MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2615–2623 Vol. 17, No. 5
0270-7306/97/$04.00+0

Characterization of a Serum Response Factor-Like Protein in *Saccharomyces cerevisiae*, Rlm1, Which Has Transcriptional Activity Regulated by the Mpk1 (Slt2) Mitogen-Activated Protein Kinase Pathway

YASUYUKI WATANABE,[1] GIICHI TAKAESU,[1] MASATOSHI HAGIWARA,[2] KENJI IRIE,[1] AND KUNIHIRO MATSUMOTO[1]*

Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01,[1] and Department of Anatomy and Dermatology, Nagoya University School of Medicine, Showa-ku, Nagoya 466,[2] Japan

Received 15 October 1996/Returned for modification 14 November 1996/Accepted 27 January 1997

The Mpk1 (Slt2) mitogen-activated protein (MAP) kinase has been implicated in several biological processes in *Saccharomyces cerevisiae*. The Rlm1 protein, a member of the MADS box family of transcription factors, functions downstream of Mpk1 in the pathway. To characterize the role of Rlm1 in mediating the transcriptional activation by the Mpk1 pathway, we constructed a LexA-Rlm1ΔN chimera in which sequences, including the MADS box domain of the Rlm1 protein, were replaced by the LexA DNA binding domain and tested the ability of this chimera to activate a LexA operator-controlled reporter gene. In this assay, the Rlm1 protein was found to activate transcription in a manner regulated by the Mpk1 pathway. The Mpk1 protein kinase phosphorylated Rlm1ΔN in vitro and the LexA-Rlm1ΔN chimera protein was phosphorylated in vivo in a Mpk1-dependent manner. These results suggest that Mpk1 regulates the transcriptional activity of Rlm1 by directly phosphorylating it. We identified a Mpk1-like protein kinase, Mlp1, as an Rlm1-associated protein by using the yeast two-hybrid system. Overexpression of *MLP1* suppresses the caffeine-sensitive phenotype of the *bck1*Δ mutation. The additivity of the *mlp1*Δ defect with the *mpk1*Δ defect with regard to the caffeine sensitivity, combined with the results of genetic epistasis experiments, suggested that the activity of Rlm1 is regulated independently by Mpk1 MAP kinase and the Mlp1 MAP kinase-like kinase.

Extracellular molecules that regulate cell proliferation and differentiation in eukaryotes exert their effects through pathways that detect signals at the cell surface and transmit them through the cytoplasm to the nucleus. Transcription factors in the nucleus are typically the ultimate targets of these signaling pathways, and they function to elicit alterations in gene expression that in turn regulate cellular events. One mechanism for transmitting these signals involves a protein phosphorylation cascade leading to activation of mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) (1, 19, 20). Thus, these enzymes are thought to function as intermediaries between membrane-associated signaling molecules and the nucleus. MAPKs/ERKs are activated through protein kinase cascades that are comprised of three highly conserved core components. The MAPKs are activated in response to dual phosphorylation on tyrosine and threonine residues catalyzed by a family of dual-specificity protein kinases, termed MEK (MAPK or ERK kinase) or MAPKK (MAPK kinase). MAPKK/MEK itself is phosphorylated and activated by upstream regulators, MAPKK kinases (MAPKKKs), that include Raf, MEK kinase (MEKK), and Mos (19). Each of these upstream components also functions in multiple cell signaling processes.

Defining the targets of MAPKs is of fundamental importance for understanding at a molecular level how intracellular processes are dramatically altered in response to environmental signals. The targets of MAPKs include a number of transcription factors, and it is presumed that it is by phosphorylation of these factors that extracellular stimuli regulate transcription. One promoter motif that mediates the response to ERKs is the c-*fos* serum response element (SRE). The c-*fos* SRE binds a ternary complex comprised of the SRF (serum response factor [31, 32]) and TCF (ternary complex factor [31, 32]) transcription factors. Recent findings indicate that phosphorylation of TCF occurs in response to activation of the MAPK pathway and that regulation of TCF activity is an important mechanism by which promoters containing the SRE respond to growth factor signals (9, 12, 15). However, although progress has been made in identifying possible targets of MAPKs, much remains to be learned about the downstream events caused by MAPK activation in vivo.

Through the selection of mutants and cloning of the genes involved, yeast molecular genetics offers a powerful tool for dissecting the components of signal transduction pathways. One of the MAPK pathways in the budding yeast *Saccharomyces cerevisiae* is mediated by the yeast protein kinase C homolog, Pkc1. This pathway consists of Bck1 (Slk1) and Mkk1/Mkk2, which are proposed to catalyze a protein phosphorylation cascade culminating in the activation of the Mpk1 MAPK homolog. The upstream activators of Mpk1 are the functionally redundant kinases Mkk1 and Mkk2, which share high sequence identity with MEK (11). Furthermore, the Bck1 kinase, which has been demonstrated to function upstream of Mkk1/Mkk2, is closely related to MEKK. Thus, Bck1, Mkk1/Mkk2, and Mpk1 kinases act, in that order, in the Pkc1-mediated pathway (11, 16, 17). This pathway is essential for integrity of the cell wall and also participates in nutrient sensing, growth

* Corresponding author. Mailing address: Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan. Fax: 81-52-789-2589 or 81-52-789-3001. E-mail: g44177a@nucc.cc.nagoya-u.ac.jp.

TABLE 1. Strains used

Strain	Genotype
1788	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1*
DL456-3D	*MAT*α *mpk1*Δ*::TRP1 ura3 leu2 trp1 his4 can1*
GMY80	*MAT***a** *mlp1*Δ*::URA3 ura3 leu2 trp1 his4 can1*
GMY81	*MAT***a** *mlp1*Δ*::LEU2 ura3 leu2 trp1 his3 ade2 can1*
GMY90-1A	*MAT***a** *ura3 leu2 trp1 his4 can1*
GMY90-1B	*MAT*α *mpk1*Δ*::TRP1 ura3 leu2 trp1 his4 can1*
GMY90-1C	*MAT*α *mlp1*Δ*::URA3 mpk1*Δ*::TRP1 ura3 leu2 trp1 his4 can1*
GMY90-1D	*MAT***a** *mlp1*Δ*::URA3 ura3 leu2 trp1 his4 can1*
L40	*MAT***a** *leu2 trp1 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*
TNP44	*MAT***a** *bck1*Δ*::HIS3 ura3 leu2 trp1 his3 ade2 can1*
TNP46	*MAT***a** *mpk1*Δ*::HIS3 ura3 leu2 trp1 his3 ade2 can1*
W303-1A	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1*

control, and actin organization. Identification of the targets of Mpk1 would contribute to an understanding of how the Pkc1-Mpk1 pathway directs downstream events.

We have previously identified the *RLM1* gene from mutants resistant to growth inhibition caused by overexpression of *MKK1*^{P386}, an activated mutation of *MKK1*. *RLM1* encodes a protein belonging to the MADS box family of transcription factors. Genetic and phenotypic analyses suggest that Rlm1 functions as a transcription factor downstream of Mpk1 and is activated by the Mpk1 MAPK pathway. Consistent with this possibility, an interaction between Mpk1 and Rlm1 in vivo was observed with the yeast two-hybrid system (35). It is therefore possible that activation of Rlm1 by the Mpk1 pathway enables it to regulate expression of specific genes involved in the response. However, the role of Rlm1 in the Mpk1 pathway has been unclear, owing to the lack of a suitable functional assay for Rlm1 in vivo.

As a first step toward assaying Rlm1 function in the Mpk1 pathway, we constructed a fusion protein, LexA-Rlm1ΔN, in which the NH_2-terminal MADS box domain of Rlm1 was replaced by the DNA binding domain of LexA. We tested whether this LexA-Rlm1ΔN chimera would activate transcription and confer Mpk1-mediated regulation of a LexA site reporter gene. In this study, we present evidence suggesting that transcriptional activation by Rlm1 is regulated by the Mpk1 pathway via direct phosphorylation by Mpk1. Furthermore, we identified a Mpk1-like protein kinase which interacts with Rlm1 by the yeast two-hybrid screening method.

MATERIALS AND METHODS

Strains and general methods. *Escherichia coli* DH5α was used for DNA manipulation (7). Another *E. coli* strain, BL21, was used as the host for expression of heterologous protein. The yeast strains used in this study are listed in Table 1. Standard procedures for yeast manipulations (13) were followed. The media used in this study included rich medium (YEPD), synthetic complete medium (SC), synthetic minimal medium (SD), and sporulation medium (13). SC lacking amino acids or other nutrients (e.g., SC-Leu lacks leucine) was used to score auxotrophies and to select transformants. Yeast cells were transformed by the lithium acetate method, using single-strand DNA as the carrier (6). Other recombinant DNA procedures were carried out as described by Sambrook et al. (28).

Plasmids. Plasmid YEp351[MPK1::HA] directs the expression of Mpk1 tagged at its carboxyl terminus with an epitope from the influenza virus hemagglutinin (HA) protein (Mpk1-HA), and plasmid YEp351[mpk1-T190A, Y192F::HA] expresses a version of Mpk1-HA that lacks the two phosphorylatable residues required for its activation. Both plasmids were the kind gifts of David E. Levin (14). Plasmid YS116 is a YEp-based *URA3* plasmid harboring the *lacZ* reporter gene containing LexA DNA binding sites in its promoter (25). Plasmid YEpMLP1 is YEplac181 carrying the 2.1-kb *Nru*I fragment of the *MLP1* gene. The NH_2-terminal portion of the *MLP1* coding sequence was amplified by PCR using the 5′ primer 5′-CCCGGATCCAAATGGCGACTGACACCGAGAG-3′, which incorporates a *Bam*HI site, and the 3′ primer 5′-CTTCCCGGGATATTACCGAACTGAA-3′. An 830-bp *Bam*HI-*Sma*I fragment generated by PCR was inserted into pBluescript SK+ (SK+MLP1-1). Then a 400-bp *Bam*HI-*Hin*dIII fragment obtained from SK+MLP1-1 and a 1.3-kb *Hin*dIII-*Sal*I fragment containing the COOH-terminal portion of *MLP1* were inserted into the *Bam*HI-*Sal*I gap of pACTII (18) to generate pACT-MLP1. In pACT-MLP1, the transcriptional activation domain of Gal4 is fused to the NH_2 terminus of Mlp1. pACT-MLP1 rescued the caffeine sensitivity of *bck1*Δ. To create LexA-Rlm1 chimera deletion plasmids, the 1.6-kb *Bgl*II-*Cla*I (amino acids 1 to 525), the 1.3-kb *Bgl*II-*Ssp*I (amino acids 1 to 445), and the 730-bp *Bgl*II-*Hin*dIII (amino acids 1 to 245) fragments of pACT-RLM1 were cloned into pBTM116. pACT-MPK1 was isolated from an *ACT* cDNA library (provided as a gift by S. J. Elledge), using *MKK1* as a bait. pACT-MPK1 expresses a fusion protein between the activation domain of Gal4 (GAD) and the full-length coding sequence of the *SLT2*/*MPK1* gene.

Cloning of *MLP1*. Plasmid pYW61 (35) contains a 2.6-kb *Bgl*II-*Pst*I fragment of pACT-RLM1 containing the full-length coding sequence, which was cloned into the *Bam*HI-*Sal*I gap of pBTM116 (35). The LexA-Rlm1 fusion protein strongly activated transcription of *HIS3* and *lacZ* reporter genes containing LexA operators in the reporter strain L40 (35). Then, the LexA-Rlm1ΔC plasmid (pYW62) was constructed by removing the 1-kb *Cla*I-*Cla*I fragment from pYW61. pYW62 expresses the LexA-Rlm1 fusion protein lacking the COOH-terminal 151 amino acids. The LexA-Rlm1ΔC fusion protein moderately activated expression of reporter genes, but histidine auxotrophy of transformants with pYW62 could be obtained by growing cells in the presence of 130 mM 3-aminotriazole (AT), a chemical inhibitor of the *HIS3* gene product, imidazole glycerol phosphate dehydrogenase. Strain L40 was transformed simultaneously with both pYW62 and one of the libraries of genomic DNA fragments (3). After 3 to 4 days of growth on SC-Leu-Trp plates, Leu^+ Trp^+ transformants were replica plated to SC-His-Leu-Trp plates containing 130 mM AT. After false-positive clones were eliminated by curing plasmids, two transformant clones remained. Two plasmids were rescued by transformation into *E. coli*. They allowed cells to grow on SC-His containing 130 mM AT when cotransformed with just pYW62. Both plasmids contained the entire coding region of a gene designated *YKL615*, as determined by restriction site mapping. The nucleotide sequences of both strands of the 430-bp *Hin*dIII-*Sma*I fragment of the plasmid were determined by dideoxy-chain termination sequencing (29).

Construction of yeast strains containing *MLP1* deletion alleles. Deletion alleles of *MLP1* were constructed by the one-step gene replacement method (27). The 1.0-kb *Eco*RI-*Eco*RV fragment of *MLP1* was replaced with the 1.1-kb *Sal*I-*Eco*RI fragment of *URA3* or the 1.6-kb *Sal*I-*Eco*RI fragment of *LEU2*, after appropriate conversion of restriction sites. The deletion removes 338 amino acids of the open reading frame (ORF) and 23 bp of the 5′ flanking sequences. The DNA containing the entire *mlp1*Δ*::URA3* or *mlp1*Δ*::LEU2* construction was used to transform the yeast strain; this was followed by selection for Ura^+ or Leu^+ transformants. Restriction mapping and Southern analysis of genomic DNAs from the resulting transformants confirmed that transplacement had occurred.

Site-directed mutagenesis of *MLP1*. A mutation of *MLP1* (*mlp1*^{Y192F}) containing phenylalanine instead of tyrosine at position 192 was prepared by using the PCR-based overlap extension technique (10). An 840-bp fragment was amplified by PCR using the 5′ primer 5′-CTTGCTGCAGTAAAAAGCGC-3′ (oligo-A) and the 3′ primer 5′-TATCGAGGTTATAAAACCCTTAATGAA-3′ (encoding Y192F). A 450-bp fragment was also amplified by using the 5′ primer 5′-TTCATTAAGGGTTTTATAACCTCGATA-3′ (encoding Y192F) and the 3′ primer 5′-CGAATTCGAATCTAAAGGTC-3′ (oligo-B). These two overlapping mutant fragments were joined by mixing and performing a PCR amplification in the presence of oligo-A as the 5′ primer and oligo-B as the 3′ primer. PCR amplification generated a 1.3-kb fragment that was digested with *Pst*I and *Bst*BI and inserted into the *Pst*I-*Cla*I gap of pBluescript SK+ (SK+MLP1-2). The 430-bp *Hin*dIII-*Sma*I fragment of SK+MLP1-2 was substituted for the corresponding wild-type DNA fragment contained in YEpMLP1 to generate YEpMLP1-Y192F. Incorporation of the mutation was verified by DNA sequencing.

Two-hybrid assays. Two-hybrid assays were performed as described previously (3, 5, 35).

β-Galactosidase assays. To create a hybrid protein containing the LexA DNA binding domain inserted in place of the MADS box domain of Rlm1, the internal region of *RLM1* was amplified by PCR using the 5′ primer 5′-AAGGATCCTTATGTACAATCTTAACCAGCCTTCAT-3′, which incorporates a *Bam*HI site, and the 3′ primer 5′-ACATCGATATAGAAGAAACATTTGG-3′. A 910-bp *Bam*HI-*Cla*I fragment generated by PCR and a 550-bp *Cla*I-*Sna*BI fragment encoding the COOH-terminal region of *RLM1* were inserted into the *Bam*HI-*Sma*I gap of pBluescript SK+ (SK+RLM1-1). The 1.5-kb *Bam*HI-*Sal*I fragment of SK+RLM1-1 was cloned into the *Bam*HI-*Sal*I gap of pBTM116 (34) to generate pYW71, expressing the chimera LexA-Rlm1ΔN. The transactivation activity of LexA-Rlm1ΔN was measured by using a *lacZ* reporter gene downstream of a promoter containing LexA DNA binding sites. β-Galactosidase assays were performed as described previously (13).

Expression of *RLM1* in bacteria. To express *RLM1* in *E. coli*, the *Bam*HI-*Sal*I 1.5-kb fragment of SK+RLM1-1 was inserted into the *Bam*HI-*Sal*I gap of pGEX-5X-1 (Pharmacia Biotech) to produce pGEX-RLM1ΔN. To create COOH-terminal deletion derivatives of glutathione *S*-transferase (GST)–Rlm1ΔN, the 0.7-kb

*Bam*HI-*Ssp*I (amino acids 222 to 445) fragment and the 0.3-kb *Bam*HI-*Eco*RI (amino acids 222 to 328) fragment of SK+RLM1-1 were cloned into pGEX-5X-1 to produce pGEX-RLM1ΔN445Δ and pGEX-RLM1ΔN328Δ, respectively. *E. coli* BL21 transformed with pGEX-RLM1ΔN and its derivatives were grown in L broth at 37°C to an optical density of 0.7 and induced for an additional 6 h in the presence of 0.2 mM isopropylthiogalactopyranoside. GST-Rlm1ΔN fusion proteins were purified as previously described (21).

Preparation of extracts and immunoprecipitations. Yeast cells grown to an optical density at 600 nm of 0.5 to 1.0 in SC-Leu at 25°C were diluted 1:1 with fresh medium prewarmed to 55°C and then maintained at 39°C for 30 min. Control cells were diluted with medium at 25°C. After the treatments, yeast cultures were quickly chilled, and cells were collected by rapid centrifugation. The cell pellet was washed twice with Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and then suspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM NaF, 1 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (0.4- to 0.6-mm diameter) was added to this suspension, and cells were broken by vigorous vortexing for 5 min at 4°C. The beads and cell debris were removed by centrifugation at 10,000 × *g* at 2°C, and the supernatant was further clarified by centrifugation at 100,000 × *g* at 2°C. Cell extracts (100 mg of protein) were incubated at 4°C for 2 h with 40 μl of protein A-Sepharose beads (Sigma) containing covalently coupled mouse monoclonal 12CA5 anti-HA immunoglobulin (Boehringer Mannheim). Immune complexes were washed three times with lysis buffer and once with kinase buffer (100 mM Tris-HCl [pH 7.5], 50 mM $MgCl_2$). Protein concentrations of cell extracts were measured with Bio-Rad protein determination reagent as described previously (2).

In vitro phosphorylation assays. Immunoprecipitated Mpk1-HA was suspended in 20 μl of kinase buffer with 1 μg of GST-Rlm1ΔN fusion proteins. The mixture was preincubated for 3 min at 30°C, after which the reaction was initiated by adding ATP to a final concentration of 0.1 mM along with 10 μCi of [γ-^{32}P]ATP. After incubation for 30 min at 30°C, the reaction was terminated by adding sodium dodecyl sulfate (SDS) sample buffer, and samples were boiled and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12% acrylamide gels. After electrophoresis, gels were stained with Coomassie brilliant blue R250 and washed in 45% isopropanol–10% acetic acid. Dried gels were subjected to autoradiography.

Immunoblots. Immunoprecipitated complexes were subjected to SDS-PAGE on 12% acrylamide gels followed by electroblotting onto Immunobilon-P membranes (Millipore Corporation). Blots were blocked by incubation for 1 h at room temperature in TBS-M (Tris-buffered saline with 5% nonfat dry milk). Blots were then incubated with monoclonal antibody 12CA5 diluted 1:1,000 in TBS-M for 20 h at 4°C. After three washes with TBS-M, blots were incubated for 2 h with peroxide-linked secondary antibody (Amersham) diluted 1:2,500 in TBS-M. After four final washes with TBS-M, blots were developed with an enhanced chemiluminescence detection kit (Amersham).

Electrophoretic mobility shift assay. Cells carrying LexA-Rlm1ΔN were lysed as described above except that the lysis buffer contained phosphatase inhibitors (50 mM glycerol-2-phosphate, 1 mM sodium orthovanadate, and 0.1 μM okadaic acid). Then cell extracts were incubated at 4°C for 2 h with 40 μl of protein A-Sepharose beads containing covalently coupled rabbit polyclonal anti-LexA antibody. Immunoprecipitated complexes were subjected to SDS-PAGE on 7% acrylamide gels, and immunoblot analysis was performed with anti-LexA antibody. In dephosphorylation reactions, calf intestine alkaline phosphatase (CIP; 1.8 U/μl) was added to the beads containing the LexA-Rlm1ΔN immune complex and incubated at 37°C for 30 min. Control reactions contained dephosphorylation buffer (50 mM Tris-HCl [pH 8.5], 1 mM EDTA) alone.

Phosphoamino acid analysis. ^{32}P-labeled phospho-Rlm1 (GST-Rlm1ΔN) eluted from gels was hydrolyzed in vacuo for 90 min at 110°C in 6 N HCl. Phosphoamino acids were separated by electrophoresis at pH 3.5 (1.5 kV, 45 min) on thin-layer cellulose plates. Marker phosphoamino acids (1 mg of each) were identified by spraying the plates with ninhydrin stain and gentle heating. The isotope-labeled phosphoamino acids were detected by autoradiography.

RESULTS

Transcriptional activation by Rlm1. We tested the idea that Rlm1 serves as a transcription factor regulated by the Mpk1 pathway. A plasmid (pYW71) was constructed to direct expression of a hybrid protein in which a portion of Rlm1, including the MADS box domain, was replaced with the DNA binding region of the bacterial LexA repressor (Fig. 1A). In the LexA-Rlm1ΔN construct, the fusion protein is bound to DNA through LexA. The ability of the LexA-Rlm1ΔN protein to activate transcription was monitored by using a *lacZ* reporter gene containing *lexA* DNA binding sites in its promoter. Expression of the LexA DNA binding domain alone failed to activate transcription of the reporter. However, the LexA-Rlm1ΔN fusion protein caused significant expression of the reporter gene, resulting in β-galactosidase activity that was much higher than the background level observed in the same host strain expressing the LexA DNA binding domain alone (Fig. 1B). Thus, expression of β-galactosidase is absolutely dependent on Rlm1ΔN. This result demonstrates that Rlm1ΔN lacking its MADS box domain can activate transcription when it is bound to DNA by virtue of its attachment to the LexA DNA binding domain.

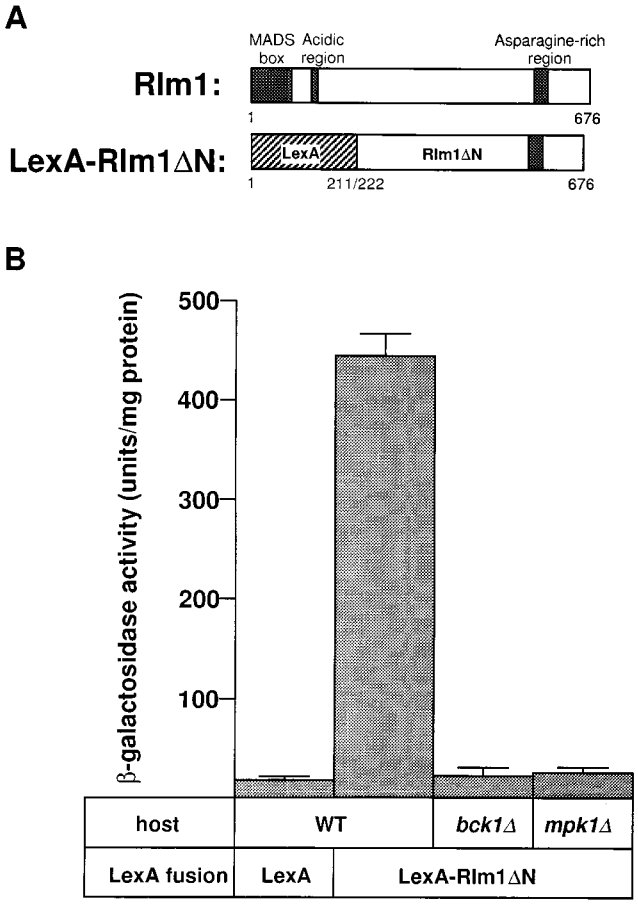

FIG. 1. Transcriptional activation by Rlm1ΔN. (A) LexA-Rlm1ΔN fusion construct. The LexA DNA binding region was fused to residues 222 to 676 of Rlm1. (B) Cells harboring the *LexA-lacZ* reporter gene were transformed with pBTM116 (LexA) or pYW71 (LexA-Rlm1ΔN). Cells were grown at 25°C in synthetic medium containing the appropriate amino acids and 2% glucose and were assayed for β-galactosidase activity as described previously (13). The units shown are the averages of two or three experiments. Strains: wild-type (WT), W303-1A; *bck1*Δ, TNP44; *mpk1*Δ, TNP46.

To test whether the Mpk1 pathway could modulate the ability of Rlm1 to activate transcription, we also quantified expression of the *lacZ* reporter gene directed by the LexA-Rlm1ΔN fusion protein in *bck1*Δ and *mpk1*Δ mutant cells. Plasmid pYW71 was transformed along with the *LexA-lacZ* reporter gene into *bck1*Δ (TNP44) (23) and *mpk1*Δ (TNP46) (23) strains, and transformants were tested for β-galactosidase activity (Fig. 1B). In these *bck1*Δ and *mpk1*Δ mutant strains, the LexA-Rlm1ΔN fusion protein was unable to activate transcription of the reporter gene. These data show that the ability of Rlm1 protein to regulate transcription is dependent on the activation of the Mpk1 pathway.

The Mpk1 pathway is activated in response to mild heat shock (14). Since the results described above indicated that

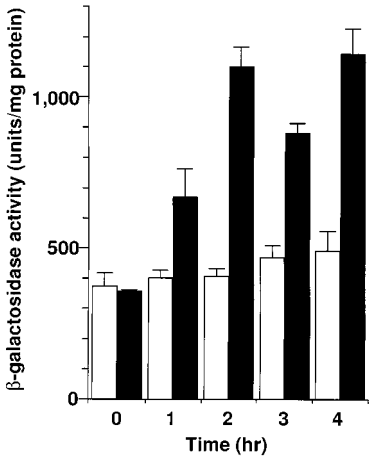

FIG. 2. Effect of mild heat shock on Rlm1ΔN transcriptional activity. Wild-type cells (W303-1A) carrying the *LexA-lacZ* reporter gene and pYW71 (LexA-Rlm1ΔN) were grown to mid-log phase (5×10^7) at 20°C in SC-Trp-Ura. Cells were diluted with fresh medium prewarmed to 37°C and shifted to 37°C (solid bar). Control cells were diluted with medium at 20°C and grown at 20°C (open bar). Aliquots were taken at 1 h intervals and assayed for β-galactosidase activity.

Rlm1 could be a downstream target of the Mpk1 pathway, we asked whether expression of the reporter directed by LexA-Rlm1ΔN could be stimulated by an increase in growth temperature. Wild-type cells carrying pYW71 (LexA-Rlm1ΔN) and the *LexA-lacZ* reporter gene were grown at 20°C and then shifted to 37°C. As shown in Fig. 2, a time-dependent 2.5- to 3-fold increase was observed following the temperature shift. The results support the possibility that signaling through Mpk1 regulates Rlm1 transcriptional activity. Thus, the extent of activation of the Mpk1 pathway can be monitored by using a *LexA-lacZ* reporter gene directed by the LexA-Rlm1ΔN fusion protein.

In vitro phosphorylation of Rlm1ΔN by Mpk1. The foregoing results suggest that the Rlm1 protein contains a transcriptional activation domain the activity of which is modulated by the Mpk1 protein kinase. To test whether the Rlm1ΔN portion of the LexA-Rlm1ΔN fusion protein is phosphorylated by Mpk1 in vitro, Rlm1ΔN was expressed as a GST fusion in *E. coli* and purified on a GST column. Immune complexes isolated from yeast cells expressing Mpk1-HA were used for kinase assays. The Mpk1-HA protein contains Mpk1 tagged at the COOH terminus with an influenza virus HA epitope and is biologically functional (14). Kamada et al. (14) have shown that the Mpk1 protein kinase is strongly activated by mild heat shock using bovine myelin basic protein as the substrate: Mpk1-HA from cells growing at 23°C showed very weak kinase activity against this substrate, while the enzyme from cells grown at 30°C was moderately active, and the enzyme from cells grown at 37°C was strongly active. Consistent with these results, phosphorylation of Rlm1ΔN increased when Mpk1-HA was immunoprecipitated from cells that had been grown at 39°C for 30 min compared with Mpk1-HA immunoprecipitated from cells grown at 25°C (Fig. 3). In a control experiment with unfused GST protein, no phosphorylation was observed (data not shown), indicating that the phosphorylation of GST-Rlm1ΔN is actually on Rlm1ΔN.

To verify that phosphorylation of Rlm1ΔN was mediated by Mpk1-HA, rather than a contaminating protein kinase in the immune precipitates, a mutant form of Mpk1, Mpk1-TAYF, that lacks the two phosphorylatable residues predicted to be required for activation was tested for protein kinase activity,

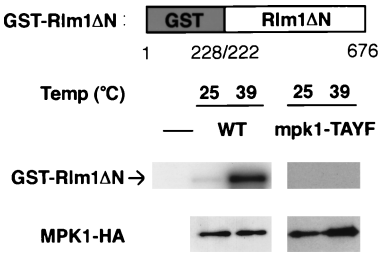

FIG. 3. In vitro phosphorylation of Rlm1ΔN by Mpk1. Wild-type cells (strain 1788) were transformed with YEp351[MPK1::HA] or YEp351[mpk1-T190A, Y192F::HA]. Mpk1-HA (WT [wild type]) and Mpk1-TAYF-HA (mpk1-TAYF) were immunoprecipitated from extracts of cultures subjected to temperature shift at 25°C for 30 min or 39°C for 30 min (see Materials and Methods). In vitro protein kinase assays were conducted with GST-Rlm1ΔN (top) as a substrate, and the reaction mixtures were subjected to SDS-PAGE on a 12% acrylamide gel as described in Materials and Methods (middle). A parallel set of immune complexes was subjected to immunodetection of Mpk1-HA (bottom).

using GST-Rlm1ΔN as the substrate. Mutation of both Thr-190 to Ala and Tyr-192 to Phe inactivates the biological function of Mpk1 (14). Mpk1-TAYF–HA immune complexes displayed no detectable protein kinase activity toward Rlm1ΔN above that found in immunoprecipitates from a control strain that did not express HA-tagged Mpk1 (Fig. 3). Taken together, these results clearly indicate that Rlm1ΔN is phosphorylated by Mpk1 MAPK in vitro.

The nature of the phosphorylation sites was determined by phosphoamino acid analysis of in vitro-labeled GST-Rlm1ΔN. This experiment revealed that phosphate was incorporated onto both serine and threonine residues (Fig. 4A). The Rlm1ΔN protein contains 10 Ser/Thr-Pro motifs that form the core potential MAPK sites. To map the regions responsible for phosphorylation by Mpk1, we tested the phosphorylation of a set of Rlm1ΔN deletion mutants (Fig. 4B). Deletion mutants of Rlm1ΔN were fused to GST for bacterial expression, and the GST-Rlm1ΔN truncation derivatives were phosphorylated in

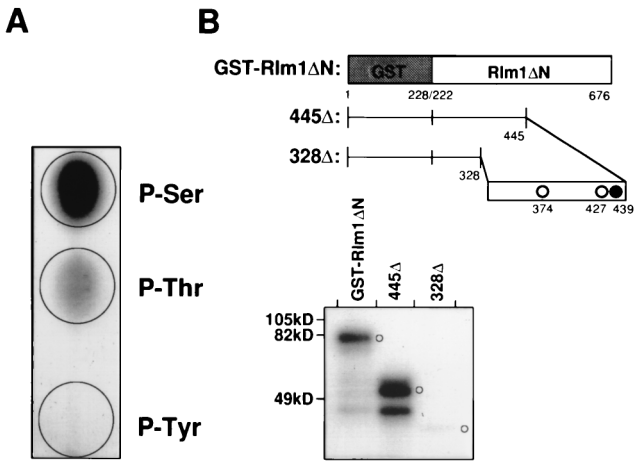

FIG. 4. Characterization of Rlm1ΔN as substrate for Mpk1. GST-Rlm1ΔN was phosphorylated in vitro by Mpk1 as described in the legend to Fig. 3. (A) Phosphoamino acid analysis of GST-Rlm1ΔN. Phosphoamino acid analysis was done as described in Materials and Methods. Encircled areas represent the location of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) based on ninhydrin staining. (B) The COOH-terminal deletion derivatives of GST-Rlm1ΔN were tested by the in vitro phosphorylation assay. The structures of the mutants used are shown above; open and shaded circles indicate conserved Ser-Pro and Thr-Pro motifs, respectively, with their amino acid positions. The bands corresponding to these GST-Rlm1ΔN derivative proteins are marked with circles.

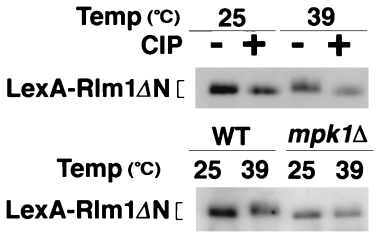

FIG. 5. Modification of Rlm1ΔN by phosphorylation. Wild-type (WT) cells (W303-1A) harboring pYW71 (LexA-Rlm1ΔN) were treated with mild heat shock as described in the legend to Fig. 3. LexA-Rlm1ΔN was immunoprecipitated from extracts of cultures, and the immunoprecipitates were incubated with (+) or without (−) CIP. The samples were analyzed by SDS-PAGE (7% gel) followed by immunoblotting with anti-LexA antibodies (top). Wild-type (W303-1A) and *mpk1*Δ (TNP46) cells harboring pYW71 were treated with mild heat shock as described above. LexA-Rlm1ΔN immune complexes were subjected to SDS-PAGE. Immunodetection was performed with anti-LexA antibodies (bottom).

vitro by Mpk1 immunoprecipitated from cells that had been grown at 39°C for 30 min. Each phosphorylation reaction was then fractionated by SDS-PAGE. Deletion of the COOH-terminal 231 amino acids (446 to 676) had little effect on the level of phosphorylation. In contrast, phosphorylation was absent from the GST-Rlm1ΔN (amino acids 222 to 328) protein (Fig. 4B). These results suggest that potential Mpk1 phosphorylation sites are likely to reside within Rlm1 amino acids 329 to 445. This region contains three Ser/Thr-Pro motifs, Ser-374, Ser-427, and Thr-439.

The LexA-Rlm1ΔN chimera is phosphorylated in vivo. To test whether the LexA-Rlm1ΔN fusion protein is phosphorylated in vivo, modification of the LexA-Rlm1ΔN protein was examined by immunoblotting with a LexA antibody. When cell extracts from wild-type strains harboring plasmid pYW71 (LexA-Rlm1ΔN) were subjected to immunoblot analysis, we detected a protein immunoreactive with the LexA antibody. The strains were then tested for the response of LexA-Rlm1ΔN to mild heat shock. Immunoblots of cell extracts grown at 39°C showed that the LexA-Rlm1ΔN protein was shifted substantially to a higher, more slowly migrating band upon treatment with mild heat shock (Fig. 5). To test the possibility that the mobility shift of LexA-Rlm1ΔN results from phosphorylation, we treated lysates with CIP and assayed the mobility shift by immunoblot analysis. Phosphatase treatment resulted in a shift to a lower LexA-Rlm1ΔN band (Fig. 5), demonstrating that the changes in LexA-Rlm1ΔN mobility were due to phosphorylation.

To determine whether the phosphorylation of LexA-Rlm1ΔN is dependent on the Mpk1 pathway, heat shock-induced in vivo phosphorylation of LexA-Rlm1ΔN was examined in *mpk1*Δ mutant cells expressing the LexA-Rlm1ΔN protein. In the *mpk1*Δ mutant, no change in LexA-Rlm1ΔN pattern was seen upon exposure to 39°C (Fig. 5). Taken together, these observations suggest that phosphorylation of LexA-Rlm1ΔN in response to mild heat shock occurs through the Mpk1-dependent pathway.

Isolation of Rlm1-interacting proteins. One of the hallmarks of MADS box proteins is their potential to interact with other regulatory proteins to control gene transcription. The yeast two-hybrid system was used to screen for proteins that interact with Rlm1. Plasmid pYW62 expresses a truncated form of Rlm1 (Rlm1ΔC) lacking its COOH-terminal 151 amino acids as a fusion to the DNA binding domain of LexA (see Materials and Methods). The yeast reporter strain L40 containing pYW62 was then transformed with plasmid libraries consisting of fusions between the GAD and yeast genomic DNA fragment (3). Nine positive clones were obtained from 1.2×10^5 transformants screened. Two clones (5 and 9) were found to strongly interact with the LexA-Rlm1ΔC fusion protein and contained the same gene (Fig. 6A). This gene was designated *MLP1* (Mpk1-like protein kinase; see below).

The *MLP1* gene encodes a Mpk1-like protein kinase. Sequence analysis revealed that *MLP1* is identical to a sequence designated YKL615 cloned by the chromosome XI sequencing project (33). The *MLP1* gene encodes a 433-residue protein that contains the 11 conserved subdomains shared by protein kinases and is most similar to Mpk1. Sequence alignment shows 61% identity over a 388-amino-acid overlap (Fig. 7). However, a characteristic regulatory domain most highly conserved among MAPKs (Thr-X-Tyr, where X is Glu, Pro, or Gly) was not present in Mlp1. Instead, a related sequence in which the residues corresponding to Thr-X-Tyr were replaced by Lys-Gly-Tyr in Mlp1 was found. Therefore, by sequence criteria, Mlp1 does not represent a member of the MAPK family but may share enzymatic properties with Mpk1. Two other nearly invariant residues are also replaced in Mlp1: arginine in place of lysine at position 54 in subdomain II and asparagine in place of aspartate at position 171 in subdomain VII (Fig. 7). However, the sequence conservation between the Mpk1 and Mlp1 domains argues that Mlp1 is nevertheless functional.

To examine the phenotypic defect associated with loss of

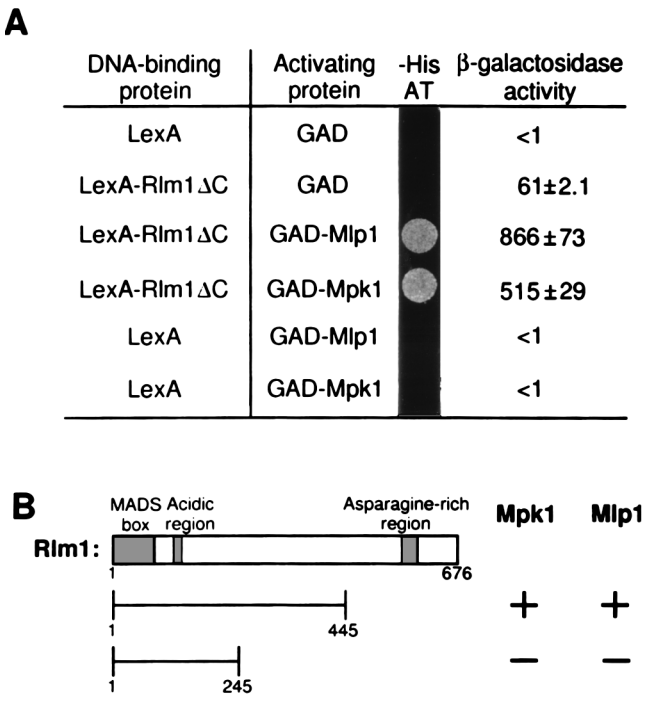

FIG. 6. Two-hybrid interaction. (A) The reporter strain L40 (*LYS2::LexA-HIS3 URA3::LexA-lacZ*) was transformed with various plasmids as indicated. Approximately 3×10^5 cells of each transformant were spotted onto SC-His medium containing 130 mM AT. Plates were incubated at 30°C for 4 days. Transformants were assayed for β-galactosidase activity as described previously (13). Plasmids: LexA, pBTM116; LexA-Rlm1ΔC, pYW62; GAD, pACTII; GAD-MLP1, pACT-MLP1; GAD-MPK1, pACT-MPK1. β-Galactosidase activity is expressed in units/milligram of protein. (B) To decide which region of Rlm1 is responsible for interacting with Mpk1 or Mlp1, various truncated derivatives of Rlm1 were introduced into the L40 two-hybrid assay system. The interactions between Mpk1 or Mlp1 and Rlm1 COOH-terminal deletions were examined by growth on SC-His medium containing 10 mM AT.

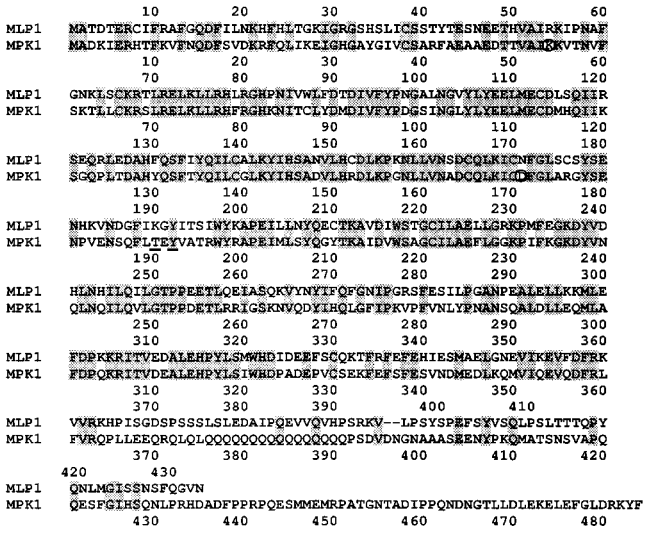

FIG. 7. Homology between the amino acid sequences of Mlp1 and Mpk1. Comparison of amino acid sequence between Mlp1 and Mpk1 is shown. Identical amino acids are indicated by shaded boxes. Critical threonine and tyrosine resides (The-X-Tyr motif) which are conserved among all MAPKs are underlined. Conserved Lys and Asp residues in protein kinases are marked with circles.

MLP1 function, a deletion mutant of *MLP1* was constructed in vitro. A 1.0-kb *Eco*RI-*Eco*RV region containing the *MLP1* ORF was replaced with the *S. cerevisiae URA3* gene (see Materials and Methods). The deletion allele (*mlp1Δ::URA3*) was then introduced into wild-type diploid yeast strains. Transformants were tested for possession of the deletion allele by Southern blot analysis (data not shown). Heterozygous diploid transformants were sporulated, and progeny from individual tetrads were dissected onto plates and analyzed. Four spores from each tetrad gave rise to colonies. The *mlp1Δ::URA3* segregants grew normally at all temperatures ranging from 14°C to 38°C and were not sensitive to caffeine (data not shown).

Similar regions of Rlm1 are required for binding to Mpk1 and Mlp1. The Rlm1 sequences required for binding to Mlp1 were examined to determine whether they coincided with the Mpk1-binding domains. DNA fragments from *RLM1* which encode various truncated versions of Rlm1 were fused with the LexA DNA binding domain, and these LexA-Rlm1 deletion chimeras were transformed into the yeast reporter strain L40 carrying GAD-Mlp1 or GAD-Mpk1. As shown in Fig. 6B, Mlp1 and Mpk1 interacted with the 200-amino-acid region (246 to 445) of Rlm1. Thus, similar regions of the Rlm1 protein are required for binding to both Mpk1 and Mlp1. These results suggest that the Mpk1 and Mlp1 binding domains may be a target for phosphorylation signals generated in response to the Mpk1 pathway. In fact, this region contains major Mpk1 phosphorylation sites (Fig. 4B).

To test whether this region of Rlm1 acts as a transcriptional activation domain, we generated a series of deletion mutants and examined their abilities to activate the LexA operator-controlled reporter gene as described above. Deletion of the COOH-terminal region (amino acids 526 to 676) severely reduced transcriptional activation by the fusion protein; further deletions resulted in a complete loss of transcriptional activity (data not shown). These results suggest that the COOH-terminal region of Rlm1 is required for transcriptional activation.

Genetic interaction of Mlp1 with the Mpk1 pathway. Since the Mpk1 and Mlp1 proteins associated with Rlm1, we expected that Mlp1 may affect the Mpk1 pathway. This possibility was examined by several genetic tests. First, we tested the effects of overexpressing Mlp1 in *bck1Δ* and *mpk1Δ* mutants. As shown in Fig. 8A, a multicopy plasmid encoding *MLP1* (YEpMLP1) suppressed the caffeine-sensitive phenotype of *bck1Δ* cells but failed to suppress the temperature-sensitive growth defect. However, the caffeine sensitivity of *mpk1Δ* was not suppressed by *MLP1* overexpression. Since Mlp1 associates with Rlm1, we imagined that this result may be due to the regulation of Rlm1. To explore this possibility, we examined the effect of *MLP1* overexpression in *bck1Δ rlm1Δ* double mutants and found that overexpression failed to suppress the caffeine-sensitive phenotype of *bck1Δ rlm1Δ* cells (data not shown). This result indicates that suppression of *bck1Δ* by *MLP1* overexpression requires Rlm1 activity.

If Mlp1 operates in a pathway parallel to the Mpk1 pathway, then disruption of *mlp1* would be expected to enhance the severity of the defects observed in *mpk1Δ* mutants. We further examined the relationship between the Mpk1 pathway and Mlp1 by constructing *mpk1Δ mlp1Δ* strains. Analysis of colony growth at 30 and 33°C (a semipermissive temperature for *mpk1Δ*) revealed that the *mpk1Δ mlp1Δ* double mutants grew as well as the *mpk1Δ* mutants (data not shown). As expected,

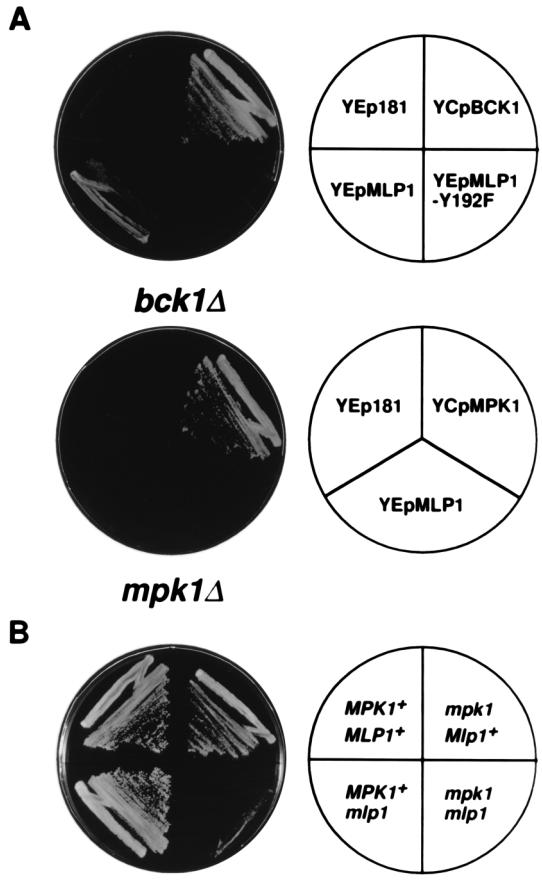

FIG. 8. Genetic interaction of Mlp1 with the Mpk1 pathway. (A) Cells were transformed with the indicated plasmids, and then transformants were streaked onto YEPD containing 2 mM caffeine. Plates were incubated for 2 days at 25°C. Strains: *bck1Δ*, TNP44; *mpk1Δ*, TNP46. (B) Caffeine sensitivity of *mlp1Δ mpk1Δ* double mutants. DL456-3D (*mpk1Δ::TRP1*) was crossed with GMY80 (*mlp1Δ::URA3*). The resulting diploid was sporulated, and the tetrads were dissected. Each strain was streaked onto YEPD containing 1.1 mM caffeine (left). The plate was incubated for 2 days at 25°C. Strains: *MPK1*$^+$ *MLP1*$^+$, GMY90-1A; *MPK1*$^+$ *mlp1Δ*, GMY90-1D; *mpk1Δ MLP1*$^+$, GMY90-1B; *mpk1Δ mlp1Δ*, GMY90-1C.

TABLE 2. Effects of the *mlp1*Δ mutation on Rlm1ΔN transcriptional activity[a]

Genotype	β-Galactosidase activity (U)	
	0 h	2 h at 37°C
MLP1$^+$	269 ± 21	1,538 ± 39
*mlp1*Δ	93 ± 27	889 ± 6

[a] Wild-type (*MLP1*$^+$; W303-1A) and *mlp1*Δ (GMY81) cells harboring the *LexA-lacZ* reporter gene were transformed with pYW71 (LexA-Rlm1ΔN). Cells were grown at 20°C, then shifted to for 2 h, and subsequently assayed for β-galactosidase activity.

the *mpk1*Δ *mlp1*Δ cells failed to grow at 37°C, and this growth defect was rescued when the medium was osmotically stabilized by 1 M sorbitol. Thus, the presence of the *mlp1*Δ mutation did not enhance the temperature-sensitive growth defect of *mpk1*Δ. However, additivity of the *mlp1*Δ and *mpk1*Δ mutations was observed with regard to caffeine sensitivity. Although *mpk1*Δ mutants are sensitive to caffeine, they grow if the caffeine concentration is less than 2 mM. As shown in Fig. 8B, *mpk1*Δ *mlp1*Δ double mutants failed to grow in the presence of 1.1 mM caffeine. As Rlm1 determines caffeine sensitivity mediated by the Mpk1 pathway, these results suggest that the Mlp1 protein kinase affects the Mpk1 pathway through the Rlm1 transcription factor.

The effect of the *mlp1*Δ mutation on the transactivation potential of LexA-Rlm1ΔN was investigated in the context of the *LexA* operator as described above. Plasmid pYW71 (LexA-Rlm1ΔN) was transformed along with the *LexA-lacZ* reporter gene into the *mlp1*Δ strain, and transformants were tested for β-galactosidase activity in response to mild heat shock. The *mlp1*Δ mutation slightly reduced the activity of Rlm1ΔN (Table 2), whereas the *mpk1*Δ mutation resulted in a complete loss of transcriptional activity of LexA-Rlm1ΔN (Fig. 1B). These data demonstrate that transcriptional activation by the LexA-Rlm1ΔN fusion protein is mainly regulated by Mpk1.

Effect of the *mlp1*^{Y192F} mutation on its ability to suppress *bck1*Δ. Activation of MAPKs requires phosphorylation of a tyrosine and a threonine residue. These sites lie in the phosphorylation lip between subdomains VII and VIII of the protein kinases. In Mlp1 the residue corresponding to Thr is Lys-190, while that corresponding to Tyr is Tyr-192 (Fig. 7). To test whether Tyr-192 is important for Mlp1 function, we generated a mutation leading to a Phe substitution for Tyr-192 (*mlp1*^{Y192F}). Mutation of Tyr-192 to Phe severely diminished the biological activity of *MPK1* (18). A multicopy plasmid encoding *mlp1*^{Y192F} (YEpMLP-Y192F) was unable to suppress the caffeine-sensitive phenotype exhibited by the *bck1*Δ mutant (Fig. 8A). This result suggests that this tyrosine residue may be a key factor in the activity of Mlp1 protein kinase.

DISCUSSION

We have previously identified Rlm1 as a downstream component of the Mpk1 pathway. The Rlm1 protein contains a MADS box domain which defines a family of transcription factors, and we envisage that Rlm1 serves as a transcriptional activator by using its DNA binding motif to recognize specific promoter elements. However, the role of Rlm1 in the Mpk1 pathway has been unclear, since we have yet to identify a specific DNA sequence which it recognizes. To develop an assay for Rlm1 function, we changed its DNA binding specificity by replacing the Rlm1-MADS box domain with the LexA DNA binding domain. We then tested whether the LexA-Rlm1ΔN fusion protein has the ability to activate transcription from a LexA reporter gene. We used this system to show that (i) Rlm1 possesses an ability to stimulate transcription, (ii) transcriptional activation by LexA-Rlm1ΔN is dependent on the Mpk1 pathway, and (iii) the COOH terminus of the Rlm1 protein is required for its transcriptional activation. Taken together with our demonstration that Rlm1ΔN can be phosphorylated in vitro and in vivo by the Mpk1 protein kinase, these results suggest that the Mpk1 MAPK directly phosphorylates Rlm1 and regulates its transcriptional activation function. Consistent with this possibility, Rlm1 associates with Mpk1 in the yeast two-hybrid assay. These findings may provide a direct link between the intracellular signaling pathway mediated by Mpk1 and the activity of the Rlm1 protein.

The yeast two-hybrid assay showed that Mpk1 associates with the middle region of Rlm1 between amino acids 246 and 445. We used various deletion mutants of Rlm1ΔN to evaluate the regions on Rlm1ΔN that became phosphorylated by Mpk1 in vitro. These experiments demonstrated that Rlm1ΔN is mainly phosphorylated by Mpk1 on its middle region between amino acids 329 and 445. Thus, the Mpk1-associated domain in Rlm1 is phosphorylated by Mpk1 in vitro. Deletion of the Rlm1 COOH-terminal domain (amino acids 526 to 676) substantially reduced transcriptional activity, indicating that the COOH-terminal region of Rlm1 is required for transcriptional activation. This domain contains a distinctive sequence of consecutive asparagines (35), and it is likely that the Asp-rich domain alters a structure involved in activation. Based on these results, it is reasonable to anticipate that the Rlm1 COOH terminus contains a strong transcriptional activation domain whose activity is potentiated by phosphorylation of multiple Ser/Thr-Pro motifs presented in the middle region. We observed that the truncated form Rlm1(87-469) failed to activate transcription but retained the ability to associate with Mpk1 (30). Rlm1(87-469) might be predicted to function as a dominant negative protein that can interfere with the activity of the wild-type Rlm1 protein by sequestering Mpk1. Indeed, overexpression of Rlm1(87-469) suppressed the lethality caused by overexpression of Mkk1^{P386} (30). This finding further suggests that the COOH-terminal activation domain of Rlm1 is critical for its function.

One of the hallmarks of MADS box proteins is their potential to interact with other regulatory proteins to control gene transcription. In mammalian cells, activation of the MAPK pathway by growth factor signals leads to phosphorylation of the SRF accessory factor TCF/Elk-1 and the activation of transcription (8, 32). Our results suggest that activation of the Mpk1 pathway leads to the phosphorylation of the Rlm1 protein and activation of transcription. However, in this study, we used a truncated form of Rlm1 (Rlm1ΔN) lacking the MADS box domain to assay Rlm1 function. Since the regions of MADS box proteins, such as SRF and yeast Mcm1, that mediate interaction with accessory proteins are located immediately adjacent to the MADS box (4, 22, 24, 26), it is possible that the intact Rlm1 protein interacts with additional cofactors in activating its target genes. Such putative partners may not be required for activity of the COOH-terminal transcription activation domain when Rlm1ΔN is fused to the DNA binding domain of LexA. Further experiments will be required to determine whether transcriptional activation of Rlm1 requires specific contacts with other proteins.

Our findings indicate that Rlm1 is a downstream target of Mpk1. However, unlike Mpk1 pathway mutants, the *rlm1*Δ cells grow normally at any temperature, grow on glycerol medium and are not sensitive to nitrogen starvation (35). One possibility is that Rlm1 is an important downstream component of the Mpk1 pathway, but additional Rlm1-like proteins

that prevent *rlm1*Δ cells from acquiring the growth defects are present. Searches of the databases with *RLM1* identified an ORF (YBR182c) on chromosome II that could encode a protein with 93% identity to Rlm1 in the first 60 amino acids. This high level of sequence identity in the MADS box domain suggested that the two might bind to the same DNA sequence and regulate the same genes. We isolated the *YBR182c* gene and prepared a strain containing a chromosomal deletion of the gene (36). The *ybr182c*Δ cells grew normally at all temperatures ranging from 14 to 38°C and were not sensitive to caffeine. The caffeine-sensitive phenotype caused by *rlm1*Δ was not enhanced in *rlm1*Δ *ybr182c*Δ double mutants. Furthermore, the *ybr182c*Δ mutation had no effect on the toxicity of *MKK1-P386* overproduction (36). These observations strongly suggest that YBR182c is not involved in the Mpk1 pathway.

The *MLP1* gene was identified by the yeast two-hybrid screening method as a gene encoding an Rlm1-binding protein. The *MLP1* gene product is highly homologous to Mpk1 and both protein kinases associate with the same region of Rlm1. This finding suggests that the Mpk1 and Mlp1 protein kinases have related functions. The following evidence suggests that Mlp1 affects the Mpk1 pathway through the regulation of Rlm1 function. First, overexpression of Mlp1 suppressed the caffeine-sensitive phenotype of *bck1*Δ mutants, while it failed to suppress its temperature-sensitive growth defect. This finding suggests that Mlp1 overproduction suppressed the *bck1*Δ mutation through Rlm1, because Rlm1 is involved in the caffeine sensitivity but not in the temperature-sensitive growth (35). Second, we found that *mpk1*Δ *mlp1*Δ double mutants were more sensitive to caffeine than the *mpk1*Δ mutant. Taken together, these observations suggest that rather than functioning in the same regulatory pathway, Mlp1 and Mpk1 perform independent but related functions in determining caffeine sensitivity through the regulation of Rlm1. Although overexpression of Mlp1 did not suppress the *mpk1*Δ mutant phenotype, this may be because the effect of Mlp1 overexpression is dependent on the basal activity of Mpk1.

Although the Mlp1 protein kinase is most similar to Mpk1, Mlp1 does not have the Thr-X-Tyr motif found in all MAPK family members but instead contains a Lys-Glu-Tyr sequence at the corresponding site. The Thr-X-Tyr motif is involved in phosphorylation and activation by dual-specificity MAPKKs, suggesting that Mlp1 may not be activated by MAPKKs. This suggests that Mlp1 may not be strictly categorized as a member of the MAPK family. However, identification of such a MAPK-like protein kinase raises some interesting possibilities. First, it would be interesting to test whether the Tyr residue in Mlp1 is phosphorylated and, if so, whether this phosphorylation regulates Mlp1 kinase activity. We showed that mutation of Tyr-192 in Mlp1 to Phe ($mlp1^{Y192F}$) eliminates its ability to suppress the *bck1*Δ mutation. This finding supports the possibility that Tyr-192 in Mlp1 is important for its function. Second, what is the upstream activator(s) of Mlp1? One possibility is that an upstream activator molecule stimulates Mlp1 to autophosphorylate on a Tyr residue, resulting in its activation. Another intriguing question is the connection between the Mlp1 and Mpk1 pathways. Overexpression or disruption of *MLP1* affected the caffeine sensitivity but not the temperature-sensitive growth resulting from mutations in the Mpk1 pathway. These results indicate that Mlp1 does not recognize all targets of the Mpk1 pathway, such as components involved in temperature-sensitive growth. Thus, Mlp1 might share some substrates with Mpk1, but it might have a quite distinct specificity.

ACKNOWLEDGMENTS

We thank S. Elledge, H. Iwasaki, D. Levin, T. Miyakawa, T. Nakamura, and Y. Ohya for materials; we thank C. Brenner, M. Lamphier, and R. Ruggieri for reading the manuscript and helpful discussions.

This work was supported by the Ministry of Education, Science and Culture of Japan (K.I. and K.M.).

The first two authors contributed equally to this work.

REFERENCES

1. **Blumer, K. J., and G. L. Johnson.** 1994. Diversity in function and regulation of MAP kinase pathways. Trends Biochem. Sci. **19:**236–240.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **41:**248–254.
3. **Chien, C.-T., P. L. Bartel, R. Sternglanz, and S. Fields.** 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA **88:**9578–9582.
4. **Christ, C., and B. K. Tye.** 1991. Functional domains of the yeast transcription/replication factor MCM1. Genes Dev. **5:**751–763.
5. **Fields, S., and O.-K. Song.** 1989. A novel gene system to detect protein-protein interactions. Nature (London) **340:**245–246.
6. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
7. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
8. **Hill, C. S., and R. Treisman.** 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell **80:**199–211.
9. **Hill, C. S., J. Wynne, and R. Treisman.** 1994. Serum-regulated transcription by serum response factor (SRF): a novel role for the DNA binding domain. EMBO J. **13:**5421–5432.
10. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
11. **Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki, K. Matsumoto, and Y. Oshima.** 1993. *MKK1* and *MKK2*, encoding *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. Mol. Cell. Biol. **13:**3076–3083.
12. **Janknecht, R., W. H. Ernst, V. Pingoud, and A. Nordheim.** 1993. Activation of TCF Elk-1 by MAP kinases. EMBO J. **12:**5097–5104.
13. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Kamada, Y., U. S. Jung, J. Piotrowski, and D. E. Levin.** 1995. The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. Genes Dev. **9:**1559–1571.
15. **Kortenjann, M., O. Thomae, and P. E. Shaw.** 1994. Inhibition of v-*raf*-dependent c-*fos* expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. Mol. Cell. Biol. **14:**4815–4824.
16. **Lee, K. S., and D. E. Levin.** 1992. Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. Mol. Cell. Biol. **12:**172–182.
17. **Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto, and D. E. Levin.** 1993. A yeast MAP kinase homolog (MPK1) mediates signalling by protein kinase C. Mol. Cell. Biol. **13:**3067–3075.
18. **Li, L., S. J. Elledge, C. A. Peterson, E. S. Bales, and R. J. Legerski.** 1994. Specific association between the human DNA repair proteins XPA and ERCC1. Proc. Natl. Acad. Sci. USA **91:**5012–5016.
19. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. Curr. Opin. Genet. Dev. **4:**82–89.
20. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.
21. **Millar, J. B. A., C. H. Mcgowan, G. Lenaers, R. Jones, and P. Russell.** 1991. $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. EMBO J. **10:**4301–4309.
22. **Mueller, C. G., and A. Nordheim.** 1991. A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. EMBO J. **10:**4219–4229.
23. **Nakamura, T., T. Ohmoto, D. Hirata, E. Tsuchiya, and T. Miyakawa.** 1996. Genetic evidence for the functional redundancy of the calcineurin- and Mpk1-mediated pathways in the regulation of cellular events important for growth in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **251:**211–219.
24. **Norman, C., M. Runswick, R. Pollock, and R. Treisman.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the *c-fos* serum response element. Cell **55:**989–1003.
25. **Ogawa, N., H. Saitoh, K. Miura, J. P. V. Magbanua, M. Bun-ya, S. Harashima, and Y. Oshima.** 1995. Structure and distribution of specific cis-elements for transcriptional regulation of *PHO84* in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **249:**406–416.

26. **Pollock, R., and R. Treisman.** 1991. Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev. **5:**2327–2341.
27. **Rothstein, R.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–211.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Takaesu, G., K. Irie, and K. Matsumoto.** Unpublished data.
31. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Semin. Cancer Biol. **1:**47–58.
32. **Treisman, R.** 1994. Ternary complex factors: growth factor regulated transcription activators. Curr. Opin. Genet. Dev. **4:**96–101.
33. **Vandenbol, M., P.-A. Bolle, C. Dion, D. Portetelle, and F. Hilger.** 1994. DNA sequencing of a 36.2 kb fragment located between the *FAS1* and *LAP1* loci of chromosome XI of *Saccharomyces cerevisiae*. Yeast **10:**S35–S40.
34. **Vojtec, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
35. **Watanabe, Y., K. Irie, and K. Matsumoto.** 1995. Yeast *RLM1* encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway. Mol. Cell. Biol. **15:** 5740–5749.
36. **Watanabe, Y., K. Irie, and K. Matsumoto.** Unpublished data.