A Yeast Mitogen-Activated Protein Kinase Homolog (Mpk1p) Mediates Signalling by Protein Kinase C

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Mitogen-activated protein (MAP) kinases are activated in response to a variety of stimuli through a protein kinase cascade that results in their phosphorylation on tyrosine and threonine residues. The molecular nature of this cascade is just beginning to emerge. Here we report the isolation of a *Saccharomyces cerevisiae* gene encoding a functional analog of mammalian MAP kinases, designated *MPK1* (for MAP kinase). The *MPK1* gene was isolated as a dosage-dependent suppressor of the cell lysis defect associated with deletion of the *BCK1* gene. The *BCK1* gene is also predicted to encode a protein kinase which has been proposed to function downstream of the protein kinase C isozyme encoded by *PKC1*. The *MPK1* gene possesses a 1.5-kb uninterrupted open reading frame predicted to encode a 53-kDa protein. The predicted Mpk1 protein (Mpk1p) shares 48 to 50% sequence identity with *Xenopus* MAP kinase and with the yeast mating pheromone response pathway components, Fus3p and Kss1p. Deletion of *MPK1* resulted in a temperature-dependent cell lysis defect that was virtually indistinguishable from that resulting from deletion of *BCK1*, suggesting that the protein kinases encoded by these genes function in a common pathway. Expression of *Xenopus* MAP kinase suppressed the defect associated with loss of *MPK1* but not the mating-related defects associated with loss of *FUS3* or *KSS1*, indicating functional conservation between the former two protein kinases. Mutation of the presumptive phosphorylated tyrosine and threonine residues of Mpk1p individually to phenylalanine and alanine, respectively, severely impaired Mpk1p function. Additional epistasis experiments, and the overall architectural similarity between the *PKC1*-mediated pathway and the pheromone response pathway, suggest that Pkc1p regulates a protein kinase cascade in which Bck1p activates a pair of protein kinases, designated Mkk1p and Mkk2p (for MAP kinase-kinase), which in turn activate Mpk1p.

Mitogen-activated protein kinases (MAP kinases) comprise a family of serine/threonine-specific protein kinases that are activated in various cell types in response to mitogenic stimuli such as insulin, epidermal growth factor, and phorbol esters (21, 42, 44). These enzymes are also stimulated in specialized cell types undergoing meiosis (18, 41), differentiation (4, 5, 16, 19, 35), or various stress responses (13, 51). The signalling pathways leading to stimulation of MAP kinases under different conditions are activated variously by tyrosine kinases, protein kinase C (PKC), or G proteins, but the mechanisms by which these pathways act remain largely obscure. MAP kinases are unique in requiring both tyrosine and threonine phosphorylation to become active (1), and a single protein kinase responsible for both phosphorylation events has been isolated from several species (10, 26, 36, 45, 48). The MAP kinase activator, or MAP kinase-kinase (MAPKK), is itself phosphorylated on threonine upon activation, suggesting that another protein kinase acts upstream of this enzyme in what appears to be a cascade of protein phosphorylation.

Several homologs of vertebrate MAP kinases exist in the budding yeast *Saccharomyces cerevisiae*. Vertebrate MAP kinases share 48 to 50% amino acid sequence identity with the yeast *FUS3* (12), *KSS1* (8), and *MPK1* (*SLT2*) (54) gene products. The *FUS3*- and *KSS1*-encoded protein kinases

mediate mating pheromone signal transduction through a pathway activated by a heterotrimeric G protein. Here we present evidence that *MPK1* participates in a protein kinase cascade mediated by *PKC1* (32), which encodes a homolog of the Ca²⁺-dependent subtypes of mammalian PKC.

MATERIALS AND METHODS

Strains, growth conditions, transformations, and nucleic acid manipulations. The yeast strains used in this study (Table 1) were derivatives of EG123, *MAT α leu2-3,112 ura3-52 trp1-1 his4 can1⁺* (49), except where indicated. Yeast cultures were grown in YEP (1% yeast extract, 2% Bacto Peptone) supplemented with either 2% glucose or 2% galactose as required. Synthetic minimal medium (47) supplemented with the appropriate nutrients was employed to select for plasmid maintenance and gene replacements. Yeast transformation was by the lithium acetate method (25). General genetic manipulation of yeast cells was carried out as described previously (47). Library screens were done by replicate plating transformants grown at 30°C on synthetic minimal medium supplemented with 1 M sorbitol to YEP-glucose at 37°C for 2 days. Plasmids were rescued from colonies arising at the restrictive temperature. Genomic yeast DNA and plasmids were isolated and prepared for restriction endonuclease digestion and hybridization as described previously (30). Nick translation, hybridization, and

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
EG123	<i>MATα leu2-3,112 ura3-52 trp1-1 his4 can1^r</i>	I. Herskowitz
1783	<i>MATα EG123</i>	I. Herskowitz
1788	<i>MATα/MATα; isogenic diploid of EG123</i>	I. Herskowitz
DL251	<i>MATα/MATα 1788 <i>bck1Δ::URA3/bck1Δ::URA3</i></i>	30
DL438	<i>MATα/MATα 1788 <i>bck1Δ::URA3/bck1Δ::URA3</i> (YEpl3[<i>MPK1</i>])</i>	This study
DL443	<i>MATα <i>lyt2 leu2-3,112 ura3-52 trp1-1</i></i>	This study
DL453	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/MPK1</i></i>	This study
DL454	<i>MATα EG123 <i>mpk1Δ::TRP1</i></i>	This study
DL455	<i>MATα EG123 <i>mpk1Δ::TRP1</i></i>	This study
DL456	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i></i>	This study
DL461	<i>MATα EG123 <i>MPK1::pUC18[URA3]</i></i>	This study
DL465	<i>MATα/MATα 1788 <i>bck1Δ::URA3/bck1Δ::URA3 mpk1Δ::TRP1/mpk1Δ::TRP1</i></i>	This study
DL604	<i>MATα/MATα 1788 <i>bck1Δ::URA3/bck1Δ::URA3</i> (YEpl3)</i>	This study
DL605	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (YEpl352)</i>	This study
DL606	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (YEpl352[<i>BCK1</i>])</i>	This study
DL607	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (pRS316[<i>BCK1</i>-20])</i>	This study
DL672	<i>MATα EG123 <i>MPK1::pUC18[URA3] SSD1::pUC18[TRP1]</i></i>	This study
DL694	<i>MATα/MATα 1788 <i>ssd1Δ::LEU2/SSD1</i></i>	This study
DL696	<i>MATα EG123 <i>ssd1Δ::LEU2</i></i>	This study
DL730	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1 ssd1Δ::LEU2/ssd1Δ::LEU2</i></i>	This study
DL750	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (YCp50-<i>LEU2</i>[<i>MPK1</i>])</i>	This study
DL769	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (YCp50-<i>LEU2</i>)</i>	This study
DL770	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (YCp50[<i>mpk1T190A</i>])</i>	This study
DL771	<i>MATα/MATα 1788 <i>mpk1Δ::TRP1/mpk1Δ::TRP1</i> (YCp50[<i>mpk1Y192F</i>])</i>	This study
L3-21	<i>MATα <i>lyt2 gal2</i></i>	E. Cabib
51.2.2	<i>MATα <i>cup1Δ::URA3 trp1-1 ura3-50 met13 gal1 can1</i> (Ade⁻ His⁻)</i>	V. Culotta
EY692	<i>MATα <i>fus3-6::LEU2 kss1 leu2-3,112 ura3-52 his3Δ200 ade2</i></i>	E. Elion
EEX167-8D	<i>MATα <i>fus3-1 kss1 leu2-3,112 ura3-52 his4-34 trp1Δ</i></i>	E. Elion
15D α	<i>MATα <i>leu2-3,112 ura3 his2 trp1Δ ade1</i></i>	S. I. Reed

DNA sequence analysis were also carried out as described previously (32).

Bacterial strains DH5 α (20), HB101 (6), and TG1 (46) were used for the propagation of all plasmids and phage. Phages M13mp18 and M13mp19 (37) were used to generate single-stranded template DNA for sequence determination. *Escherichia coli* cells were cultured in Luria broth or YT medium and transformed or infected with M13 by standard methods (33).

Gene replacements and integrative mapping. For construction of the *mpk1* deletion allele, a 2.3-kb *SalI*-*EcoRI* fragment bearing nearly the entire *MPK1* gene (54) was cloned into vector pBR322. After digestion of this plasmid with *HindIII* to eliminate 1.3 kb of the *MPK1* coding sequence, the ends were made flush with Klenow fragment and dephosphorylated with calf intestinal alkaline phosphatase. A *SmaI* fragment bearing the *TRP1* gene (from pUC18[*TRP1*]) was ligated into the *HindIII* site of the pBR322 construction. The resulting 2.1-kb *SalI*-*EcoRI* fragment, bearing the *TRP1* gene flanked by *MPK1* sequences (*mpk1 Δ ::TRP1*), was isolated and used to transform diploid strain 1788 to tryptophan prototrophy. For construction of the *ssd1/srk1* deletion allele, a 7-kb *BamHI*-*SalI* fragment bearing the entire *SSD1/SRK1* gene (55) was cloned into vector pUC18. After digestion with *XbaI* and *HpaI* to remove the 5'-most 2.3 kb of coding sequence, the ends were made flush and dephosphorylated as described above. An *HpaI* fragment bearing the *LEU2* gene (from YEpl3) was ligated into the blunt-end site of this pUC18 construction. The resulting 5.5-kb *BamHI*-*BglII* fragment, bearing the *LEU2* gene flanked by *SSD1/SRK1* sequences (*ssd1/srk1 Δ ::LEU2*), was used to transform strain 1788 to leucine prototrophy. Gene replacements were confirmed by restriction and hybridization analysis.

A 1-kb *EcoRI*-*SstI* fragment of *MPK1* was cloned into pUC18[*URA3*] (30) for the purpose of integrative mapping. This plasmid (pUC18[*MPK1::URA3*]) was used to transform strain EG123, yielding strain DL461. A 7-kb *BamHI*-*SalI* fragment bearing the *SSD1/SRK1* gene was cloned into pUC18[*TRP1*] for the same purpose. This plasmid (pUC18[*SSD1/SRK1::TRP1*]) was used to transform strain DL461, yielding strain DL672. Integration at the proper sites was confirmed genetically in crosses against the corresponding deletion mutants. Finally, strains DL461 and DL672 were used in crosses with DL443 (*lyt2*).

Expression of X-MAPK in *S. cerevisiae*. The open reading frame of *Xenopus* MAP kinase (X-MAPK) (17) was amplified from a cDNA by polymerase chain reaction with the 5' primer 5'-CCGGATCCCCATGGCAGCGGCAGGAGCTGCGTCT-3' and the 3' primer 5'-CGGGATCCGTCAGTACCCTGGCTGGAATCTAGCG-3'. These primers introduced *BamHI* sites just before the start codon and after the stop codon. The amplified X-MAPK fragment was cloned into the *BamHI* site of pBluescriptII KS(-) (Stratagene). The recombinant X-MAPK expressed from this construction had the capacity to autophosphorylate on tyrosine and to be activated by purified X-MAPK activator (unpublished data). The wild type and the K57D mutant of X-MAPK were cloned as *BamHI* fragments into yeast expression vector pAA7 (provided by T. Fukasawa, Keio University), containing the galactose-inducible *GAL7* promoter, and into pKT10, containing the constitutive *TDH3* promoter.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis of X-MAPK was done as described previously (27) with the mutagenic primer 5'-CGAGTTGCTATCGATAAAATCAGCCC-3' to yield MAPK57D. This mutagenesis was confirmed by the presence of a newly introduced *ClaI* site.

FIG. 1. Comparison of X-MAPK and yeast Mpk1p sequences. Roman numerals denote subdomains conserved in protein kinases. Dashes indicate gaps introduced to optimally align the sequences. The phosphorylated threonine and tyrosine residues of MAP kinases are marked with asterisks. The DNA sequences of *MPK1* and X-MAPK can be found in references 54 and 17, respectively.

The *MPK1* gene (carried entirely within a 2.7-kb *SphI-EcoRI* fragment) was subjected to mutagenesis with the mixed primer 5'-CAGTCAATTTTGA/GCGGAGTA/TCGTGGC CACTAG-3' to yield *mpk1IT190A* and *mpk1Y192F*. These mutational events were confirmed by DNA sequence analysis, and the *mpk1* mutants were cloned as *SphI-EcoRI* fragments into the yeast shuttle vector YCp50-*LEU2* for expression.

Chromosomal mapping of *MPK1*. *MPK1* was assigned to a position on chromosome VIII linked to *CUP1* by hybridization of a nick-translated probe, derived from *MPK1* (the 2.7-kb *SphI-EcoRI* fragment), to a set of λ clone grid filters (provided by Maynard Olson, Washington University). Meiotic mapping with a *cup1*-tagged strain confirmed this position. Strain 51.2.2 (*cup1 Δ ::URA3*; provided by V. Culotta, Johns Hopkins University) was crossed with DL454 (bearing *mpk1 Δ ::TRP1*).

RESULTS

Loss of *PKC1* function results in a cell lysis defect (31, 38) that is due to a deficiency in cell wall construction (unpublished observations) and that is suppressed by osmotic stabilizing agents. Mutational activation of a gene designated *BCK1* (for bypass of C kinase) alleviates the requirement for *PKC1* (30). The *BCK1* gene is predicted to encode a protein kinase that is most closely related to the *STE11*-encoded protein kinase, which functions within the mating pheromone response pathway (43). Deletion of *BCK1* results in a temperature-dependent cell lysis defect that is suppressed by osmotic stabilizers, supporting a model in which *BCK1* functions downstream of *PKC1* (30).

Isolation of dosage-dependent suppressors of a *bck1* deletion mutant. To identify additional components of the *PKC1*-mediated pathway that act downstream of *BCK1*, we isolated dosage-dependent suppressors of a *bck1* deletion mutant. A genomic yeast library, cloned in the multicopy shuttle vector YEpl3 (containing *S. cerevisiae LEU2*; provided by C. Guthrie, University of California), was used to transform a strain bearing the *bck1 Δ ::URA3* mutation

(DL251 [30]) to leucine prototrophy; transformation was followed by a screen for growth at the restrictive temperature. A total of 9 transformants, from among 5,000 screened (approximately five genomic equivalents), were capable of growth at 37°C. The plasmids recovered from these yeast transformants were of two classes. Six contained the *BCK1* gene, and three contained overlapping sequences corresponding to a locus distinct from *BCK1*. Deletion analysis of this locus revealed a 2.3-kb *SalI-EcoRI* fragment that was essential for suppression of *bck1 Δ ::URA3* (data not shown). DNA sequence analysis of this fragment revealed that it is identical to a previously described gene, designated *SLT2* (for suppressor of *lyt2* [54]). The *SLT2* gene was isolated through its ability to suppress a temperature-dependent cell lysis defect, designated *lyt2*, when maintained on either high- or low-copy-number plasmids. We obtained the *lyt2* mutant (strain L3-21; provided by E. Cabib, National Institutes of Health) and found that the *lyt2*-associated defect is the result of two unlinked mutations and that *SLT2* is allelic to one of these (see below). For this reason, and for the results described below, we have redesignated the *SLT2* gene *MPK1* (for MAP kinase). The *MPK1* gene encodes a protein kinase that is most closely related to vertebrate MAP kinases (48% identical to X-MAPK [Fig. 1]) and the yeast *FUS3*- and *KSS1*-encoded protein kinases, which function within the mating pheromone response pathway (34).

Deletion of *MPK1*. To examine the phenotypic effect of loss of *MPK1* function, a deletion mutant of *MPK1* was constructed in vitro. A 1.3-kb fragment of *MPK1*, which includes nearly all of the coding sequence, was replaced with the *S. cerevisiae TRP1* gene (see Materials and Methods). This deletion allele (*mpk1 Δ ::TRP1*) was transplanted into a diploid strain (1788) with multiple auxotrophic markers by selection for tryptophan prototrophy. Transformants were tested for possession of the *mpk1 Δ ::TRP1* allele by restriction and hybridization analysis (Fig. 2A). Two independently derived diploids, heterozygous at *MPK1* (*mpk1 Δ ::TRP1*/*MPK1*), were induced to sporulate, and tetrads were dissected. The phenotypic defect associated with the *mpk1 Δ ::TRP1* mutation (in strain DL456) was similar to that of the

FIG. 2. Deletion of the *MPK1* gene and suppression of *bck1Δ* by *MPK1*. (A) Sequences encoding the predicted catalytic domain of *MPK1* were replaced with the yeast *TRP1* gene. Genomic DNA was isolated from (left to right) an *MPK1*⁺ strain (1788), a diploid strain heterozygous for the *mpk1Δ* mutation (*mpk1Δ::TRP1/MPK1*⁺; DL453), and the haploid segregants of a representative tetrad from DL453 (top; grown at 30°C). DNA (5 μg) was digested with *Xba*I and prepared for hybridization with a nick-translated 1.7-kb *Xba*I fragment from *MPK1*. The smaller hybridizing species is derived from the deleted allele. (B) Diploid yeast strains were streaked onto either YEP-glucose medium or YEP-glucose supplemented with 1 M sorbitol and incubated for 3 days at the indicated temperature. Strains are (clockwise from top) wild type (1788), *bck1Δ* (DL251), *mpk1Δ* (DL456), and a *bck1Δ mpk1Δ* double mutant (DL465). Diploid strains were used to prevent the accumulation of recessive extragenic suppressor mutations. (C) Diploid strains were streaked onto YEP-glucose medium and incubated for 3 days at the indicated temperature. Strains are (clockwise from top) wild type (1788), *bck1Δ* bearing the multicopy plasmid YEp13 (DL604), *bck1Δ* bearing YEp13[*MPK1*] (DL438), *mpk1Δ* bearing the multicopy plasmid YEp352 (DL605), *mpk1Δ* bearing YEp352[*BCK1*] (DL606), and *mpk1Δ* bearing the low-copy-number plasmid pRS316[*BCK1*-20] (DL607).

bck1Δ mutation (in strain DL251). Loss of *MPK1* resulted in slow growth at 30°C (Fig. 2A) and cell lysis at 37°C, as judged by failure to grow at the elevated temperature (Fig. 2B) and by the appearance of a high frequency (approximately 70%) of nonrefractile ghosts (data not shown). This defect was suppressed by incorporation of sorbitol to 1 M into the medium for osmotic support. The *mpk1Δ::TRP1*-associated lysis defect was slightly less severe than that of the *bck1Δ::URA3* mutation as judged by growth rates at reduced temperatures (data not shown). Deletion of both *BCK1* and *MPK1* resulted in a defect that was similar to that observed for deletion of either gene alone. The nonadditivity of the *bck1Δ* and *mpk1Δ* defects suggests that these genes function within the same pathway. Overexpression of *MPK1* from a multicopy plasmid nearly completely suppressed the *bck1Δ* defect (Fig. 2C; doubling time at 37°C of 135 min for DL438 versus 100 min for wild-type strain 1788). In contrast, expression of an activated allele of *BCK1* (*BCK1*-20), or maintenance of wild-type *BCK1* in multiple copies, failed to suppress the *mpk1Δ* defect. These results indicate that *MPK1* functions within the *PKC1*-mediated pathway at a point downstream of *BCK1*.

Mutagenesis of *MPK1*. Activation of vertebrate MAP kinases requires phosphorylation of a tyrosine and a threonine residue. Both of the phosphorylated residues in activated MAP kinases are conserved in the Mpk1 protein (Mpk1p) (Fig. 1). We created mutant alleles of *MPK1* that were

altered at its putative phosphate acceptor sites. Mutation of threonine 190 to alanine (T190A) resulted in an allele of *MPK1* that was incapable of complementing the *mpk1Δ::TRP1* mutation when expressed from a low-copy-number vector (Fig. 3). Mutation of tyrosine 192 to phenylalanine (Y192F) resulted in a partially functional allele which poorly complemented the *mpk1Δ::TRP1* mutation. When expressed from a multicopy vector, *mpk1Y192F* (but not *mpk1T190A*) complemented the *mpk1Δ::TRP1* mutation well (data not shown).

X-MAPK complements the *mpk1Δ* defect. To determine whether the structural similarity between yeast Mpk1p and vertebrate MAP kinases is indicative of functional analogy, we asked whether X-MAPK could complement the *mpk1Δ* mutant. We designed a high-copy-number plasmid (pAA7-MAP) in which a cDNA encoding X-MAPK (see Materials and Methods) is expressed under the control of the galactose-inducible yeast *GAL7* promoter. Additionally, a mutant X-MAPK derivative with a lysine-to-aspartic-acid substitution at amino acid position 57 (K57D) was constructed (pAA7-MAPK57D). This mutation, residing within the putative ATP-binding site, inactivates the protein kinase (unpublished data). The ability of X-MAPK to complement the growth defect associated with the *mpk1Δ::TRP1* allele (in strain DL456) was tested on galactose-containing medium at the restrictive temperature. Control DL456 cells transformed with pAA7 were defective for growth at 37°C, but

FIG. 3. Mutation of potential phosphorylation sites of Mpk1p. Strain DL456 (*mpk1Δ::TRP1*) was transformed with low-copy-number (centromere-bearing) plasmids with wild-type *MPK1*, *mpk1T190A*, or *mpk1Y192F*. Representative transformants were streaked onto YEP-glucose medium and incubated at 37°C for 3 days. Plasmids are (clockwise from top) YCp50[*mpk1Y192F*] (in DL771), YCp50[*mpk1T190A*] (in DL770), YCp50-*LEU2* (in DL769), and YCp50-*LEU2*[*MPK1*] (in DL750).

DL456 cells transformed with pAA7-MAP were able to grow at this temperature, although at a slower rate than DL456 cells carrying an *MPK1*-bearing plasmid (Fig. 4A). Moreover, DL456 transformed with pAA7-MAPK57D failed to grow at 37°C, suggesting that the protein kinase activity of

X-MAPK is required for complementation of the *mpk1Δ* defect.

We next tested the ability of X-MAPK to replace the mating pheromone response functions of *FUS3* and *KSS1*. The *FUS3* gene positively regulates the pheromone response pathway in parallel with *KSS1* by activation of the *STE12*-encoded transcription factor (12). Strains defective in both *FUS3* and *KSS1* do not respond to mating pheromone and are sterile. The ability of a *MATa fus3Δ kss1* mutant strain (EY692) expressing X-MAPK to mate with an appropriate *MATa* partner was tested. The mating defect of EY692 cells was not corrected by expression of X-MAPK (data not shown), indicating that X-MAPK is unable to replace the functions of either *FUS3* or *KSS1*.

In addition to their roles in the regulation of mating-specific functions, *FUS3* and *KSS1* provide distinct functions in pheromone-induced control of the cell cycle. The *FUS3* gene promotes arrest of the cell cycle in G_1 by inactivating G_1 cyclins required for activity of the Cdc28 protein kinase (12). The *KSS1* gene promotes adaptation to pheromone, resulting in reentry of the cell cycle after G_1 arrest (8). Mutations in the *SST2* gene, which render strains defective in adaptation to pheromone, are suppressed by overexpression of the *KSS1* gene. In contrast, expression of X-MAPK failed to suppress an *sst2* mutation (data not shown). The *fus3-1* allele is defective only in the G_1 arrest-promoting function of *FUS3*. We examined the ability of X-MAPK to complement the G_1 arrest deficiency of *fus3-1* in a *MATa fus3-1 kss1* mutant strain (EEX167-8D). Upon exposure to mating pheromone, EEX167-8D cells expressing X-MAPK still failed to arrest in G_1 . In fact, EEX167-8D cells expressing X-MAPK from the yeast *TDH3* promoter were

FIG. 4. Effect of X-MAPK expression on mutant yeast cells. (A) Independent isolates of strain DL456 (*mpk1Δ::TRP1*), transformed with the indicated plasmids, were streaked onto agar plates containing (from left to right) YEP-glucose supplemented with 1 M sorbitol, YEP-glucose, or YEP-galactose and incubated at 37°C for 3 days. (B) Strain EEX167-8D (*MATa fus3-1 kss1*), transformed with the indicated plasmids, was plated on synthetic glucose medium lacking uracil. Sterile filter disks were placed on the nascent lawn, and synthesized α -factor was pipetted onto the disks (left, 0.5 μ g; right, 5 μ g). Plates were incubated at 30°C for 3 days.

even more resistant to pheromone-induced arrest than those carrying the control plasmid (Fig. 4B). One explanation for this result is that X-MAPK may interact nonproductively with a component (a substrate or activator) of the pheromone response pathway and thus interfere with this process when the activity of Fus3p-Kss1p is weak. This interpretation is supported by the observation that expression of the inactive K57D allele of X-MAPK also conferred resistance to pheromone-induced arrest of EEX167-8D cells.

SSD1/SRK1 contributes to the cell lysis defect associated with loss of MPK1. To determine the nature of the previously described *lyt2* defect (54), we first backcrossed the *lyt2* mutant (strain L3-21) against a wild-type strain (1783). Although the *lyt2* defect was initially reported to result from a single mutation, we found that the temperature-dependent cell lysis defect required the cosegregation of two unlinked mutations because only approximately 25% of the segregants from the backcross displayed the *lyt2* defect. Because the *SLT2* (*MPK1*) gene was reported to suppress the *lyt2* defect when maintained in a low-copy-number plasmid, we explored the possibility that *MPK1* was allelic to one of the mutations responsible for this defect. We created a plasmid bearing the *MPK1* gene linked to the *S. cerevisiae* *URA3* gene (see Materials and Methods) and selected for integration of this plasmid (pUC18[*URA3::MPK1*]) at the genomic *MPK1* locus of wild-type strain EG123. An integrant (strain DL461) was crossed to a *lyt2* mutant (strain DL443), and segregation analysis was conducted on the resulting diploid strain. A total of 17 lysis mutants arose from 15 tetrads analyzed (approximately 25% of segregants). None of these mutants possessed the *URA3* marker, indicating tight linkage of *MPK1* to one of the determinants of the *lyt2* defect. The *mpk1* allele associated with the *lyt2* defect must be only partially defective, because when separated from the second determinant it does not result in the temperature-dependent growth defect observed for *mpk1Δ::TRP1* strains.

To isolate the other determinant of the *lyt2* defect, we screened a low-copy-number genomic yeast library (in YCp50; provided by Mark Rose) for complementation of *lyt2*. This library was used to transform an *lyt2* mutant (strain DL443) to uracil prototrophy; transformation was followed by a screen for growth at the restrictive temperature. A total of 21 transformants, from among 10,000 screened (approximately 10 genomic equivalents), were capable of growth at 37°C. Of the plasmids recovered from 16 transformants, 8 could suppress *lyt2* upon secondary transformation. Restriction analysis revealed that five of these were isolates of *MPK1*, and three contained overlapping sequences corresponding to another locus (data not shown). Deletion analysis of this locus revealed a 1.3-kb *XbaI-EcoRI* fragment that was essential for suppression of *lyt2*. DNA sequence analysis of this fragment revealed that it is identical to the previously described *SSD1/SRK1* gene (52, 55). To determine whether this locus is allelic with the second *lyt2* determinant, we created a plasmid bearing *SSD1/SRK1* linked to the *TRP1* gene and selected for integration of this plasmid (pUC18[*TRP1::SSD1/SRK1*]) at the genomic *SSD1/SRK1* locus in strain DL461 (bearing pUC18[*MPK1::URA3*]). The doubly tagged strain (DL672) was crossed to the *lyt2* mutant (strain DL443), and segregation analysis was conducted on the resulting diploid strain. A total of 14 lysis mutants arose from 15 tetrads analyzed. None of these mutants possessed either of the selectable markers, indicating that mutations in both *MPK1* and *SSD1/SRK1* are responsible for the *lyt2* defect.

To determine the phenotypic effect of loss of both *MPK1*

and *SSD1/SRK1*, we constructed a deletion mutant of *SSD1/SRK1*. The 5'-most 2.3 kb of coding sequence of this gene was replaced by the *LEU2* gene (see Materials and Methods) and transplanted into wild-type strain 1788. Possession of the deletion allele (*ssd1/srk1Δ::LEU2*) was confirmed by restriction and hybridization analysis (data not shown). A haploid segregant bearing this mutation (DL696), which grew normally over a range of temperatures, was crossed to an *mpk1Δ::TRP1* mutant. Double mutants grew poorly at 23°C, and growth was negligible at 30°C (data not shown), a defect more severe than that resulting from either mutation alone. This defect was fully suppressed by incorporation of osmotic stabilizing agents into the medium.

Mapping the chromosomal location of MPK1. To determine the chromosomal map position of *MPK1*, a probe derived from the *MPK1* coding sequence was hybridized to a set of λ clone grid filters (provided by M. Olson, Washington University). The *MPK1* probe hybridized with clone no. 6665 and 7082, which correspond to an overlapping region of chromosome VIII, approximately 65 to 85 kb centromere proximal to *CUP1* (37a). The *MPK1* gene was found to be genetically linked to *CUP1*, confirming this map position. The segregation pattern of 24 parental:2 nonparental:27 tetratype yields a map distance of 37 centimorgans between *MPK1* and *CUP1*, as calculated by the method of Perkins (40). These results are in contrast to the previous assignment of *SLT2* to chromosome V by chromosome hybridization (54). The most likely explanation for this discrepancy is that chromosomes V and VIII generally migrate closely to each other on chromosome separation gels, facilitating misassignment by this technique.

DISCUSSION

Isolation of the MPK1 gene. The yeast *BCK1* gene encodes a protein kinase that has been proposed to act on one branch of a bifurcated pathway mediated by the PKC isozyme encoded by *PKC1* (30). The *BCK1*-encoded protein is most closely related to the yeast *STE11*-encoded protein kinase, which mediates a step in the mating pheromone response pathway. A yeast strain bearing a deletion in the *BCK1* gene displays a temperature-dependent cell lysis defect. This strain was used to isolate dosage-dependent suppressors of the *bck1* deletion defect. The only suppressor locus isolated encodes a protein kinase that shares 48 to 50% amino acid sequence identity with vertebrate MAP kinases and was therefore designated *MPK1* (for MAP kinase). The *MPK1*-encoded protein is also closely related to the protein kinases encoded by the *FUS3* and *KSS1* genes (54), MAP kinase homologs which function within the pheromone response pathway. Deletion of *MPK1* resulted in a cell lysis defect that was virtually indistinguishable from that associated with deletion of *BCK1*. Neither expression of an activated allele of *BCK1* nor overexpression of wild-type *BCK1* suppressed the defect associated with loss of *MPK1* function. These results support a model in which *MPK1* functions downstream of *BCK1*.

Mutational activation of another gene, designated *MKK1* (for MAPKK), suppresses both the *pkc1* deletion defect and the *bck1* deletion defect (unpublished data). The *MKK1* gene overlaps functionally with a closely related gene, designated *MKK2*—deletion of both genes results in a temperature-dependent cell lysis defect similar to that associated with deletion of either *BCK1* or *MPK1* (24). The *MKK1/2* genes are predicted to encode protein kinases that are most closely related to the *STE7*-encoded protein kinase (53) which, like

STE11, *FUS3*, and *KSS1*, functions within the pheromone response pathway. An activated allele of *MKK1* fails to suppress the *mpk1Δ* defect, but overexpression of *MPK1* suppresses the cell lysis defect associated with deletion of both *MKK1* and *MKK2* (unpublished data). These results, taken in the aggregate, indicate that the *MKK1/2* genes function between *BCK1* and *MPK1*.

The cell lysis defect associated with deletion of *MPK1* was suppressed by expression of X-MAPK, suggesting functional conservation between the yeast and vertebrate enzymes. Additionally, both of the residues that are phosphorylated in activated MAP kinases (39) are conserved in yeast Mpk1p, raising the possibility that the activity of this yeast MAP kinase homolog may be regulated in a way that is analogous to that of vertebrate MAP kinases. Mutants of *MPK1*, altered at the predicted phosphate acceptor residues, displayed severely diminished capacity to complement an *mpk1* deletion mutant, indicating the importance of these residues in Mpk1p function.

MAP kinase activation by protein kinase cascades. Vertebrate MAP kinase activators have been purified and found in each case to be a single protein kinase responsible for both tyrosine and threonine phosphorylation (10, 26, 36, 45, 48). In addition, MAPKK is itself phosphorylated on threonine upon activation, suggesting that another protein kinase acts upstream of this enzyme.

Our findings, together with recently established relationships among the components of the pheromone response pathway, suggest the nature of the protein kinases that act upstream of MAP kinases. First, the protein kinases functioning within the pheromone response pathway have been shown to act in a cascade, such that Ste11p phosphorylates Ste7p, which in turn phosphorylates Fus3p and Kss1p (14, 15, 50, 57). This cascade is presumed to be a mechanism for rapid signal amplification. Second, the protein kinases that mediate the *PKC1*-activated pathway are closely related to those operating within the pheromone response pathway—Bck1p shares 45% identity with Ste11p (through their catalytic domains [30]), Mkk1p and Mkk2p share 39% identity with Ste7p (24), and Mpk1p shares 46% identity with Fus3p and Kss1p (54), their closest known respective relatives. Additionally, Leberer et al. have isolated a novel yeast gene that functions within the pheromone response pathway (designated *STE20*), at a point upstream of *STE11* but downstream of the G protein (29). The *STE20* gene encodes a protein kinase that shows some similarity within its catalytic domain to isozymes of PKC (33% identical to rat PKCε). Third, epistatic interactions among *BCK1*, *MKK1/MKK2*, and *MPK1* indicate an order of function analogous to that observed for their respective homologs in the pheromone response pathway. The architectural conservation between the *PKC1*-mediated pathway and the pheromone response pathway suggests that the Bck1p, Mkk1/2p, and Mpk1p kinases also act in a cascade (Fig. 5). Fourth, the sequences of MAPKKs isolated from *Xenopus* (26), mouse (9), and rat (56) cells reveal a high degree of similarity to the yeast Mkk1/2p and Ste7p kinases. These observations suggest that Mkk1/2p and Ste7p are yeast analogs of vertebrate MAPKKs, and that Bck1p and Ste11p are activators of these enzymes (MAPKK kinases). They further indicate the existence of functionally distinct but architecturally related pathways in *S. cerevisiae* that have apparently evolved as modules. Although we have found that components of one module can be coerced to substitute for the analogous components in the other module (e.g., *STE11* overexpression suppresses loss of *BCK1* [unpublished data]), wild-type

FIG. 5. Architectural similarity between the *PKC1*-mediated pathway and the pheromone response pathway. Shaded boxes denote structurally conserved protein kinases. The *MKK1* gene (24) was isolated independently as a dosage-dependent suppressor of an *smg3* mutation (22) and a *pkc1^{ts}* mutation (unpublished data). The *MKK2* gene was isolated by molecular hybridization techniques using probes derived from *MKK1* (24). The *BCK1* (*SSP31*) gene was isolated independently as a dominant extragenic suppressor of *pkc1Δ* (30) and as a dosage-dependent suppressor of *smg3* (23). *STE5* functions downstream of the G protein and upstream of *STE11* (3), as does *STE20*, which encodes a protein kinase that is most closely related to isozymes of PKC, but only through its catalytic domain (33% identical to rat PKCε [29]). Other details are given in the text.

components expressed at normal levels do not appear to function in the heterologous pathway.

The protein kinases encoded by the *c-raf-1* proto-oncogene and the *v-raf* oncogene are capable of activating purified MAPKK in vitro (11, 28). Because the *raf*-encoded protein kinases are not closely related to either Bck1p or Ste11p, the existence of multiple classes of MAPKK activators is suggested, perhaps integrating signals from different pathways. Signal integration at some level in vertebrates is also suggested by the wide array of extracellular signals capable of activating vertebrate MAP kinases. Alternatively,

a mammalian Bck1p-Stellp homolog may copurify with MAPKK, implicating *raf* as an activator of the former.

***SSD1/SRK1* contributes to the cell lysis defect associated with loss of *PKC1*-mediated pathway components.** We found that the temperature-sensitive *lyt2* mutant initially described by Torres et al. (54) possesses two unlinked mutations. One is a partial defect in the *MPK1* gene, and the other is a recessive defect in the *SSD1/SRK1* gene. Deletion of both genes resulted in a severe growth defect even at reduced temperatures. The *SSD1/SRK1* gene is predicted to encode a protein related to the Dis3 protein of *Schizosaccharomyces pombe* (52, 55), which has been implicated in protein phosphatase function. The former has been identified previously as a suppressor of defects associated with activation of the *RAS*/cyclic AMP pathway (55), defects in the G_1 -S transition (52), and loss of *BCK1* function (7). Moreover, several components of the *PKC1*-mediated pathway have been isolated recently through a screen for mutations that are synthetically lethal (at 30°C) with a deletion of *SSD1/SRK1* (2). The *SSD1/SRK1* gene may function on a separate branch of the *PKC1* pathway from that of the protein kinases (Bck1p, Mkk1/2p, and Mpk1p). However, the relationships between *SSD1/SRK1* and pathways other than that mediated by Pkc1p suggest a more general role for this gene.

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