mkk1 and Mkk2 is highly divergent, a high degree of similarity was observed in their first 23 residues, which was highly conserved in *Saccharomyces spp.*, *Candida glabrata*, *Kluyveromyces lactis*, and *Ashbya gossypii* (Fig. 7A). An N-terminal PXLXL signature pre-

ceded by basic residues, reminiscent of higher eukaryotic MAPKKs, was also conserved in the orthologous MAPKKs of more distant fungi such as *Magnaporthe*, *Podospora*, *Neurospora*, and *Ustilago*. Thus, although it does not completely fit the consensus for a MAPK-docking domain, the MAPKKs of fungal CWI pathways have an N-terminal LXL signature preceded by basic Lys or Arg residues that might constitute a MAPK-docking domain.

To study the function of this putative docking domain located in the first 23 amino acids of Mkk1 and Mkk2, we generated two sorts of mutants in Mkk2: a truncated version lacking this region and a double L20A,L22A mutant. Mkk2(L20A,L22A) had a reduced ability to complement *mkk1 mkk2* phenotypes compared with wild type Mkk2, whereas Mkk2(Δ1–23) was virtually unable to produce complementation (Fig. 6). This indicates that the N-terminal conserved region is important for signaling through the pathway. To check whether defective function of these mutants was due to lack of interaction with the Slt2 MAPK, we tested both mutations for interaction with Slt2 in the two-hybrid system. The L20A,L22A mutation was sufficient to eliminate Mkk2-Slt2 interaction (Fig. 7B), confirming that the putative docking domain described here is functional.

To investigate the contribution of this docking domain to Mkk2 retrophosphorylation by Slt2, we studied the mobility of the mutant forms both in basal and activation conditions in comparison to wild type and the Mkk2(S42A,S50A) mutant. As shown in Fig. 7C, Mkk2(Δ1–23) showed no hyperphosphorylation when expressed as the only MAPKK. This is likely due to its limited ability to activate Slt2, because restoring the pathway by the presence of Mkk1 led to its phosphorylation. However, even in the latter conditions, Mkk2(Δ1–23) did not shift to the Ser⁵⁰-dependent hyperphosphorylated band of lowest mobility. Interestingly, the Mkk2(L20A,L22A) mutant behaved as Mkk2(S42A,S50A), also lacking the band of lowest mobility (Fig. 7C). This implies that the docking domain of Mkk2 is essential for the phosphorylation of Ser⁵⁰ by Slt2.

DISCUSSION

Mkk1 and Mkk2, the MAPKKs that operate in the CWI MAPK pathway in *S. cerevisiae* were reported to be functionally redundant when first uncovered (40). Among the MAPK cascades described in *S. cerevisiae*, the CWI pathway is the only one that displays two MAPKKs. A search for hypothetical protein sequences from other yeast species on the basis of their homology to Mkk1 and Mkk2 on the growing number of fungal genomes available in databases reveals that Mkk homologs exist

in related organisms. However, only species phylogenetically close to *S. cerevisiae*, such as *Saccharomyces paradoxus*, and even more distant species of the genus, like *Saccharomyces castellii*, had duplicated Mkks, whereas all other genera explored using yeast and filamentous fungi, such as *Candida*, *Kluyveromyces*, *Ashbya*, *Yarrowia*, *Schizosaccharomyces*, *Ustilago*, *Aspergillus*, *Neurospora*, *Magnaporthe*, *Podospora*, *Blumeria*, or *Pneumocystis* apparently had only one Mkk ortholog. In consequence, duplication of the cell integrity MAPKKs seems characteristic of the *Saccharomyces* genus and is not a common feature of yeast or other fungi. Consultation of the YGOB tool (Yeast Gene Order Browser (48)) to determine syntenic relationships reveals that Mkk1 and Mkk2 are an ohnologous pair, generated from an ancestral whole genome duplication (49, 50). In this report, we confirm genetically by using isogenic *mkk1* and *mkk2* mutants that either MAPKK can function to efficiently maintain signaling through the CWI pathway. Furthermore, we prove that chimerical proteins obtained by interchange of their relatively divergent N-terminal regulatory region also preserve function. However, we present evidence that their post-translational modification by phosphorylation displays features unique to each kinase, suggesting that subtle differences in their regulation may exist.

We report here that Mkk1 and Mkk2 are phosphorylated by their downstream target in the CWI pathway, the MAPK Slt2. Retrophosphorylation of MAPKKs by their corresponding MAPKs is known to exist in the mammalian MEK-ERK pathway and in the yeast mating pheromone pathway. However, the functional implications of this phosphorylation event are obscure. In the MEK-ERK pathway, retrophosphorylation seems to involve a Pro-rich motif located near the C terminus of the MAPKK (18–20), but such a motif is not conserved in yeast MAPKKs. Rather, our analysis of the mobility in gel of chimerical Mkk proteins made by interchanging Mkk1 and Mkk2 non-kinase N-terminal extensions suggests that Slt2-dependent phosphorylation mainly targets this region. A Pro-rich motif that obeys to the consensus found in Src homology domain 3-binding proteins is conserved between Mkk1 (residues 80–91) and Mkk2 (residues 83–92). However, mutation of the two Pro residues described to eliminate interaction with Src homology 3 domains in other proteins had no effect in retrophosphorylation or function of Mkk1 and Mkk2 (data not shown). An extensive study have been made by Errede and Ge (28) and Maleri *et al.* (29) on the retrophosphorylation of the MAPKK Ste7 dependent on downstream MAPKs Fus3 and Kss1 of the yeast pheromone-response and invasive growth pathways, respectively. The possibility of a negative role for such retrophosphorylation was suspected from the fact that the hyperphosphorylated form of Ste7 seemed to associate less efficiently with Ste5, the scaffolding protein of the pathway (51). However, the analysis of Ste7 mutations that abrogated the feedback phosphorylation as well as phosphomimetic substitutions in the phosphorylation sites led these authors to conclude that Ste7 retrophosphorylation rather contributes to signaling specificity toward one MAPK or the other (29), a situation that cannot be conveyed to the cell integrity pathway, which has one only MAPK output.

We have defined Ser⁵⁰ in Mkk2 as one of the target residues for its Slt2-mediated retrophosphorylation. Although Ser⁵⁰ seems the chief recipient of the phosphorylation event that leads to the band of lowest electrophoretic mobility, we cannot fully discard the participation of Ser⁴², because the double Mkk2^{S42A,S50A} mutation led to a sharper elimination of such a band than the single Mkk2^{S50A}. Peculiarly, neither Ser⁴² nor Ser⁵⁰ are conserved in the sequence of Mkk1, and mutation of Thr⁵¹ within the closest Ser/Thr-Pro motif in Mkk1 relative to Mkk2 Ser⁵⁰ does not have any effect in Slt2-dependent phosphorylation of Mkk1 (data not shown). This suggests that, although Slt2 phosphorylates both MAPKKs, the phosphorylated residues targeted are specific for each kinase, in agreement with the different gel mobility pattern displayed by Mkk1 and Mkk2 in the presence of stimulus. In contrast to Mkk2, which mostly shifts to an intermediate level of Slt2-dependent phosphorylation that is independent of its Ser/Thr-Pro sites, Mkk1 is fully shifted to a band of low mobility under stimulation conditions. There are 5 other (Ser/Thr)-Pro sites in the N-terminal extension of Mkk1 that have not been investigated that could partially or totally account for this behavior.

As judged by the enhanced competence of the Ser⁵⁰ phosphorylation-deficient Mkk2 to complement a double *mkk1 mkk2* mutant as compared with the wild type, we concluded that phosphorylation of this residue by Slt2 negatively regulates Mkk2 function. Yet we ignore the biological relevance of the phosphorylation of Ser⁵⁰. Neither elimination of this phosphorylation site by change to Ala nor its substitution for a putative phosphomimetic Glu residue (data not shown) led to significant changes in the ability of Mkk2 to phosphorylate Slt2 nor to discernable alterations in its localization or stability. As speculated by Maleri *et al.* (29), who reported similar results for the Ste7 MAPKK, retrophosphorylation of Mkks by Slt2 may fine-tune their function by modulating signaling complex formation.

It was unexpected to find that the elimination of all Ser/Thr-Pro residues in Mkk2 does not fully abrogate Slt2-dependent retrophosphorylation. In contrast to our results on Mkk2, changing to Ala 7 Ser or Thr residues within (Ser/Thr)-Pro motifs in Ste7 led to a total elimination of its MAPK-dependent phosphorylation (29). So the major Slt2-dependent phosphorylation event on Mkk2, primary to phosphorylation of Ser⁵⁰, occurs in sites different from an (Ser/Thr)-Pro motif. Other yeast MAPKs, namely Hog1, Fus3, and Kss1, do not seem to exert cross-phosphorylation on CWI MAPKKs, because we observed that the shift still happened in both *pbs2* and *ste11* mutants, which are incapable of activating their respective downstream MAPKs (data not shown). We cannot fully discard that autophosphorylation of the MAPKKs plays a role in this phenomenon, but its strict dependence on Slt2 kinase activity and the fact that direct phosphorylation of Ser⁵⁰ does not seem to be a key for the other phosphorylation event, make it hard to conceive that it may be so. If this was the case, our data with kinase-dead *mkk2* mutant in the presence of Mkk1 and vice versa would imply that it is a trans-phosphorylation and that Mkk1 and Mkk2 could phosphorylate each other. A second possibility is that an intermediate kinase, itself activated by Slt2, is involved. PAK kinases, for instance, have been reported to

Slt2 Retrophosphorylates Mkk1 and Mkk2 MAPKKs

regulate MAPKKs in mammalian cells by phosphorylation (52). However, analysis of Mkk1 and Mkk2 phosphorylation in yeast mutants defective for Cla4, Ste20, and Skm1 PAKs indicated that these proteins are not involved in this event (data not shown). Although other types of Slt2-regulated kinase might operate at this level, its possible identity cannot be anticipated because no kinase targets such as mammalian RSK, MSK, or MAPKAPs (reviewed in Refs. 1 and 53) have been detected so far for Slt2. Finally, Slt2 could phosphorylate Mkk2 in Ser or Thr residues that do not fit an (Ser/Thr)-Pro consensus. Although this is not a common behavior for a MAPK, the observation that the Mkk2^{S42A,S50A} mutant protein is still able to be phosphorylated by Slt2 *in vitro*, although much less efficiently than the wild type, seems to favor this hypothesis.

We also prove that retrophosphorylation of Mkk2 Ser⁵⁰ requires the existence of a MAPK-docking domain at its N-terminus that does not exactly match the conventional "D-site" (K/R)₂₋₃X₁₋₆(L/I)X(L/I) motif found in MAPK activators and targets (13, 15), although it keeps the LXL signature. We show here that this motif is conserved in the cell integrity MAPKKs of several fungi, despite the limited degree of conservation among the sequences of their non-kinase N-terminal extensions. The function of this docking domain in the CWI MAPKKs is not limited to retrophosphorylation, but our data point toward a role for MAPKK-MAPK interaction via this motif in the activation of the MAPK. Mutation of Leu²⁰ and Leu²² in Mkk2 eliminated Mkk2-Slt2 interaction, and led to a mutant protein with a slightly reduced functionality. This is reminiscent of the phenotype of the equivalent mutations in Ste7, that reduce but do not eliminate signaling through the mating pheromone pathway (14). Remarkably, Mkk2 function as the Slt2 activator is seriously compromised by deletion of its first 23 amino acids. Levels of the Δ1–23 truncated protein are comparable with those of the wild type, so the lack of function is not due to problems of stability of the mutant protein. Although we cannot rule out that the truncated protein might be catalytically less competent, we can speculate from our data that Leu²⁰ and Leu²² in Mkk2 cooperate with their preceding residues in constituting a MAPK-binding site. In Ste7, a combination of mutations in the MAPK-docking site with mutations that impair binding to the Ste5 scaffold impede signaling (14), so the N-terminal region of Mkk2 might as well couple different interaction events for the formation of complexes to allow proper function of the pathway. D-sites are key to understanding the molecular mechanisms involved in MAPK signaling (13, 15). The N-terminal MAPK-binding site of mammalian MEK1 has also been shown to mediate both transmission of the signal toward ERK2 and retrophosphorylation of the Pro-rich domain of MEK1 by ERK2 (54). The N-terminal extension of JNK1, the MAPKK of the mammalian JNK pathway, has been reported to compete for binding to its activator, MEKK1, and its downstream target JNK, establishing a mechanism of sequential interactions for the transmission of the signal through the pathway (46). This illustrates the mechanistic importance of MAPKK non-catalytic N-terminal extensions studied here. Our finding of an unconventional but functional MAPK-docking domain in Mkk1 and Mkk2 establishes a novel argument on the paral-

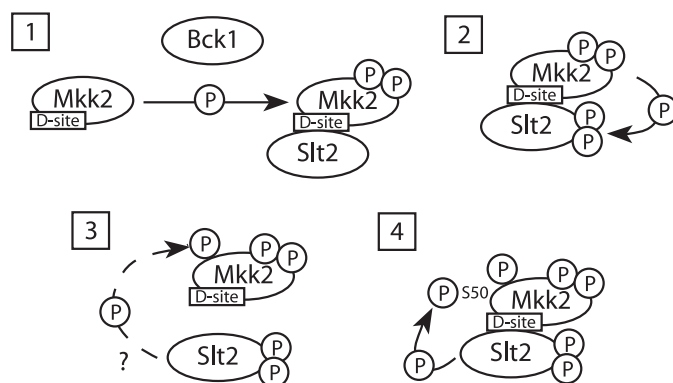


FIGURE 8. Model for the Slt2-dependent retrophosphorylation events that operate on Mkk2. Sequential activation of components of the MAPK module showing the role of a MAPK-docking domain in Mkk2 and two layers of Slt2-dependent phosphorylation of Mkk2 described in this work (see text for details).

lelism between mammalian ERK pathways and the fungal CWI MAPK pathway.

The sequence of events suggested by our results is depicted in the model in Fig. 8. Briefly, phosphorylation of Mkk by Bck1 (step 1) would lead to an active Mkk kinase able to interact with Slt2 via its D-site and to phosphorylate it in the activation loop (step 2). In the case of Mkk2, active Slt2 would trigger two different retrophosphorylation layers: a major D-site independent phosphorylation of Mkk2 outside consensus (Ser/Thr)-Pro motif, that may be indirect (step 3), and a direct and D-site-dependent phosphorylation of Ser⁵⁰ that negatively modulates the pathway (step 4). In summary, our data underscore the importance of the N-terminal extension of yeast CWI MAPKKs and infer the existence or complex feedback mechanisms exerted by Slt2 on its immediate upstream activators.

Acknowledgments—We are grateful to D. Levin, M. A. Rodríguez-Gabriel, H. Martin, C. Moreno, and all members of our laboratory for strains, plasmids, suggestions, and discussions throughout this work. We acknowledge M. I. García Sáez, T. Aparicio, and R. Torremocha of the Unidad de Genómica y Proteómica (Parque Científico de Madrid/Universidad Complutense de Madrid) for DNA sequencing.

REFERENCES

1. Raman, M., Chen, W., and Cobb, M. H. (2007) *Oncogene* **26**, 3100–3112
2. Garrington, T. P., and Johnson, G. L. (1999) *Curr. Opin. Cell Biol.* **11**, 211–218
3. Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) *Physiol. Rev.* **79**, 143–180
4. Yoon, S., and Seger, R. (2006) *Growth Factors* **24**, 21–44
5. Bardwell, L., Cook, J. G., Inouye, C. J., and Thorner, J. (1994) *Dev. Biol.* **166**, 363–379
6. Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998) *Microbiol. Mol. Biol. Rev.* **62**, 1264–1300
7. Ferrell, J. E., Jr. (1996) *Trends Biochem. Sci.* **21**, 460–466
8. Schwartz, M. A., and Madhani, H. D. (2004) *Annu. Rev. Genet.* **38**, 725–748
9. Sette, C., Inouye, C. J., Stroschein, S. L., Iaquina, P. J., and Thorner, J. (2000) *Mol. Biol. Cell* **11**, 4033–4049
10. Bhattacharyya, R. P., Remenyi, A., Good, M. C., Bashor, C. J., Falick, A. M., and Lim, W. A. (2006) *Science* **311**, 822–826
11. Whitmarsh, A. J., and Davis, R. J. (1998) *Trends Biochem. Sci.* **23**, 481–485
12. Posas, F., and Saito, H. (1997) *Science* **276**, 1702–1705

13. Grewal, S., Molina, D. M., and Bardwell, L. (2006) *Cell Signal.* **18**, 123–134
14. Bardwell, A. J., Flatauer, L. J., Matsukuma, K., Thorner, J., and Bardwell, L. (2001) *J. Biol. Chem.* **276**, 10374–10386
15. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) *Nat. Cell Biol.* **2**, 110–116
16. Remenyi, A., Good, M. C., Bhattacharyya, R. P., and Lim, W. A. (2005) *Mol. Cell* **20**, 951–962
17. Adachi, M., Fukuda, M., and Nishida, E. (1999) *EMBO J.* **18**, 5347–5358
18. Mansour, S. J., Resing, K. A., Candi, J. M., Hermann, A. S., Gloor, J. W., Herskind, K. R., Wartmann, M., Davis, R. J., and Ahn, N. G. (1994) *J. Biochem. (Tokyo)* **116**, 304–314
19. Gardner, A. M., Vaillancourt, R. R., Lange-Carter, C. A., and Johnson, G. L. (1994) *Mol. Biol. Cell* **5**, 193–201
20. Brunet, A., Pages, G., and Pouyssegur, J. (1994) *FEBS Lett.* **346**, 299–303
21. Coles, L. C., and Shaw, P. E. (2002) *Oncogene* **21**, 2236–2244
22. Eblen, S. T., Slack, J. K., Weber, M. J., and Catling, A. D. (2002) *Mol. Cell Biol.* **22**, 6023–6033
23. Eblen, S. T., Slack-Davis, J. K., Tarcsfalvi, A., Parsons, J. T., Weber, M. J., and Catling, A. D. (2004) *Mol. Cell Biol.* **24**, 2308–2317
24. Chen, R. E., and Thorner, J. (2007) *Biochim. Biophys. Acta* **1771**, 353–404
25. Errede, B., and Levin, D. E. (1993) *Curr. Opin. Cell Biol.* **5**, 254–260
26. Sabbagh, W., Jr., Flatauer, L. J., Bardwell, A. J., and Bardwell, L. (2001) *Mol. Cell* **8**, 683–691
27. Dohlman, H. G., and Slessareva, J. E. (2006) *Sci. STKE* **2006**, cm6
28. Errede, B., and Ge, Q. Y. (1996) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351**, 143–148
29. Maleri, S., Ge, Q., Hackett, E. A., Wang, Y., Dohlman, H. G., and Errede, B. (2004) *Mol. Cell Biol.* **24**, 9221–9238
30. Bardwell, L., and Thorner, J. (1996) *Trends Biochem. Sci.* **21**, 373–374
31. Levin, D. E. (2005) *Microbiol. Mol. Biol. Rev.* **69**, 262–291
32. van Drogen, F., and Peter, M. (2002) *Curr. Biol.* **12**, 1698–1703
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J., and Struhl, K. (2007) *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., New York
34. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* **10**, 1793–1808
35. Flandez, M., Cosano, I. C., Nombela, C., Martin, H., and Molina, M. (2004) *J. Biol. Chem.* **279**, 11027–11034
36. Soler, M., Plovins, A., Martin, H., Molina, M., and Nombela, C. (1995) *Mol. Microbiol.* **17**, 833–842
37. Wang, W., and Malcolm, B. A. (1999) *BioTechniques* **26**, 680–682
38. Martin, H., Arroyo, J., Sanchez, M., Molina, M., and Nombela, C. (1993) *Mol. Gen. Genet.* **241**, 177–184
39. Guarente, L. (1983) *Methods Enzymol.* **101**, 181–191
40. Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K., and Oshima, Y. (1993) *Mol. Cell Biol.* **13**, 3076–3083
41. Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000) *J. Biol. Chem.* **275**, 1511–1519
42. Sheu, Y. J., Santos, B., Fortin, N., Costigan, C., and Snyder, M. (1998) *Mol. Cell Biol.* **18**, 4053–4069
43. Jung, U. S., and Levin, D. E. (1999) *Mol. Microbiol.* **34**, 1049–1057
44. Garcia, R., Bermejo, C., Grau, C., Perez, R., Rodriguez-Pena, J. M., Francois, J., Nombela, C., and Arroyo, J. (2004) *J. Biol. Chem.* **279**, 15183–15195
45. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) *EMBO J.* **16**, 1901–1908
46. Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. (1998) *Genes Dev.* **12**, 3369–3381
47. Paravicini, G., and Friedli, L. (1996) *Mol. Gen. Genet.* **251**, 682–691
48. Byrne, K. P., and Wolfe, K. H. (2005) *Genome Res.* **15**, 1456–1461
49. Kellis, M., Birren, B. W., and Lander, E. S. (2004) *Nature* **428**, 617–624
50. Wolfe, K. H., and Shields, D. C. (1997) *Nature* **387**, 708–713
51. Choi, K. Y., Satterberg, B., Lyons, D. M., and Elion, E. A. (1994) *Cell* **78**, 499–512
52. Park, E. R., Eblen, S. T., and Catling, A. D. (2007) *Cell Signal.* **19**, 1488–1496
53. Gaestel, M. (2006) *Nat. Rev. Mol. Cell Biol.* **7**, 120–130
54. Xu, B., Wilsbacher, J. L., Collisson, T., and Cobb, M. H. (1999) *J. Biol. Chem.* **274**, 34029–34035
55. Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin, D. E. (1993) *Mol. Cell Biol.* **13**, 3067–3075