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Yeast MEK-Dependent Signal Transduction: Response Thresholds and Parameters Affecting Fidelity

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Ste7p and Mkk1p are MEK (MAPK/ERK kinase) family members that function in the mating and cell integrity signal transduction pathways in *Saccharomyces cerevisiae*. We selected *STE7* and *MKK1* mutations that stimulated their respective pathways in the absence of an inductive signal. Strikingly, serine-to-proline substitutions at analogous positions in Ste7p (position 368) and Mkk1p (position 386) were recovered by independent genetic screens. Such an outcome suggests that this substitution in other MEKs would exhibit similar properties. The Ste7p-P^{368} variant has higher basal enzymatic activity than Ste7p but still requires induction to reach full activation. The higher activity associated with Ste7p-P^{368} allows it to compensate for defects in the cell integrity pathway, but it does so only when it is overproduced or when Ste5p is missing. This behavior suggests that Ste5p, which has been proposed to be a tether for the kinases in the mating pathway, contributes to Ste7p specificity.

Extracellular molecules that regulate cell proliferation and differentiation in eukaryotes depend on pathways that detect signals at the cell surface and transmit them through the cytoplasm to nuclear and other intracellular targets. One mechanism for transmitting these signals involves a phosphorylation cascade that is conserved in organisms as diverse as *Saccharomyces cerevisiae* and humans. The three sequentially acting enzymes of the conserved cascade are called MEK kinases (MEKKs), MAPK/ERK kinases (MEKs), and mitogen-activated protein kinases (MAPKs).

Presently, there is evidence for five separate but structurally related MAPK activation pathways that mediate distinct responses to different extracellular or cell autonomous signals in the budding yeast *S. cerevisiae* (for a review, see reference 33). Two of these pathways figure prominently in the work described here (Fig. 1). One controls the mating response, in which pheromones inform cells of the proximity of a mating partner and induce differentiation into a mating-competent state. The second regulates cell wall construction, a process that is especially important at regions of polarized growth during cell proliferation or mating differentiation (11, 32, 40).

Activation of the mating response pathway occurs when the peptide pheromone secreted by one cell type binds to the specific integral membrane receptor on the opposite cell type (Fig. 1). This cell surface interaction stimulates the dissociation of a heterotrimeric G protein into its $G_{\beta\gamma}$ and G_{α} subunits. Free $G_{\beta\gamma}$ stimulates a phosphorylation cascade composed of protein kinases that function in the order Ste20p, Ste11p, Ste7p, and Fus3p/Kss1p (for a review, see reference 13). The mechanisms underlying activation of Ste20p and Ste11p are still not known. However, Ste7p is directly phosphorylated and activated by Ste11p (40). The activation of Ste7p appears to involve two residues (Ser-359 and Thr-363) which are located in a region analogous to the phosphorylation lip of MAPK (17, 40, 63). Ste7p activates Fus3p and Kss1p by phosphorylating them at the Thr and Tyr residues of the signature TEY motif within the MAPK phosphorylation lip (12, 16). Fus3p and Kss1p are redundant for pheromone-induced signal transmission leading to transcriptional activation of genes whose products are required for the process of mating (9). Fus3p has an additional role in promoting arrest of the cell cycle at the G_1 phase (43, 57).

Ste5p is also essential for mating competence and transmission of the pheromone-induced signal, but until recently, its molecular function has been enigmatic (21, 34). Deductions from genetic epistasis analyses place its first execution point after the G protein and before or at the level of Ste11p (for a review, see reference 13). However, results from two-hybrid analyses showed that Ste5p interacts with Ste11p, Ste7p, Kss1p, and Fus3p. The multiple interactions are consistent with Ste5p having a role in several steps along the MAPK activation pathway (3, 44, 36). Additional biochemical evidence showed that Ste5p and these four kinases are present in a macromolecular assembly (3). These findings suggest a model in which Ste5p is a scaffold that promotes interactions between the kinases in the mating response pathway.

The phosphorylation cascade that is part of the cell integrity pathway includes Pkc1p (the yeast protein kinase C homolog), Bck1p (a MEKK), the redundant Mkk1p and Mkk2p enzymes (MEKs), and Mpk1p (a MAPK) (Fig. 1). Genetic approaches identified these components and established their order of function (13). The finding that Pkc1p phosphorylates Bck1p in vitro provides biochemical corroboration for the relationship between these two components (58).

Only more recently has there been evidence for multiple MAPK activation pathways in vertebrates (8). One pathway mediates responses to mitogenic agents and uses Raf-1, Mek1, and Mek2 and the p42 and p44 MAPK isozymes (37). A more recently identified pathway mediates stress responses to agents

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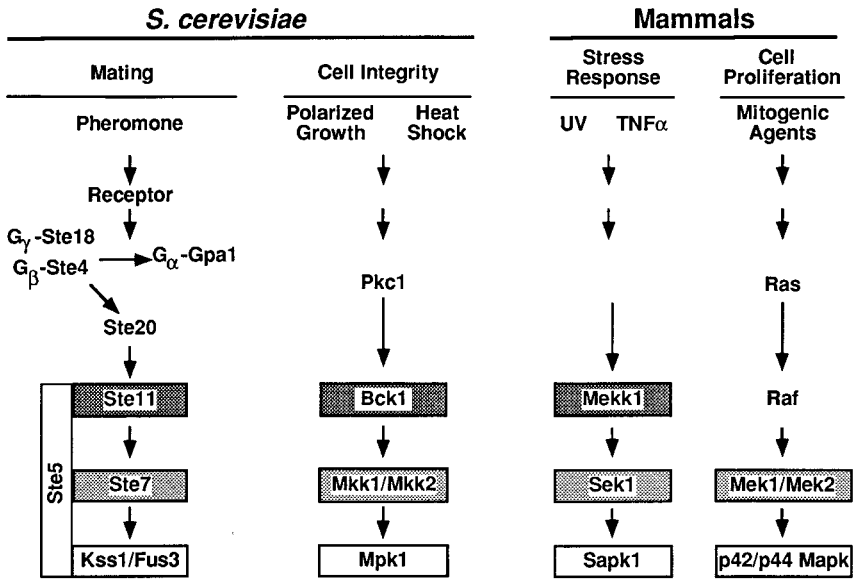

FIG. 1. MAPK-dependent signal transduction pathways in *S. cerevisiae* and in mammals. Evidence supporting the depicted organization of the cell integrity, pheromone-dependent mating, and mammalian MAPK-dependent pathways has been reviewed or published recently (13, 37, 39, 47, 60). Homologous kinases are indicated within boxes at equivalent tiers in the pathways. TNFα, tumor necrosis factor alpha.

such as UV and tumor necrosis factor alpha. This pathway uses Mekk1, a mammalian MEKK family member; a MEK family member called Sek1 or JNKK1; and a MAPK family member called Sapk1 or JNK1 (39, 47, 60). Raf-1 and Mekk1 activate Mek1 and Sek1, respectively, by phosphorylating two serine residues that are analogous to the Ser-359 and Thr-363 residues implicated in the activation of Ste7p (Fig. 2) (1, 60, 63).

Alanine substitutions at Ser-218 and Ser-222 of mammalian MEK were generated by site-directed mutagenesis and confirmed the essential role of these regulatory sites in determining the catalytic activity of MEK (1, 6, 35, 41, 49, 63). Additionally, glutamate or aspartate substitutions that can mimic phosphorylated residues were made at these positions in Mek1. The resulting variants have constitutive catalytic activity and promoted proliferation and differentiation responses associated with MEK-dependent signal pathways (6, 35, 41, 49).

Such a mutagenic approach opens a promising avenue for dissecting the physiological responses of mammalian cells associated with MEK isoforms. This challenge will become more daunting given the likely prospect that there will be many mammalian MEK isoforms and that these will be involved in a plethora of cellular processes. To know what constraints may apply to this approach, we still need a better understanding of the signal thresholds that trigger different pathway responses and of the parameters that determine the fidelity of signal transmission.

Toward this end, we sought to identify and characterize mutations in the yeast Ste7p and Mkk1p MEK family members that stimulate biological responses in the absence of inductive signal. We uncovered such mutations and took advantage of them to define the levels of MEK activity needed for different biological responses. These analyses led us to unanticipated but important insights into parameters that limit a given MEK's ability to cross-function in other MAPK activation pathways.

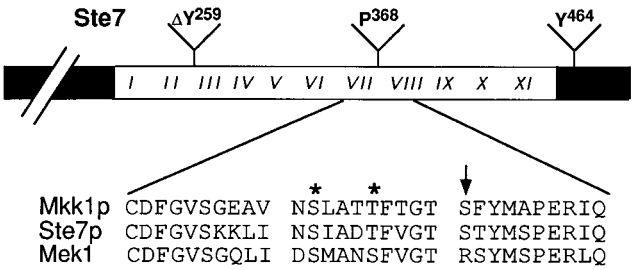

FIG. 2. Gain-of-function variants of Ste7p and Mkk1p. The diagram represents the nonconserved regions (solid boxes) and the catalytic domain (open box) of a generalized MEK. The roman numerals refer to the subdomains described by Hanks and Quinn (20). The substituted residues that generated gain-of-function variants of Ste7p are indicated above the diagram; the amino acid residues in subdomains VII and VIII of Mkk1p, Ste7p, and Mek1 are aligned below (7, 24, 56). The positions of sites in Sek1 or Mek1 that are phosphorylated by Mekk1 or Raf are indicated by asterisks (*), and the positions of the substituted serine residues in Ste7p-P^{368} and Mkk1p-P^{386} are indicated by the arrow (40, 41, 63).

MATERIALS AND METHODS

Plasmids. Two reporter gene constructs for targeted integration in *S. cerevisiae* were made for the purpose of monitoring activity of the mating response pathway. Plasmid pDH17 is a pRS306 (integrating *URA3*)-based vector that contains the *mfa2*::*FUS1-lacZ* allele (51). This allele includes the 6.7-kb *Pst*I-*Sal*I fragment containing the *FUS1-lacZ* reporter gene from pSL553 flanked by 2.1 kb of *MFa2* 5′ noncoding sequences which extend upstream from the *Sph*I site specified in pSM23 and a 550-bp *Bam*HI-*Eco*RI fragment containing *MFa2* 3′ noncoding sequences (19, 38). Plasmid pDH106 is a YIp5-based plasmid that contains the *HIS3*::*FUS1*::*HIS3* allele, which can be targeted to integrate at the *HIS3* locus (55). In this allele, a 2.5-kb *Eco*RI-*Bst*BI fragment containing *HIS3* 5′ noncoding sequences flanks 1.2 kb of *FUS1* promoter sequences fused 22 bp upstream of the *HIS3* coding region. The fusion junction between the *FUS1* promoter and *HIS3* coding sequences is identical to that described by Stevenson et al. (54).

Several plasmids that carry *MKK1* or *STE7* alleles were used in this study. Plasmid pSPC32 has the 1.9-kb *Bam*HI-*Pst*I fragment encompassing the *MKK1* promoter and coding sequence inserted in the low-copy (*CEN4 URA3*) vector YCplac33 (18, 24). Plasmid pSPC32-39 is identical to pSPC32 except for the single base substitution that generated the *MKK1-P368* allele (see below). Plasmid pSTE7.2 is a genomic library isolate that carries a 1.9-kb *STE7* fragment in the high-copy (2μm *LEU2*) vector YRp7 (55, 56). Plasmids pNC110 and pSL1951 carry a 2.1-kb *Hin*dIII fragment encompassing the *STE7* promoter and coding sequence inserted in the *Hin*dIII site of the low-copy (*CEN4 URA3*)

TABLE 1. Yeast strains

Strain	Relevant genotype	Source or reference
SYT11-12A	*MAT***a** *stt1-1* (*pkc1*^{ts}) *ade8 his3 leu2 met3 ura3*	61
SYT32L-2C	*MAT*α *stt1-1* (*pkc1*^{ts}) *mkk1*Δ::*LEU2 his3 leu2 trp1 ura3*	K. Irie
KAN-4C	*MAT***a** *bck1*Δ::*LEU2 ura3 leu2 trp1*	24
3233-1A	*MAT*α *mkk1*Δ::*LEU2 his3 leu2 trp1 ura3*	24
3233-1B	*MAT***a** *mkk1*Δ::*LEU2 mkk2*Δ::*HIS3 his3 leu2 trp1 ura3*	24
3233-4A	*MAT*α *mkk1*Δ::*LEU2 mkk2*Δ::*HIS3 his3 leu2 trp1 ura3*	24
3233-1B-D14	*MAT***a** *mkk1*Δ::*LEU2 mkk2*Δ::*HIS3 his3 leu2 trp1 ura3* *MAT*α *mkk1*Δ::*LEU2 mkk2*Δ::*HIS3 his3 leu2 trp1 ura3*	K. Irie
DL247[a]	*MAT***a** *bck1*Δ::*URA3 can1 his4 leu2-3,112 trp1-1 ura3-52* *MAT*α *BCK1 can1 his4 leu2-3,112 trp1-1 ura3-52*	31
DL251-1B[a]	*MAT***a** *bck1*Δ::*URA3 can1 his4 leu2-3,112 trp1-1 ura3-52*	R. Cade and B. Errede
DL376[a]	*MAT***a** *pkc1*Δ::*LEU2 can1 his4 leu2-3,112 trp1-1 ura3-52*	32
DL456[a]	*MAT***a** *mpk1*Δ::*TRP1 can1 his4 leu2-3,112 trp1-1 ura3-52* *MAT*α *mpk1*Δ::*TRP1 can1 his4 leu2-3,112 trp1-1 ura3-52*	30
SC3-590D[a]	*MAT*α *bck1*Δ::*URA3 ste5*Δ::*LEU2 can1 his4 leu2-3,112 trp1-1 ura3-52*	B. Yashar
SY2003[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52*	J. Horecka and G. Sprague
SY2068[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste7*Δ*3*::*LEU2 far1*	J. Printen
SY2071[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste4*Δ::*LEU2 far1*	
SY2072[b]	*MAT***a** *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste4*Δ::*LEU2 far1 arg4*	
SY2074[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste4*Δ::*LEU2 far1 arg4* *MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste4*Δ::*LEU2 far1 ARG4*	
SY2186[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste5*Δ::*LEU2*	
SY2208[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 fus3*Δ*6*::*LEU2 kss1*Δ::*URA3*	J. Horecka and G. Sprague
SY2263[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste11*Δ::*ura3*	J. Printen
SY2595[b]	*MAT*α *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste4*Δ	
SY2810[b]	*MAT***a** *HIS3*::*FUS1*::*HIS3 mf***a***2-*Δ*1*::*FUS1-lacZ ade1 leu2-3,112 trp1-DH1 ura3-52 ste7*::*ura3*	
E929-6C-20[c]	*MAT***a** *cyc1 CYC7-H2 can1 leu2-3,112 trp1-*Δ*1 ura3-52 ste11*Δ*6*	45
E929-6C-22[c]	*MAT***a** *cyc1 CYC7-H2 can1 leu2-3,112 trp1-*Δ*1 ura3-52 bar1*Δ::*LEU2*	64
E929-6C-24[c]	*MAT***a** *cyc1 CYC7-H2 can1 leu2-3,112 trp1-*Δ*1 ura3-52 bar1*Δ::*LEU2 ste11*Δ*6*	C. Adler and B. Errede

[a] Isogenic with strain EG123.
[b] Isogenic with strain Sc252.
[c] Isogenic with strain E929-6C.

vectors YCp50 and pRS316, respectively (27, 51, 56). Plasmids pSL2002, pSL2234, and pSL2233 are identical to pSL1951 except for the *STE7-*Δ*Y259*, *STE7-P368*, and *STE7-Y464* alleles, respectively (see below). Plasmid pSL2267 was constructed by cloning a *Pst*I-*Xho*I fragment from pSL2234 into the low-copy (*CEN4 TRP1*) vector pRS314 (51).

To facilitate biochemical analysis of Ste7p-P^{368}, a serine-to-proline mutation was introduced into the *CYC1-STE7M* allele by site-directed mutagenesis of pNC318 with the mutagenic oligonucleotide 5′-GACATATAAGTGGNCGTT CC-3′ (28, 64). The resulting plasmid (pNC318-P368) expresses the substitution protein fused to an 11-amino-acid Myc epitope at the C terminus (Ste7Mp-P^{368}) under the control of the *CYC1* promoter. Plasmids pNC318, pNC318-P368, and pNC318-R220, which are low-copy (*CEN3 TRP1*) plasmids, were used for expression of Ste7Mp, Ste7Mp-P^{368}, and Ste7Mp-R^{220}, respectively, in yeast cells (64). Plasmid pGA1944 was used to express the glutathione *S*-transferase (GST)-Fus3p-R^{42} fusion in *Escherichia coli* (12).

Yeast strains and growth conditions. The genotypes of the yeast strains used in this study are given in Table 1. Strains with different mutations in the cell integrity pathway have been described before or were constructed by standard yeast genetic procedures. Strain SYT32L-2C (*pkc1*^{ts} *mkk1*Δ::*LEU2*) is a meiotic segregant of a cross between strains SYT11-12A and 3233-1A. Diploid strain 3233-1B-D14 (*MAT***a**/*MAT*α *mkk1*Δ::*LEU2*/*mkk1*Δ::*LEU2 mkk2*Δ::*HIS3*/*mkk2*Δ::*HIS3*) was constructed by mating strains 3233-1B and 3233-4A. Strain DL251-1B (*bck1*Δ::*URA3*) is a meiotic segregant of strain DL251 (31). Strain SC3-590D (*bck1*Δ::*URA3 ste5*::*LEU2*) is a meiotic segregant of a derivative of strain DL247 that had been made heterozygous for *ste5*::*LEU2* by gene replacement (see below).

Strain SY2003, which contains the *HIS3*::*FUS1*::*HIS3* and *mf***a***2*::*FUS1-lacZ* alleles integrated at the corresponding *HIS3* and *MF***a***2* loci, is a derivative of Sc252 (59). The alleles were introduced by two-step gene replacements with *Nhe*I-linearized pDH106 and *Hin*dIII-linearized pDH17, respectively. A *far1* allele was introduced into a *MAT***a** strain of the Sc252 background by selecting for a spontaneous mutation that caused the *far1* mutant phenotype and then verifying that the mutation belonged to the expected complementation group (22). This *far1* allele was introduced into the *MAT*α progenitor of strains SY2068 and SY2071 by appropriate crosses.

Strain SY2074, a homozygous *MAT*α/*MAT*α *FUS1*::*HIS3*/*FUS1*::*HIS3 FUS1-lacZ*/*FUS1-lacZ ste4*Δ::*LEU2*/*ste4*Δ::*LEU2* diploid, was created by the following steps. First, *FUS1-lacZ ste4*Δ::*LEU2* haploid strains of opposite mating types (SY2071 and SY2072) were transformed with YCpSTE4 to complement the *ste4*Δ mutation. The two strains were then crossed to give a *MAT***a**/*MAT*α diploid strain that is His$^-$ as a result of cell type-specific repression of regulatory genes required for *FUS1* promoter activity. Finally, mating type-homozygous diploid cells were selected as cells that spontaneously converted one or the other *MAT* allele. Because the *FUS1* promoter is active in diploid cells that are homozygous at the *MAT* locus and wild type for other components of the pheromone response signal pathway, the spontaneous convertants were easily identified as His$^+$ cells on medium lacking histidine and uracil. The mating phenotype of the resulting His$^+$ colonies was determined by the pheromone halo assay (53). Finally, an isolate with an α phenotype was cured of the YCpSTE4 plasmid, which carries the *URA3* selectable marker, by being passaged on medium containing 5-fluoro-orotic acid (2).

The strains with deletions of different components of the mating response pathway were constructed by gene replacement (46). Plasmids p121 (*ste4*Δ::*LEU2* [M. Whiteway, Biotechnology Research Institute, Montreal, Quebec, Canada]), pJM41 (*ste5*::*LEU2* [G. Ammerer, University of Vienna, Vienna, Austria]), pSL1094 (*ste11*::*URA3* [54]), pNC113 (*ste7*Δ*3*::*LEU2* [4]), pSL2077 (*ste7*::*URA3* [54]), pEE98 (*fus3*-Δ*6*::*LEU2* [10]), pBC65 (*kss1*Δ::*URA3* [5]), and pVZ77 (*bar1*Δ::*LEU2* [V. MacKay, Zymogenetics]) were the sources of the fragments for the indicated gene replacements.

Unless otherwise specified, we used the media and genetic procedures described by Sherman et al. (50). When derepression of the *CYC1-STE7* alleles was needed, 1% sucrose was used as the carbon source in place of glucose. Yeast strains were transformed by the procedure of Ito et al. (25). Osmotic suppression of temperature-dependent cell lysis was assayed essentially as described by Irie et al. (24). β-Galactosidase activity was measured as described by Jarvis et al. (26). For pheromone induction, *BAR1* cultures were exposed to 5 μM α-factor for 75 min, and *bar1* cultures were exposed to 50 nM α-factor for 75 min.

Isolation of *MKK1* and *STE7* gain-of-function alleles. Yeast strain SYT32L-2C was transformed with a *CEN* plasmid (pSPC32) that expresses the *MKK1* gene under the control of its own promoter and contains the *URA3* gene as a selectable marker. Spontaneously arising yeast transformants that grew at 37°C, the restrictive temperature for the conditional *pkc1* allele (*stt1-1*), were selected for further analysis (61). The dependence of the phenotype on the *MKK1* plasmid was scored as the inability to grow at the restrictive temperature in the presence of 5-fluoro-orotic acid (2). Plasmid DNA was isolated from these transformants and retransformed into a naive SYT32L-2C strain. The resulting transformants were retested to confirm plasmid-dependent growth at the restrictive tempera-

ture. The DNA sequence of *MKK1* within pSPC32-39 was determined on both strands (48).

Mutations in *STE7* were generated by passage of plasmid pSL1951 through an *E. coli mutD* strain (SB61) (15). A *MAT*α/*MAT*α *ste4*Δ/*ste4*Δ diploid strain (SY2074) was transformed with a DNA preparation made from a single colony. We used the α/α diploid in the selection scheme because recessive suppressors that stimulate the *FUS1*::*HIS3* reporter independently of the pheromone response pathway were recovered at a high frequency in a haploid *ste4*Δ strain. Transformants that grew on SD medium lacking uracil and histidine after 3 days at 30°C were recovered and tested for pheromone production as an independent measure of pathway activation. The dependence of the phenotype on the plasmid was tested by restreaking individual transformants onto medium containing 5-fluoro-orotic acid to select for plasmid loss. The resulting colonies were then scored for the inability to grow on medium lacking histidine. Plasmids were rescued from isolates that scored positively on the two secondary screens and retested in a naive SY2074 strain. The DNA sequence of the entire *STE7* coding region was determined for all gain-of-function alleles (48). Plasmids containing the mutant *STE7* alleles were designated pSL2002 (*STE7-ΔY259*), pSL2234 (*STE7-P368*), and pSL2233 (*STE7-Y464*).

Immune complex kinase assays. In vitro assays of Ste7 catalytic activity measure phosphotransfer of γ-^{32}P from ATP to the protein substrate Fus3p. These assays were performed directly on immune complexes of Ste7Mp isolated from native protein extracts as described by Errede et al. (12). The polypeptide substrate was a catalytically inactive form of Fus3p (Fus3Mp-R^{42}) prepared from a GST fusion protein produced in *E. coli*. The fusion protein was isolated on glutathione-agarose columns and treated with thrombin to release the GST polypeptide as described elsewhere (12, 52). The resulting mixture was used as the substrate preparation without further purification. Typically, the thrombin cleavage reaction does not go to completion, and the mixture consists of some fusion protein in addition to the cleaved Fus3p-R^{42} and GST polypeptides.

Kinase assays were performed directly with the Ste7p immune complex bound to protein A-Sepharose beads. For each reaction, immune complex beads (15 μl packed volume) were suspended in 6 μl of kinase assay buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2], 15 mM $MgCl_2$, 5 mM EGTA [ethylene glycol tetraacetic acid], 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 15 mM 4-nitrophenylphosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 20 μg of leupeptin per ml, 40 μg of aprotinin per ml). Approximately 250 ng of the thrombin-treated GST-FusMp-R^{42} substrate (2 to 5 μl) was added to the above suspension. The reactions were initiated by addition of an ATP mix (1 μl of 250 mM HEPES [pH 7.2], 2.0 μl of 1 mM ATP, 0.2 μl of [γ-^{32}P]ATP [10 μCi/μl], 2.4 μl of 100 mM $MgCl_2$) and water to give a final reaction volume of 20 μl. The mixtures were incubated at 37°C for 20 min, and reactions were terminated by addition of an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and boiling for 3 min (29). The resulting mixture of immune complex and substrate proteins was resolved by SDS-PAGE (29). Incorporation of ^{32}P into protein substrate was quantified by PhosphorImager analysis of the Fus3p-R^{42} polypeptide band in the dried gels.

Immunoblot analysis. Protein extracts for immunoblot analyses were fractionated on SDS–7.5% polyacrylamide gels and transferred to nitrocellulose membranes. The Myc1-9E10 monoclonal antibody (2 μg/ml) was used as the primary antibody to detect Ste7M and Ste7M variant proteins on the nitrocellulose membranes (14, 64). The Promega Protoblot immunoblot system with goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (0.1 μg/ml) was used to detect the primary antibody by colorimetric methods as described by the vendor (Promega).

RESULTS

Identification of *MKK1* gain-of-function mutations. Gain-of-function variants of Mkk1p were identified by screening for spontaneous *MKK1* mutations that suppress the cell lysis phenotype associated with loss of *PKC1* function in the cell integrity pathway (Fig. 1) (24, 61). A *pkc1*^{ts} *mkk1*Δ::*LEU2* yeast strain (SYT32L-2C) was transformed with a *CEN* plasmid containing the *MKK1* gene under the control of its own promoter (pSPC32). While expression of *MKK1* from a high-copy-number (2μm) plasmid suppresses the cell lysis defect at the restrictive temperature for *pkc1*^{ts} function (37°C), single-copy expression of wild-type *MKK1* (pSPC32) does not (24). DNA sequence analysis of the plasmid recovered from one of the yeast transformants that grew at the restrictive temperature (pSPC32-39) revealed a single-base-pair change in the *MKK1* coding sequence. This mutation at position 1156 of the nucleotide sequence generates a serine-to-proline substitution at amino acid 386 in Mkk1p (24). The mutant allele was designated *MKK1-P386*.

TABLE 2. Activity of *MKK1* alleles in the cell integrity pathway[a]

Strain	Relevant genotype	Growth on YPD at 37°C	
		MKK1	*MKK1-P386*
DL376	*pkc1*Δ	−	+
KAN-4C	*bck1*Δ	−	+
3233-1B	*mkk1*Δ *mkk2*Δ	+	+
DL456	*mpk1*Δ	−	−

[a] The plasmids used were pSPC32 (*MKK1*) and pSPC32-39 (*MKK1-P386*).

To further characterize the activity of Mkk1p-P^{386} in the cell integrity pathway, the variant was expressed in strains containing deletions of different components in this pathway (Fig. 1). While *PKC1* function is essential for cell wall integrity and growth, the cell lysis phenotype associated with loss of function of *BCK1*, *MKK1*, and *MKK2* or *MPK1* is conditional (24, 30–32). Strains containing deletions of these genes were transformed with *CEN* plasmids expressing either Mkk1p or Mkk1p-P^{386} and tested for growth at the nonpermissive temperature of 37°C. As expected from known epistatic relationships, the ability of the Mkk1p-P^{386} variant to rescue the cell lysis defect of different pathway mutants is dependent on the downstream MAPK (Mpk1p) and independent of activation events catalyzed by kinases that function upstream of Mkk1p and Mkk2p (Table 2). On the basis of this pattern of suppression, Mkk1p-P^{386} constitutively activates the cell integrity pathway.

Identification of *STE7* gain-of-function mutations. Gain-of-function *STE7* alleles were identified by a screen for isolates that could activate mating pathway transcriptional responses independently of the G protein. This screen relied on a *FUS1*::*HIS3* reporter gene in which the well-characterized pheromone-inducible *FUS1* promoter drives expression of the *HIS3* coding region (54). Expression of His3p from this promoter is dependent on the integrity of the pheromone response pathway. In uninduced wild-type cells, the basal activity of the pathway is sufficient to allow *FUS1*::*HIS3* yeast strains to grow on medium lacking histidine. Deletion of the G protein β subunit (*ste4*Δ) eliminates this basal activity and renders cells unable to grow on medium lacking histidine.

A mutagenized bank of *STE7* plasmids was transformed into a *ste4*Δ *FUS1*::*HIS3* strain (SY2074), and individual transformants were screened for growth on medium lacking histidine. This screen identified three independent *STE7* plasmid alleles (Fig. 2). Two alleles involved single-base-pair mutations that produced a serine-to-proline substitution at amino acid residue 368 and an asparagine-to-tyrosine substitution at amino acid residue 464. The third allele involved a deletion of three nucleotides, causing a loss of tyrosine residue 259. The mutant alleles were named *STE7-P368*, *STE7-Y464*, and *STE7-ΔY259*, respectively.

To characterize the activities of the Ste7p variants in the mating response pathway, we transformed plasmids expressing Ste7p, Ste7p-P^{368}, Ste7p-Y^{464}, or Ste7p-ΔY^{259} into *FUS1*::*HIS3* strains from which different pheromone response pathway components had been deleted. The transformed strains were then tested for growth on medium lacking histidine. As expected from the known relationship of pathway components, the three mutant alleles but not the wild type suppressed the transcriptional defect of the *ste4*Δ and *ste5*Δ strains but not of the *fus3*Δ *kss1*Δ strain (Table 3). Unexpectedly, none of the alleles supported *FUS1-HIS3* expression in the *ste11*Δ strain, which lacks the MEKK of this pathway. This result suggests

TABLE 3. Characterization of *STE7* gain-of-function mutations in the mating response pathway[a]

Host strain	Relevant genotype	Growth on medium lacking histidine			
		STE7	*STE7-ΔY259*	*STE7-P368*	*STE7-Y464*
SY2074	*ste4Δ*	−	+	+	+
SY2186	*ste5Δ*	−	+	+	+
SY2263	*ste11Δ*	−	−	−	−
SY2068	*ste7Δ*	+	+	+	+
SY2208	*fus3Δ kss1Δ*	−	−	−	−

[a] The plasmids used were pSL1951 (*STE7*), pSL2002 (*STE7-ΔY259*), pSL2234 (*STE7-P368*), and pSL2233 (*STE7-Y464*).

that the variants are not fully constitutive enzymes but still depend on phosphorylation for their activity.

A more quantitative comparison of pathway activation promoted by the three variants was obtained by measuring β-galactosidase activity expressed from a *FUS1-lacZ* reporter gene in a *ste7Δ* strain. In the absence of inducer, the Ste7p variants elevated β-galactosidase activity 2.4- to 6.6-fold over the amount in the strain expressing wild-type Ste7p (Table 4). Of the three variants, Ste7p-P^{368} promoted the highest basal activity, but this amount was still threefold less than the amount found under fully induced conditions with the wild-type enzyme. After pheromone induction, the amount of β-galactosidase activity supported by all three variants was the same (Table 4). These data show that the activity of the variants is still responsive to the inductive signal.

Additionally, even the most potent of the gain-of-function variants does not induce G_1 arrest in the absence of pheromone or cause supersensitivity to pheromone. Wild-type cells that are capable of G_1 arrest (*FAR1*) and express Ste7p-P^{368} have the same doubling time in the absence of pheromone as strains expressing Ste7p (data not shown). Therefore, progression through the cell cycle does not appear to be perturbed by expression of the Ste7p variant. Furthermore, Ste7p-P^{368} and Ste7p strains respond identically in halo assays, which are one measure of the G_1 arrest response to pheromone (data not shown).

Pheromone-independent activity of Ste7p-P^{368} is greater than that of the wild-type enzyme. Alignment of the Ste7p and Mkk1p protein sequences revealed that the substitutions in Mkk1p-P^{386} and Ste7p-P^{368} had changed analogous serine residues. This serine is positioned between the conserved kinase subdomains VII and VIII in a region that is highly conserved between members of the MEK family (Fig. 2). Because of this striking outcome, we reasoned that an analysis of the enzymatic properties of Ste7p-P^{368} would yield information pertinent to the analogous substitution in other MEK family members.

To express Ste7p-P^{368} for immune complex kinase assays, we cloned the *STE7-P368* allele into a yeast expression vector that overproduced the protein under the control of the *CYC1* promoter and in frame with a c-Myc epitope tag (64). The resulting Ste7Mp-P^{368} variant was isolated from cell extracts by immunoprecipitation and tested for the ability to phosphorylate catalytically inactive Fus3p-R^{42} purified from *E. coli* as a GST fusion (12). For reference, immune complex kinases assays were also performed with the wild-type Ste7Mp and catalytically inactive Ste7Mp-R^{220} enzymes.

TABLE 4. Transcriptional activity supported by *STE7* gain-of-function alleles

Plasmid in strain SY2810 (*ste7Δ*)	*STE7* allele in plasmid	β-Galactosidase activity[a]	
		Without α-factor	With α-factor
pRS316	None	1	2
pSL1951	*STE7*	5	98
pSL2002	*STE7-ΔY259*	12	101
pSL2234	*STE7-P368*	33	104
pSL2233	*STE7-Y464*	17	97

[a] Activities are expressed in modified Miller units (25).

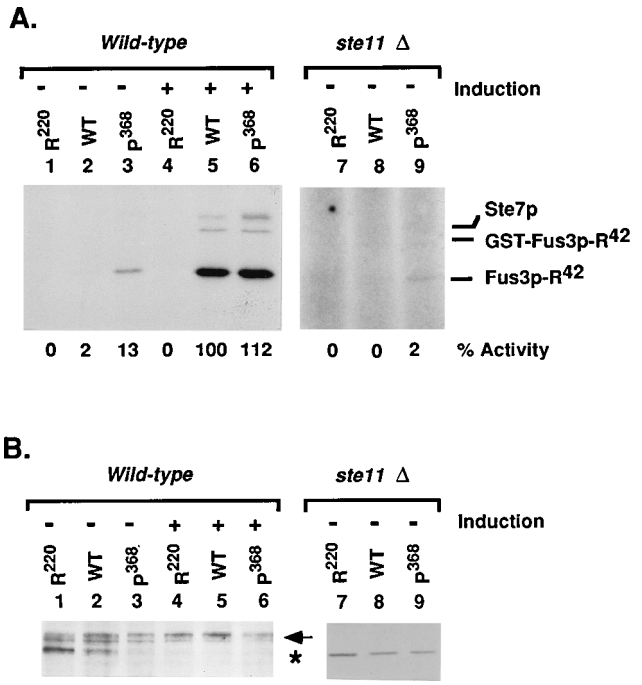

FIG. 3. Comparison of Ste7p and Ste7p-P^{368} enzyme activities. (A) Immune complex kinase assays comparing the amount of Fus3p-R^{42} phosphorylation catalyzed by Ste7p (WT), Ste7p-P^{368} (P^{368}), and Ste7p-R^{220} (R^{220}). These proteins were expressed in wild-type (E929-6C-22) and *ste11Δ* (E929-6C-24) yeast strains, as indicated. Cultures were induced by mating pheromone (+) or not induced (−) prior to extract preparation. To visualize $^{32}P_i$ incorporation into Fus3p-R^{42} by autoradiography, the panel showing assays of *ste11Δ* extracts was overexposed compared with the wild-type panel. The radioactivity incorporated into Fus3p-R^{42} in each lane was quantified by PhosphorImager analysis and is reported below the autoradiogram as a percentage of the value measured for the pheromone-induced Ste7p (WT) reaction (lane 5). Other phosphoproteins (lanes 5 and 6) are residual GST-Fus3p-R^{42} fusion protein in the substrate preparation (see text) and Ste7p, as indicated. This pheromone-induced and Ste7p-dependent phosphorylation is attributed to Fus3p and Kss1p, which coimmunoprecipitate with Ste7p (3). (B) Immunoblot detection of Ste7Mp-R^{220}, Ste7Mp, and Ste7Mp-P^{368} in extracts from wild-type and *ste11Δ* yeast cells. The hyperphosphorylated (arrow) and less phosphorylated (asterisk) forms of Ste7p are indicated.

Phosphorylation of Fus3p-R^{42} was readily detected in the immune complex kinase assays with Ste7Mp-P^{368} isolated from uninduced cells (Fig. 3A, lane 3). Phosphorylation of the substrate by Ste7Mp was weaker but could be detected by using the more sensitive PhosphorImager analysis (results not shown). Quantification of the PhosphorImager signals revealed that when the enzymes were isolated from uninduced cells, there was sixfold greater Fus3p-R^{42} phosphorylation with Ste7Mp-P^{368} than with wild-type Ste7Mp. Pheromone induction increased the activities of both Ste7Mp-P^{368} and Ste7Mp, so that each phosphorylated equivalent amounts of Fus3p-R^{42} (Fig. 3A, lanes 5 and 6). Because the reactions are within the linear range for the substrate, this result shows that under inducing conditions, the two enzyme preparations have the same activity.

From the immunoblot analyses, increased production of Ste7p-P^{368} does not cause the difference in basal activity found for Ste7p and Ste7p-P^{368} (Fig. 3B, lanes 2 and 3). Additionally, Coomassie staining patterns for the SDS-PAGE-fractionated assay samples confirmed that the amount of immune complex in each was the same (data not shown). Therefore, the serine-to-proline substitution in Ste7p-P^{368} leads to a more active preparation under basal conditions than is seen with the wild-type enzyme.

Because Ste7p-P^{368}-dependent reporter gene activation in vivo required Ste11p, we compared the activity of the variant in the presence and absence of Ste11p. In cells with a deletion of *STE11* (*ste11*Δ), Ste7Mp-P^{368} phosphorylated Fus3p-R^{42}, but its activity was five- to sixfold lower than when isolated from uninduced wild-type cells (Fig. 3A, compare lanes 3 and 9). In assays with either the wild type (Ste7Mp) or the catalytically inactive variant (Ste7Mp-R^{220}), we detected no phosphorylation of Fus3p-R^{42} by either autoradiography or PhosphorImager analysis (Fig. 3A, lanes 7 and 8, and results not shown). Thus, within the sensitivity limits of these assays, Ste11p is absolutely required for the activity of the wild-type enzyme but not Ste7p-P^{368}. Because Ste7p-P^{368} did not suppress the transcriptional defect of a *ste11*Δ *FUS1*::*HIS3* strain, this amount of Ste11p-independent Ste7p-P^{368} activity is insufficient to promote a transcriptional output from the mating pathway.

The immune complex kinase assays provided a measure of the activity of Ste7p-P^{368} in vitro. To evaluate how these variations in activity affect signal transmission in vivo, we analyzed the phosphorylation states of Ste7p-P^{368}. In response to pheromone, Ste7p is hyperphosphorylated in a Fus3p- and Kss1p-dependent reaction (64). Conversion of the protein from a less phosphorylated to a hyperphosphorylated form requires both activation of Ste7p by the inductive signal and Ste7p-promoted activation of Fus3p or Kss1p (12, 64). Thus, changes in the phosphorylation state of Ste7p serve as an internal monitor for signal flow between Ste7p and Fus3p/Kss1p.

The hyperphosphorylated and less phosphorylated forms of Ste7Mp and its variants were identified on immunoblots by their characteristic mobilities on SDS-PAGE (Fig. 3B). The migration patterns for Ste7p and Ste7p-R^{220} were as expected. In uninduced wild-type cells, the majority of both proteins was detected as a single band with an apparent molecular mass of 58 kDa (Fig. 3B, lanes 1 and 2). In contrast, the majority of Ste7Mp-P^{368} appears as a doublet at a mobility of ~70 kDa, which is characteristic of the hyperphosphorylated forms (Fig. 3B, lane 3). For reference, the proteins from pheromone-induced cultures were examined, and all three were in the hyperphosphorylated state (Fig. 3B, lanes 3 to 5). Therefore, the increased basal activity of Ste7p-P^{368} is sufficient to generate enough active Fus3p and Kss1p in vivo so that all detectable Ste7p is maintained in the hyperphosphorylated state. In extracts from *ste11*Δ cells, Ste7M, Ste7M-R^{220}, and Ste7M-P^{368} were present only in the less phosphorylated form (Fig. 3B, lanes 7 to 9). Thus, the Ste11p-independent activity of Ste7p-P^{368} is below the threshold required to generate increased steady-state amounts of active Fus3p and Kss1p in vivo.

Ste7p-P^{368} functions effectively in the cell integrity pathway if it is overproduced. We previously reported that Ste7p-P^{368} but not Ste7p mediates Raf-dependent signaling in yeast cells (23). This interaction with a heterologous MEK activator suggested to us that the more active variant might have gained the ability to stimulate responses associated with other yeast MAPK activation pathways. To test this possibility, we assessed Ste7p-P^{368} for function in the cell integrity pathway.

A strain in which the *MKK1* and the *MKK2* genes had been deleted was transformed with yeast plasmids that expressed either Ste7p-P^{368} or Ste7p. The resulting transformants were tested for growth at the restrictive temperature (37°C). No suppression of the signal defect was detected when either protein was expressed from the *STE7* promoter (Fig. 4A, *STE7* and *STE7-P368*). When Ste7p-P^{368} was overproduced from the *CYC1* promoter, it suppressed the signal defect in *mkk1*Δ *mkk2*Δ cells as effectively as Mkk1p-P^{386} (Fig. 4A, compare *CYC1-STE7-P368* and *MKK1-P386*). By contrast, when Ste7p was similarly overproduced, it rescued only ~25% of the cell population (Fig. 4A, *CYC1-STE7*). Because the amount of protein affected the phenotype, we attribute this behavior to higher plasmid copy number in a subset of individual cells. We verified that suppression was dependent on the expressed Ste7p or Ste7p-P^{368} by curing cells of the plasmids and confirming that the isolates lost their ability to grow at 37°C (results not shown). Although both the wild type and Ste7p-P^{368} could act in the cell integrity pathway, the more effective suppression by Ste7p-P^{368} suggests that the proline substitution variant is more capable of pathway cross-reaction, perhaps because of an overall increase in its enzymatic capacity.

Ste5p affects pathway specificity. Mkk1p-P^{386} suppressed the signal defect associated with the loss of upstream components in the cell integrity pathway. To determine if Ste7p-P^{368} activity in this pathway had equivalent characteristics, we compared Ste7p and Ste7p-P^{368} for the ability to suppress the signal defect associated with the loss of Bck1p. Neither Ste7p or Ste7p-P^{368} suppressed the *bck1*Δ phenotype, even when the proteins were overproduced (Fig. 4B, *STE7*, *STE7-P368*, *CYC1-STE7*, and *CYC1-STE7-P368*). The lower activity of Ste7p and Ste7p-P^{368} in the absence of MEKK modification could be responsible for this failure. Alternatively, the availability for cross-reaction could be more compromised in this circumstance because Ste7p or Ste7p-P^{368} is competing with Mkk1p/Mkk2p for the Mpk1p substrate.

The mating response pathway component Ste5p is postulated to be a scaffold that organizes the kinases into a complex (3, 36, 44). Reasoning that in this capacity, Ste5p could limit the amount of Ste7p or Ste7p-P^{368} available to function in an alternative pathway, we determined if either protein would suppress the cell lysis phenotype in a *ste5*Δ *bck1*Δ double mutant strain (SC3-590D). We found that in this double mutant strain, overproduction of Ste7p-P^{368} suppressed the signal defect in the cell integrity pathway as effectively as Mkk1p-P^{386} (Fig. 4C, *CYC1-STE7-P368* and *MKK1-P386*). More importantly, some suppression was seen even when Ste7p-P^{368} was expressed from its own promoter (Fig. 4C, *STE7-P368*). Suppression of the *bck1*Δ defect required the proline substitution in Ste7p, because expression of the wild-type Ste7p in any amount did not suppress cell lysis (Fig. 4C, *STE7* and *CYC1-STE7*). These results show that Ste7p-P^{368} functions in the cell integrity pathway with the same characteristics as Mkk1p-P^{386}. Because this ability is restricted by the presence of Ste5p, we further conclude that one of the roles of Ste5p is to limit illegitimate interactions of Ste7p with other pathways.

DISCUSSION

Conformational model for the effects of the proline 368 substitution on Ste7p activity. Ste7p-P^{368} was identified in a screen for random *STE7* mutations that stimulate mating-specific transcriptional responses in the absence of the signal-transducing G_{β} subunit. Without pheromone stimulation, the activity of Ste7p-P^{368} is sixfold higher than that of the wild-type enzyme, but this increased activity still depends on the upstream kinase Ste11p. Pheromone stimulation causes a further increase in Ste7p-P^{368} activity to an amount equivalent to that

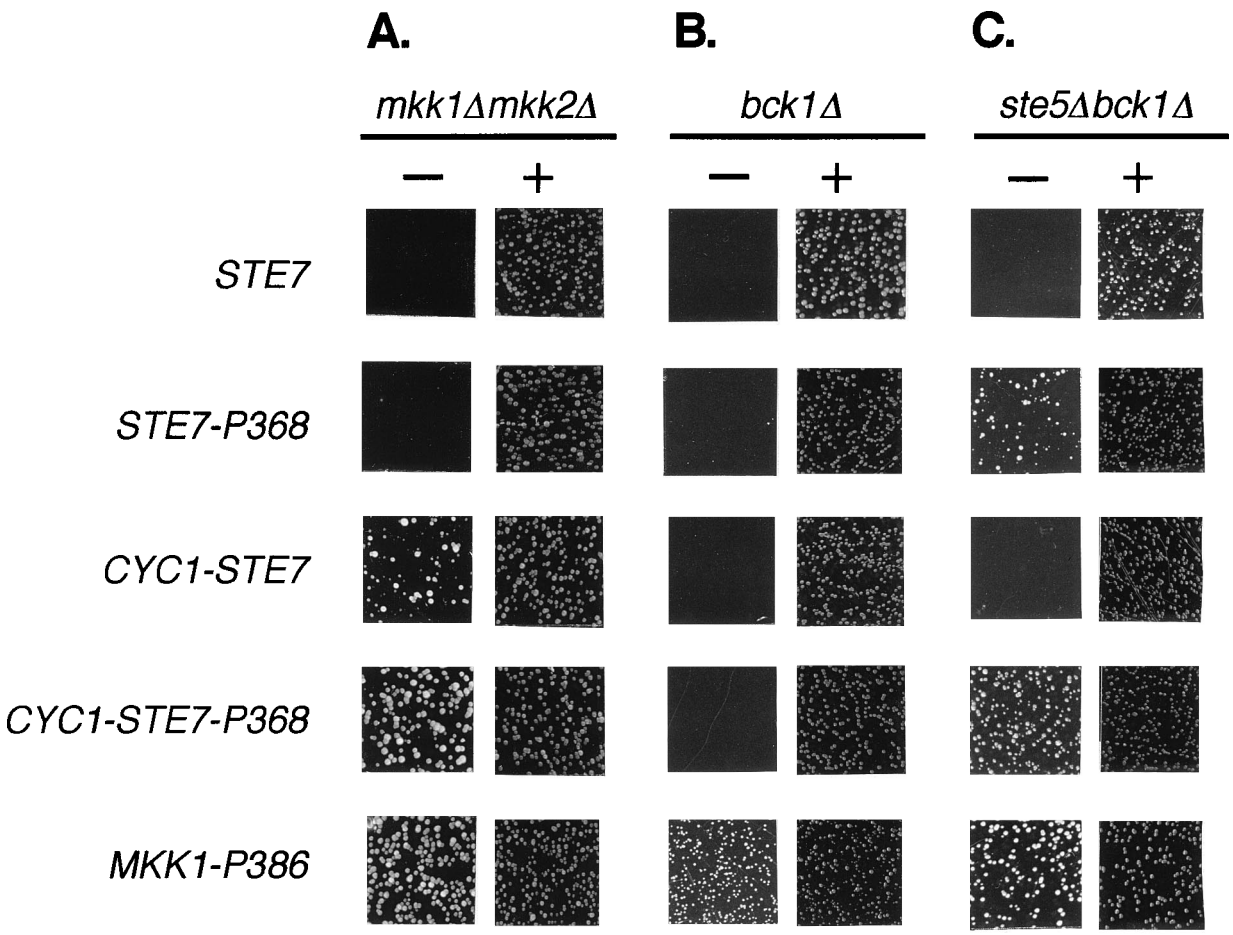

FIG. 4. Activity of Ste7p-P^{368} and Ste7p in the cell integrity pathway. Overnight cultures of *mkk1*Δ *mkk2*Δ (3233-1B-D14), *bck1*Δ (DL251-1B), and *ste5*Δ *bck1*Δ (SC3-590D) yeast strains containing plasmids with the indicated alleles were grown in appropriate selective medium at 22°C. Equal numbers of cells were spread onto complete medium with 2% sucrose as the carbon source and incubated at 37°C for 3 days. The plates either lacked (−) or contained 1 M sorbitol (+) as an osmotic stabilizer to compensate for the cell wall deficiency of the mutant strains. (A) The plasmids used were pNC110 (*STE7*), pSL2234 (*STE7-P368*), pNC318 (*CYC1-STE7*), pNC318-P368 (*CYC1-STE7-P368*), and pSPC32-39 (*MKK1-P386*). Note that each allele is carried on a low-copy centromeric vector. (B and C) The plasmids used were the same as in panel A except that the high-copy 2μm plasmid pSTE7.2 (*STE7*) was used instead of pNC110.

of the fully activated Ste7p. Our interpretation of these results is that Ste7p-P^{368} requires phosphorylation for catalytic activity, but compared with the wild-type enzyme, a greater fraction of Ste7p-P^{368} is present in the modified and active forms under basal conditions.

Recent insights derived from the crystal structure of ERK2 suggest some plausible models for how the proline substitution might affect Ste7p in the way that we suggest (62). The regulatory Thr-183 and Tyr-185 sites of ERK2 are located within a "phosphorylation lip" that is part of a loop between protein kinase subdomains VII and VIII. The unphosphorylated Tyr-185 is buried in a hydrophobic pocket and is not readily accessible for modification by MEK. Therefore, it has been proposed that conformational changes must occur in the lip region upon binding to MEK for catalysis and also to accommodate the resulting phosphotyrosine side group.

Serine 368 of Ste7p lies within an analogous region between subdomains VII and VIII in proximity to the phosphate acceptor residue(s) that becomes modified upon activation by Ste11p (Fig. 2) (17, 40, 63). Assuming that a mechanism similar to that for ERK2 applies to Ste7p, the serine-to-proline substitution could alter the conformation of the phosphorylation lip in a way that makes the regulatory site(s) more accessible to MEKKs (62). An alternative and not mutually exclusive model for the increased basal activity of Ste7p-P^{368} is that the proposed conformational alteration in the lip region causes the variant to be more resistant to a deactivation event, such as might be catalyzed by a phosphatase.

Regardless of the underlying mechanism, the proline substitution does alleviate restrictions on Ste7p interactions with heterologous MEK kinases, such as mammalian t-Raf. This characteristic allowed us to reconstitute Raf-1-dependent signal transduction in yeast cells and link it to the output of a convenient reporter gene (23). The genetic potential of this reconstituted signal pathway has already been exploited and led to the identification of Bmh1p, a 14-3-3 protein family member, as one factor that stimulates Raf-1 activation by Ras (23). Ste7p-P^{368} and analogous genetic strategies may be useful in future applications to identify activators of other heterologous MEKKs.

Various levels of MEK activity trigger different pathway responses. We correlated different levels of Ste7p activity with the transcriptional and G_1 arrest responses that contribute to the establishment of mating competence. An amount of Ste7p or Ste7p-P^{368} activity that is 2% of the fully induced enzyme was insufficient to elicit either response. However, a sixfold

increase above this amount, such as occurred when Ste7p-P^{368} was coexpressed with the unstimulated form of Ste11p, surpassed the signal threshold for a transcriptional response, but this activity, which is still only 12% of the fully induced amount, did not cause G_1 arrest. Either this amount of Ste7p activity is below the signal threshold needed, or G_1 arrest requires a pheromone-dependent input from an as yet undefined branch of the pathway. Alternatively, the persistent elevated activity caused by Ste7-P^{368} could trigger an adaptive response which counteracts the inhibitory effects of pathway activity on cell cycle progression.

The only assay presently available for evaluating signal activity in the cell integrity pathway does not give a quantitative measure of the relative activities of Mkk1p-P^{386} and Mkk1p. Yet it is clear that the variant has higher activity than the wild-type protein, because even without overproduction, Mkk1p-P^{386} suppresses the growth defects associated with loss of upstream components in this pathway. From our findings with Ste7p-P^{368}, it would be surprising if the constitutive activity of Mkk1p-P^{386} is comparable to that of the fully active form. Instead, we expect that its modification by Bck1p is still essential for full activation but that the simple growth assay employed here does not discriminate between the two levels of activity.

Superficially, the Mkk1p and Ste7p serine-to-proline variants might be thought to behave differently, because the former compensates for a deficiency in its upstream MEKK but the latter does not. Yet two observations suggest that this difference reflects a difference in the sensitivities of the bioassay for cell integrity compared with those for mating and the associated transcriptional and cell cycle arrest responses to pheromone. First, biochemical assays show that Ste7p-P^{368} does have a low but detectable level of activity in the absence of its upstream MEKK, Ste11p (Fig. 3). Yet this activity is insufficient for mating differentiation responses. Second, the Ste7p and Mkk1p variants have the same properties when compared in similar bioassays. For example, even with overexpression, neither Ste7p-P^{368} nor Mkk1p-P^{386} caused any detectable mating differentiation responses in a *ste11*Δ background (data not shown), yet either gain-of-function variant suppressed the cell lysis defect of a *bck1*Δ strain. These observations support the notion that a lower signal threshold is needed to support cell integrity than is needed to promote mating differentiation.

Parameters important for pathway specificity of MEK function. Through the analysis of yeast gain-of-function MEK mutations, we gained some insights into the in vivo parameters that are important for fidelity of signaling in MAPK activation pathways. Specifically, Ste7p-P^{368} is more able than Ste7p to compensate for deficiencies in the cell integrity pathway, yet the illegitimate interactions did not occur unless Ste5p was missing or Ste7p-P^{368} was overexpressed and presumably exceeded the amount of Ste5p. There are two plausible models to account for this behavior. One is that Ste5p may be an important element for feedback reactions that attenuate the activity of Ste7p (12). Another is that the architecture of these pathways may be a key determinant of Ste7p specificity. Recent work suggests that Ste5p provides a scaffold in the mating response pathway that promotes interactions between Ste11p, Ste7p, Fus3p, and Kss1p (3, 36, 44). This work suggested that such a framework is important in facilitating both activating and attenuating interactions between the kinases. Our studies suggest that another function for this organization could be minimizing Ste7p cross-interactions with other pathways.

Our results with Ste7-P^{368} have broad ramifications for the use of gain-of-function MEK mutations in other systems. Recent evidence makes it clear that multiple MAPK activation pathways transduce signals from distinct stimuli in vertebrates (8, 39, 47, 60). Because the architecture of MAPK activation pathways ensured the specificity of MEK gain-of-function mutations in *S. cerevisiae*, we anticipate that similar mechanisms might maintain the specificity of signaling by newly identified MEK family members. If this prediction is valid, expression of gain-of-function MEK mutations in vertebrates offers a powerful approach for dissecting the physiological responses linked to separate pathways. However, one constraint is that the variant MEKs need to be expressed at normal levels. Otherwise, overproduction of the enzyme may allow cross-pathway interactions and lead to ambiguities in the interpretation of results.

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