Molecular Biology of the Cell
Vol. 6, 889–909, July 1995

Phosphorylation and Localization of Kss1, a MAP Kinase of the *Saccharomyces cerevisiae* Pheromone Response Pathway

Doreen Ma,* Jeanette Gowen Cook, and Jeremy Thorner†

Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3202

Submitted January 18, 1995; Accepted May 3, 1995
Monitoring Editor: Keith R. Yamamoto

Kss1 protein kinase, and the homologous Fus3 kinase, are required for pheromone signal transduction in *Saccharomyces cerevisiae*. In *MAT***a** haploids exposed to α-factor, Kss1 was rapidly phosphorylated on both Thr183 and Tyr185, and both sites were required for Kss1 function in vivo. De novo protein synthesis was required for sustained pheromone-induced phosphorylation of Kss1. Catalytically inactive Kss1 mutants displayed α-factor–induced phosphorylation on both residues, even in *kss1*Δ cells; hence, autophosphorylation is not obligatory for these modifications. In *kss1*Δ *fus3*Δ double mutants, Kss1 phosphorylation was elevated even in the absence of pheromone; thus, cross-phosphorylation by Fus3 is not responsible for Kss1 activation. In contrast, pheromone-induced Kss1 phosphorylation was eliminated in mutants deficient in two other protein kinases, Ste11 and Ste7. A dominant hyperactive allele of *STE11* caused a dramatic increase in the phosphorylation of Kss1, even in the absence of pheromone stimulation, but required Ste7 for this effect, suggesting an order of function: Ste11 → Ste7 → Kss1. When overproduced, Kss1 stimulated recovery from pheromone-imposed G_1 arrest. Catalytic activity was essential for Kss1 function in signal transmission, but not for its recovery-promoting activity. Kss1 was found almost exclusively in the particulate material and its subcellular fractionation was unaffected by pheromone treatment. Indirect immunofluorescence demonstrated that Kss1 is concentrated in the nucleus and that its distribution is not altered detectably during signaling.

INTRODUCTION

Mammalian cells adjust their division rate, their pattern of gene expression, and their morphology in response to numerous peptide hormones and growth factors. These external cues act through cell surface receptors that stimulate intracellular signal transduction pathways leading to an appropriate cellular response (reviewed in Johnson and Vaillancourt, 1994). Receptor-mediated signal transduction appears to be an evolutionarily ancient mode of intercellular communication because even unicellular eukaryotes, like *Saccharomyces cerevisiae*, release and respond to extracellular peptides. In this yeast, the two haploid cell types (**a** cells and α cells) secrete peptide pheromones (**a**-factor and α-factor, respectively) that bind to cognate G-protein–coupled receptors and trigger a response pathway (for reviews, see Sprague and Thorner, 1992; Bardwell *et al.*, 1994; Oehlen and Cross, 1994). In response to pheromone, the pattern of gene expression changes, progression through the cell cycle is blocked, and the cell wall and cytoskeleton are reorganized leading to a transient developmental transformation of the haploids into cells that possess the characteristics of gametes. The physiological and morphological changes that are elicited allow for the efficient conjugation ("mating") of the haploids to form a diploid cell. Over the last decade, genetic and

* Current address: Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285.
† Corresponding author: Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, Room 401, Barker Hall, Corner of Hearst & Oxford Streets, Berkeley, CA 94720-3202.

© 1995 by The American Society for Cell Biology

biochemical studies have identified at least five protein kinases that are essential components of the pheromone response pathway (for reviews, see Ammerer, 1994; Blumer and Johnson, 1994; Herskowitz, 1995). These enzymes are, in order of discovery, the products of the *STE7* (Teague *et al.*, 1986), *KSS1* (Courchesne *et al.*, 1989), *FUS3* (Elion *et al.*, 1990), *STE11* (Rhodes *et al.*, 1990), and *STE20* (Leberer *et al.*, 1992; Ramer and Davis, 1992) genes.

KSS1 and *FUS3* encode relatively short protein kinases (368 and 353 residues, respectively) that are quite similar to each other (56% amino acid sequence identity). Indeed, genetic analysis has shown that the *KSS1* and *FUS3* gene products serve partially overlapping functions in the pheromone signaling pathway; *kss1* and *fus3* single mutants mate at a readily detectable frequency, whereas *kss1 fus3* double mutants are completely sterile (Elion *et al.*, 1991a,b; Ma and Thorner, unpublished results). Paradoxically, overexpression of *KSS1*, but not *FUS3*, promotes recovery from pheromone-imposed G_1 arrest (Courchesne *et al.*, 1989). In addition, Fus3 is expressed exclusively in haploid cells, whereas Kss1 is expressed in both haploids and diploids. Kss1 is also involved in a second signaling pathway that is required for invasive growth by haploid cells. Other components of the mating signal transduction network also participate in the invasive growth pathway, but Fus3 appears to be an inhibitor of invasive growth (Roberts and Fink, 1994).

Kss1 and Fus3 are highly related (>50% sequence identity) to mitogen-activated or messenger-activated protein kinases (MAPKs), also dubbed extracellular-signal–regulated kinases (ERKs) (Boulton *et al.*, 1990; Cobb *et al.*, 1991). This class of enzymes becomes activated in animal cells in response to a wide variety of growth-stimulatory or differentiation-inducing stimuli, depending on the cell type examined and the stimulus presented (for reviews, see Pelech and Sanghera; 1992; Johnson and Vaillancourt, 1994; Marshall, 1994). Activated MAPKs purified from stimulated animal cells are phosphorylated on both threonine and tyrosine residues (Ray and Sturgill, 1988; Rossomando *et al.*, 1991; Tobe *et al.*, 1991). Treatment with either phosphatase 2A, a phospho-Ser/Thr–specific phosphatase, or CD45, a phospho-Tyr–specific phosphatase, inactivates MAPKs in vitro (Anderson *et al.*, 1990), indicating that both modifications are required for catalytic function. These phosphorylation sites have been mapped (Payne *et al.*, 1991) to a -TEY- site situated between two segments of primary sequence, referred to as subdomains VII and VIII, that are highly conserved in protein kinases (Hanks and Quinn, 1991).

Multiple isoforms of MAPK exist in mammalian cells (Boulton *et al.*, 1991b); in addition to Kss1 and Fus3, at least three other MAPK homologues–Mpk1 (Lee *et al.*, 1993), Hog1 (Brewster *et al.*, 1993), and Smk1 (Krisak *et al.*, 1994)–have been identified in *S. cerevisiae*. MAPKs are also involved in signaling pathways in *Drosophila melanogaster* (Brunner *et al.*, 1994), *Caenorhabditis elegans* (Lackner *et al.*, 1994), and *Xenopus laevis* (Haccard *et al.*, 1993; Waskiewicz and Cooper, 1993). All of these enzymes act as downstream components of a conserved signaling cascade that is composed of the MAPK (ERK), a MAPK/ERK kinase (MEK) that is homologous to *S. cerevisiae* Ste7, and a MEK kinase (MEKK), which in some systems is homologous to *S. cerevisiae* Ste11 and in other systems is homologous to mammalian c-Raf. Although it is clear that MAPK activation is required for a variety of different signaling processes, the mechanisms required for the maintenance of specificity from stimulus to cellular response in the face of such a highly conserved signaling module has yet to be fully explored (Cano and Mahadevan, 1995). It is possible that different isoforms of MAPK are activated in response to different extracellular signals, or are confined to certain subcellular locations. Alternatively, or in addition, the kinetics or extent of activation of particular MAPKs may carry some information for specificity (Qui and Green, 1992; Traverse *et al.*, 1992).

Recently, a number of genetic and biochemical studies of Fus3 have provided considerable insight about the activation and action of this particular yeast MAPK (Gartner *et al.*, 1992; Elion *et al.*, 1993; Errede *et al.*, 1993; Tyers and Futcher, 1993; Zhou *et al.*, 1993; Brill *et al.*, 1994; Kranz *et al.*, 1994; Neiman and Herskowitz, 1994). Little is known, however, about the activation of Kss1 and whether regulation of Kss1 differs from that of Fus3. Here we have applied a combined biochemical and genetic analysis of Kss1 to examine the subcellular distribution of this protein and to define the kinetics of phosphorylation of this enzyme in response to pheromone induction. We also used a similar approach to determine the nature of the components necessary for this activation in vivo and to explore the basis for the ability of Kss1, when overproduced, to promote the resumption of cell cycling after pheromone-induced G_1 arrest.

MATERIALS AND METHODS

Strains and Growth Conditions

The yeast strains used in this work, and their construction, are described in Table 1. For some experiments, the *kss1Δ::HIS3* null mutation was used. Another null allele (*kss1Δ::hisG*) was constructed by introducing a *Bam*HI site at nucleotide 343, according to the numbering of Courchesne *et al.* (1989), using an appropriate oligonucleotide primer and a commercial kit (Amersham, Arlington Heights, IL) for site-directed mutagenesis in bacteriophage M13. A 788-bp *Bam*HI–*Xba*I fragment was removed from the resulting construct and replaced by a fragment containing the *URA3* gene flanked by direct repeats of the *Salmonella typhimurium hisG* gene (Alani *et al.*, 1987). A linear fragment containing the resulting *kss1Δ::hisG-URA3-hisG* construct was released from its host plasmid by restriction enzyme digestion and used to transform a haploid

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

Strain	Genotype	Source
YPH499	*MAT***a** *KSS1*$^+$ *FUS3*$^+$ *ade2-101*^{oc} *his3-Δ200 leu2-Δ1 lys2-801*^{am} *trp1-Δ1 ura3-52*	Sikorski and Hieter (1989)
YPH500	*MATα* (otherwise isogenic to YPH499)	Sikorski and Hieter (1989)
YDM100	YPH499 *kss1Δ::HIS3 FUS3*$^+$	This work[a]
YDM300	YPH499 *kss1Δ::HIS3 fus3-6::LEU2*	This work[b]
YDM230	YPH499 *kss1Δ::hisG fus3-6::LEU2*	This work[c]
YDM400	YPH499 *KSS1*$^+$ *FUS3*$^+$ *sst2Δ::URA3*	This work[d]
DC14[e]	*MAT***a** *his1*	J.B. Hicks
DC17[e]	*MATα his1*	J.B. Hicks
E929-6C-1[f]	*MAT***a** *KSS1*$^+$ *FUS3*$^+$ *cyc1 CYC7-H2 can1 leu2-3,112 trp1-Δ1 ura3-52 ste7-Δ3::LEU2*	Company *et al.* (1988)
JGY7	*MAT***a** *KSS1*$^+$ *FUS3*$^+$ *ade2*^{oc} *his LEU2 lys2 trp1 ura3-52 ste7*^{ts}	This work[g]
JGY11	*MAT***a** *KSS1*$^+$ *FUS3*$^+$ *ade2*^{oc} *his LEU2 lys2 trp1 ura3-52 ste11*^{ts}	This work[h]
FC180	*MAT***a**/*MAT***a** *bar1/bar1 can1/can1 cry1/cry1 cyh2/+his4*^{am}*/+ hmlα::LEU2/hm1α::LEU2 leu2/leu2 lys2*^{oc}*/lys2*^{oc} *SUP4-3(am)*^{ts}*/SUP4-3(am)*^{ts} *trp1*^{am}*/+ ura3/ura3*	F.R. Cross

[a] The *kss1Δ::HIS3* deletion-insertion mutation was excised from plasmid pBC62 (Courchesne *et al.*, 1989) by digestion with *Eco*RI and *Pvu*II, and the resulting linear fragment was used for DNA-mediated transformation of YPH499, selecting for His$^+$ transformants. Correct gene transplacement of the *KSS1* locus was confirmed by restriction enzyme digestion and Southern hybridization analysis.

[b] The *fus3-6::LEU2* allele (Elion *et al.*, 1990) was excised from plasmid pYEE98 (generously provided by E. Elion, Harvard Medical School) by digestion with *Nhe*I and *Hind*III, and the resulting linear fragment was used for DNA-mediated transformation of YDM100, selecting for Leu$^+$ transformants. Correct gene transplacement of the *FUS3* locus was confirmed by restriction enzyme digestion and Southern hybridization analysis.

[c] The *fus3-6::LEU2* mutation was introduced into strain YPH499 as described above in footnote b, and the *kss1Δ::hisG* mutation, which removes 238 of the 368 codons of the *KSS1* coding sequence, also was introduced into the same strain.

[d] To introduce a null mutation in the *SST2* gene (Dietzel and Kurjan, 1987), the two-step gene transplacement method (Scherer and Davis, 1979) was used, as follows. The 3.4-kb *Hind*III–*Sna*BI fragment of *SST2* was ligated into the 4.6-kb vector YIp5 (Struhl *et al.*, 1979) that had been digested to completion with *Hind*III and *Nru*I; the resulting 8-kb plasmid was digested with *Hpa*I to delete an internal 2.3-kb fragment of the *SST2* coding region, and religated. The resulting 5.7-kb plasmid, pBC14 (gift of Dr. W.E. Courchesne, this laboratory), cut at a unique *Nhe*I site in the remaining *SST2* sequence to direct its integration to the genomic *SST2* locus, was used to transform YPH49 by electroporation (Becker and Guarente, 1991). Stable Ura$^+$ transformants were selected on -Ura medium, and then plated on 5-fluoro-orotic acid (5-FOA) medium (Boeke *et al.*, 1984) to select for cells in which the *URA3* gene and, potentially, the normal *SST2* gene, had been excised. Ura$^-$derivatives that lost the normal *SST2* gene and retained the *sst2Δ* allele were identified by their phenotype (hypersensitivity to pheromone action) (Chan and Otte, 1982) and confirmed by restriction enzyme digestion and hybridization analysis (Southern, 1975) of their genomic DNA, isolated as described (Hoffman and Winston, 1987).

[e] From the Davenport/Delbrück Collection, Cold Spring Harbor Laboratory.

[f] Gift of B. Errede, University of North Carolina, Chapel Hill.

[g] Derived from a cross of YPH500 (*MATα*) with 381G-93F (*MAT***a** *ste7*^{ts}) (Hartwell, 1980).

[h] Derived from a cross of YPH500 (*MATα*) with 381G-44B (*MAT***a** *ste11*^{ts}) (Hartwell, 1980).

recipient by electroporation (Becker and Guarente, 1991). Stable Ura$^+$ transformants were selected and shown to contain the *kss1Δ::hisG-URA3-hisG* allele at the chromosomal *KSS1* locus by restriction mapping and Southern hybridization analysis (Southern, 1975). Ura$^-$ derivatives were then selected on medium containing 5-fluoro-orotic acid (Boeke *et al.*, 1984) to obtain derivatives (*kss1Δ::hisG*) from which the *URA3* gene had been excised.

Media were prepared as described by Sherman *et al.* (1986), except that twice the recommended level of nutritional supplements was used in synthetic minimal media. *Escherichia coli* HB101 (Boyer and Roilland-Dussoix, 1969) was used for the propagation of most plasmids, and *E. coli* TG1 (Sambrook *et al.*, 1989) was used for the preparation of single-stranded DNA for mutagenesis and nucleotide sequencing.

Plasmid Constructions and Recombinant DNA Methods

To construct a multi-copy plasmid overexpressing *KSS1*, an *Eco*RI–*Sph*I fragment containing the entire *KSS1* coding sequence and its 5′- and 3′-flanking regions (Courchesne *et al.*, 1989) was ligated into the corresponding sites of the 2 μm DNA vector, YEp352 (Hill *et al.*, 1986), generating YEp-*KSS1*. Appropriate synthetic oligonucleotides and a commercially available kit for site-directed mutagenesis (Amersham) were used, following the manufacturer's instructions and according to the method of Nakamaye and Eckstein (1986), to generate derivatives containing each of the following amino acid substitutions in the *KSS1* coding sequence: Y24F; K42R; K43R; T183A; Y185F; K42R K43R; and T183A Y185F. (Due to an error in oligonucleotide synthesis, the K42R mutant also contained a second alteration, Q45P; however, this change was not present in the K43R and K42R K43R mutants.) All mutations were confirmed by direct nucleotide sequence analysis. To generate a set of multi-copy plasmids, each expressing one of these mutant genes, the corresponding *Eco*RI–*Sph*I fragments were inserted into YEp352, as described above, generating YEp-Y24F, YEp-K42R (Q45P), YEp-K43R, and so forth. To permit regulated overexpression of *KSS1*, transcription of *KSS1* was placed under control of the *GAL1* promoter in the vector YEp352GAL (Benton *et al.*, 1990), generously provided by Dr. B. Benton (this laboratory). To facilitate this construction, site-directed mutagenesis was used to create a *Bam*HI site at the 5′-end of the *KSS1* gene at nucleotide 343 (according to the numbering of Courchesne *et al.*, 1989), by changing the original sequence (GCATCT) to GGATCC. The resulting *Bam*HI–*Sph*I fragment of

KSS1 was ligated into the corresponding sites of YEp352*GAL*, to yield YEp*GAL-KSS1*. Expression of each mutant gene was placed under *GAL1* promoter control using the same scheme. To construct a multi-copy plasmid expressing Fus3, the *FUS3* gene and its promoter were excised from plasmid pYEE81 (Elion *et al.*, 1990) by digestion with *Bam*HI and *Sal*I, and re-ligated into the corresponding sites of YEp352. Plasmid pYGU-*STE11ΔN* (Cairns *et al.*, 1992), which expresses a constitutively active form of Ste11 lacking its N-terminal domain under control of the *GAL1* promoter, was generously provided by Dr. S. Ramer (Stanford University, Stanford, CA).

To generate a Kss1-containing antigen for the preparation of antibodies, a 1.05-kb *Hin*dIII–*Sph*I fragment containing the last 308 codons of the *KSS1* open reading frame was fused in-frame to the C-terminus of the *E. coli trpE* gene in the expression vector pATH23 (Koerner *et al.*, 1991), yielding plasmid TrpE-KSS1MT. To insert an epitope tag into Kss1, a blunt-ended synthetic oligonucleotide encoding an antigenic determinant (-IEEQKLISEEDLLRKRD-) from the c-Myc oncoprotein that is recognized by monoclonal antibody (mAb) 9E10 (Evan *et al.*, 1985), was ligated in-frame with the *KSS1* coding sequence at an *Eco*RV site between codons 8 and 9. The modified *KSS1* gene was subcloned into the *CEN* plasmid pRS316, to generate pRS-*mycKSS1*, and into the multi-copy vector YEp352, to yield YEp-*mycKSS1*.

Preparation of Antiserum

A protease-deficient *E. coli* strain, CAG512, carrying plasmid TrpE-KSS1MT was treated with indole-acrylic acid to induce the expression of the 70-kDa TrpE-Kss1 fusion protein. Inclusion bodies containing the antigen were then isolated by established procedures (Koerner *et al.*, 1991) and the protein in the inclusion bodies was subjected to preparative electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The Coomassie-stained band corresponding to the fusion protein was recovered using an Elutrap elution chamber (Schleicher & Schuell, Keene, NH) and the purified protein was used as antigen to raise polyclonal antiserum in New Zealand White rabbits following standard immunization procedures (Harlow and Lane, 1988).

Hybridoma cell lines (kindly provided by J.M. Bishop, University of California, San Francisco, CA) secreting anti-c-Myc mAb 9E10, which recognizes the epitope employed in this work, were used to produce ascites fluid that was used without further purification. Recloning of hybridoma cell lines and induction of ascites tumors in mice was carried out in the Hybridoma Facility of the Cancer Research Laboratory, University of California, Berkeley, CA.

Bioassays for Pheromone Response and Mating Proficiency

Quantitative mating assays (Sprague, 1991) were conducted by mixing $\sim 10^7$ exponentially growing haploid cells of the strain to be tested with a tenfold excess of haploid cells of the opposite mating type, either DC17 or DC14 (Table 1), collecting the cells on a nitrocellulose filter, rinsing once with rich medium (YPD) (Sherman *et al.*, 1986), and incubating the filter on a YPD plate at 30°C for 5–12 h. To score the number of diploids formed, the cells were eluted from the filters and dispersed by vigorous vortex mixing in sterile water, and various dilutions were plated on medium selective for diploids. After 2 days of growth on selective medium, the number of diploid colonies was counted. Mating efficiency was defined as the number of diploids that arose divided by the number of input haploids of the strain tested. An agar diffusion (halo) assay was used to measure pheromone-induced growth arrest and recovery, as described in detail previously (Julius *et al.*, 1983; Reneke *et al.*, 1988).

Preparation of Cell Extracts and Subcellular Fractionation

Yeast cells were grown at 30°C to mid-exponential phase ($A_{600\ nm}$ = 0.5–1), either in synthetic defined (SD) medium appropriate for the maintenance of plasmids or in rich (YPD) medium. When cells were treated with pheromone, the culture was adjusted to a final concentration of 25 mM sodium succinate (pH 3.5), which decreases the rate of pheromone proteolysis (Ciejek and Thorner, 1979), split into two equal portions, and α-factor (12 μM final concentration) was added to one sample. At various times after pheromone addition, aliquots were removed, chilled on ice, harvested by brief centrifugation in a microfuge, washed twice by resuspension and resedimentation in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 3 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and resuspended in the same buffer at a concentration of $A_{600\ nm}$ = 10 per 100 μl. All subsequent steps were carried out at 4°C. Pre-chilled glass beads (0.45–0.6 mm diameter) were added to the meniscus of the cell suspension and lysis was achieved by vigorous vortex mixing for six 1-min intervals, with intermittent cooling on ice. To remove the glass beads, the bottom of the microfuge tube was punctured with a syringe needle (~0.5 mm diameter) and inserted into another tube; the lysate was collected into the fresh tube by brief centrifugation in a clinical centrifuge. The crude extract was subjected to centrifugation at 2000 rpm for 5 min in a Sorvall SS34 rotor to remove unbroken cells and large debris. The clarified lysate was then subjected to sedimentation for 20 min at 60,000 rpm (100,000 × *g*) in a tabletop ultracentrifuge (Beckman TL-100, TLA 100.3 rotor; Beckman Instruments, Fullerton, CA). The supernatant fraction (HSS) was withdrawn to a fresh tube and the pellet was resuspended in lysis buffer using a plastic pestle. The resuspended material was recentrifuged for 20 min at 60,000 rpm, yielding the final washed pellet (HSP).

When only total extract was needed, cultures were grown and harvested as described above, but were resuspended in lysis buffer containing 1% SDS before disruption with glass beads and boiling, which minimized proteolysis of Kss1.

Immunodetection of Protein Blots

For immunoblot analysis, 50–100 μg of protein were subjected to electrophoresis in a 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose was carried out by standard procedures (Towbin *et al.*, 1979). To detect Kss1, the rabbit polyclonal antiserum described above, or the 9E10 mAb-containing ascites fluid, was incubated with the filter at a dilution of 1:1000. Proteins on immunoblots were detected using a commercially available chemiluminescence detection system (ECL, Amersham), as recommended by the manufacturer.

Radiolabeling of Cells and Immunoprecipitation of Polypeptides

To label total cellular proteins, cells were grown at 30°C to mid-exponential phase in low sulfate/low phosphate medium (Julius *et al.*, 1984) supplemented with all L-amino acids except Cys and Met, resuspended at a density of $A_{600\ nm}$ = 2 per ml, and incubated for 60 min either with 30 μC_i of [^{35}S]Cys and [^{35}S]Met (Tran^{35}S-Label, ICN, Cleveland, OH) or with 200 μCi of $^{32}PO_4^{3-}$ (Dupont-NEN, Boston, MA) per $A_{600\ nm}$ of cells. To inhibit protein synthesis before labeling, cycloheximide was added to a final concentration of 10 μg/ml, and the culture was incubated for 15 min before addition of the radioisotopes. Pheromone treatment was carried out as described above. At various times after exposure to pheromone, samples of the control and pheromone-treated cultures were removed, chilled, and mixed with an equal volume of ice-cold 20 mM NaN_3 to terminate labeling. The radiolabeled cells were collected by brief sedimentation in a clinical centrifuge, washed twice by successive resuspen-

sions and recentrifugations in ice-cold 10 mM NaN_3, and finally resuspended at a density of 2 $A_{600\ nm}$/100 μl in ice-cold extraction buffer containing an assortment of phosphoprotein phosphatase inhibitors and protease inhibitors (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 3 mM dithiothreitol, 10 mM sodium pyrophosphate, 10 mM NaN_3, 10 mM NaF, 0.4 mM sodium *meta*-vanadate, 0.4 mM sodium *ortho*-vanadate, 0.1 mM β-glycerol-phosphate, 1 μg/ml phosvitin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin A).

Cell extracts were prepared and separated into the particulate fraction (HSP) and the cytosolic fraction (HSS), as described above. The particulate material was resuspended in a volume of extraction buffer equal to the volume of cytosol recovered. SDS was added to each fraction to a final concentration of 1%, and the proteins were solubilized by boiling for 5 min. For analysis of mutant Kss1 proteins, total extracts were solubilized with SDS in the same way. Before immunoprecipitation, the sample to be examined was diluted with an equal volume of 2× immunoprecipitation buffer (IP) (2× = 50 mM Tris-HCl, pH 7.5, 4 mM EGTA, 300 mM NaCl, 10 mM NaF, 2% NP-40, 1% deoxycholate). The following pre-treatments were used to remove proteins that associate nonspecifically with immune complexes. First, IgG-Sorb (The Enzyme Center, Boston, MA) was added (20 μl of a 20% suspension in IP containing 10 mg/ml bovine serum albumin [IPB] per 200 μl of the sample). After incubation on ice for 30 min, the mixture was clarified by centrifugation for 1 min in a microfuge and the supernatant solution was withdrawn to a fresh tube. Second, pre-immune rabbit serum (4 μl) was added and, after 1 h on ice, a fresh portion of IgG-Sorb (20 μl) was added. After incubation on ice for 30 min, the mixture was clarified by centrifugation as above and the supernatant solution was removed to another fresh tube. To this pre-cleared extract, 4 μl of a rabbit polyclonal anti-Kss1 serum (#9325) was added, and the mixture was incubated for 3 to 12 h on ice. An aliquot of IgG-Sorb (20 μl), which had been pre-adsorbed with an excess of a total lysate from unlabeled cells of strain YDM230 (Table 1) was added, and the mixture was incubated for an additional 30 min. Immune complexes were collected by centrifugation and washed successively by resuspension and recentrifugation in two portions (1 ml each) of the following three solutions: IPB + 2 M urea; IPB + 250 mM NaCl; and 10 mM Tris-HCl, pH 7.5, 50 mM NaCl. The final washed pellet was resuspended in 30 μl gel electrophoresis sample buffer (Laemmli, 1970) and boiled for 5 min. Samples were clarified by centrifugation for 5 min in a microfuge, and the supernatant solution (typically ~25 μl) was applied to 10% SDS-PAGE. Fluorography of dried gels was performed with a commercially available amplifier (Amplify, Amersham) using conditions recommended by the manufacturer.

Phosphoamino Acid Analysis

Kss1 protein was labeled with $^{32}PO_4^{3-}$ in the absence and presence of pheromone, collected by immunoprecipitation, and subjected to SDS-PAGE, along with ^{14}C-labeled molecular weight standards (Amersham), by the methods described above. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane filter (Millipore, Bedford, MA) by electroblotting in 48 mM Tris base, 39 mM glycine-HCl, pH 8.6, 20% methanol, using a Multiphor II apparatus (Pharmacia-LKB, Uppsala, Sweden), as recommended by the supplier, except that the current was reduced to 0.4 mA/cm^2 and the transfer time was extended to 2 h. The prominent band corresponding to Kss1 was located by autoradiography of the PVDF filter using Kodak XAR film overnight at −70°C with an intensifying screen. The strip corresponding to Kss1 was excised and either immersed directly in freshly prepared 6 N HCl and incubated at 110°C for 1 h, or exposed to 6 N HCl vapor under vacuum at 110°C for 4 h and then eluted with four 50-μl portions of distilled deionized water (Hildebrandt and Fried, 1989). The membrane was discarded and the hydrolysate was lyophilized. The dried material was resuspended in 5 μl of pH 1.9 buffer (2.5% concentrated formic acid, 7.8% glacial acetic acid) containing 2 μg each of phosphoserine, phosphothreonine, and phosphotyrosine, and subjected to two-dimensional thin layer electrophoresis on microcrystalline cellulose plates essentially as described by Cooper *et al.* (1983). After drying, the phosphoamino acid standards were visualized by spraying the plate with 0.2% ninhydrin in ethanol and heating, and the radioactivity present was quantified using a phosphorescent screen and a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) or by autoradiography with x-ray film for 2 wk, as described above.

Indirect Immunofluorescence

FC180 cells (a gift of Fred Cross, Rockefeller University, New York, NY) containing different plasmids were grown to mid-exponential phase. The cells were then fixed by adding formaldehyde to the culture to a final concentration of 5% (w/v). The fixed cells were harvested, washed twice, and resuspended at 5 A_{600}/ml in 1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5, 50 mM β-mercaptoethanol. The cell wall was digested using Zymolyase 100T (Kirin Brewery, Japan) at 100 μg/ml for 30 min at 30°C. The digested fixed cells were then washed by resuspension and recentrifugation three times with the same buffer lacking β-mercaptoethanol and resuspended in the same solution at 2 A_{600}/ml. Samples (20 μl) of the cells were spotted into the wells of a microscope slide that had been pre-coated with poly-L-lysine and allowed to settle for 30 min. To permeabilize the cells, the slide was immersed in methanol (−20°C) for 6 min and then in acetone (−20°C) for 30 s. Cells were rehydrated with phosphate-buffered saline containing bovine serum albumin (PBSA) (170 mM NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 containing 10 mg/ml bovine serum albumin). Antibody decoration was performed essentially as described by Pringle *et al.* (1991). Ascites fluid containing anti-c-Myc mAb 9E10 was used at dilutions of 1:33, 1:100, and 1:300. The secondary antibody, purified fluorescein isothiocyanate-conjugated sheep anti-mouse Ig (Boehringer, Indianapolis, IN), was used at 1:50 and 1:200 dilutions. Slides were mounted in Citifluor after a 5-min treatment with 1 μg/ml DAPI. A Nikon Optiphot fluorescence microscope was used to examine the cells at 400×-1000× magnification. Images were recorded on Kodak Ektachrome P800/1600 color reversal film using a Nikon EX-35WA camera attached to a AFX-IIA automatic shutter control.

RESULTS

Specific Immunodetection of the KSS1 Gene Product

To monitor expression of the 368-residue *KSS1* gene product, two complementary approaches were taken. First, rabbit polyclonal antibodies were raised against a large carboxyl-terminal segment of the *KSS1* coding sequence (residues 61–368) that was expressed in bacterial cells as a fusion to *E. coli* TrpE. Upon SDS-PAGE and immunoblotting of cell extracts, this antiserum cross-reacted nonspecifically with several bands of higher apparent molecular mass; however, the most rapidly migrating species recognized by these antibodies was the Kss1 protein because this band was present in wild-type cells at a low level, was completely absent in two independently constructed *kss1* null mutants, and was markedly more prominent in cells expressing the *KSS1* gene from its own promoter on a multicopy plasmid (Figure 1A). Furthermore, the mobility of this band corresponded to an $M_r$app of 43 kDa, in excellent agreement with the calculated mo-

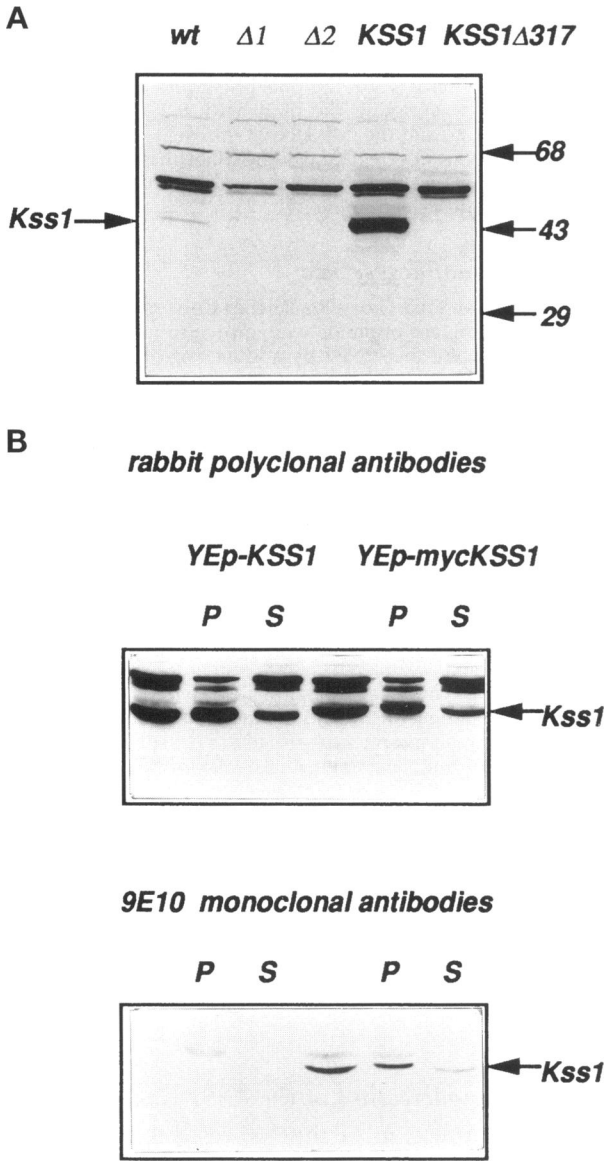

Figure 1. Specificity of anti-Kss1 antibodies. (A) Extracts were prepared from a wild-type *KSS1*$^+$ strain (YPH499; wt), and otherwise isogenic strains carrying either the *kss1Δ::HIS3* mutation (YDM100; Δ1) or the *kss1Δ::hisG* mutation (YDM230; Δ2), or the same strain transformed with a multi-copy plasmid, YEp-*KSS1*, expressing either intact Kss1 from its own promoter (KSS1) or a C-terminal truncation mutation, Kss1(Δ317–368) (KSS1Δ317), resolved by SDS-PAGE, transferred electrophoretically to a nitrocellulose filter, and probed with a rabbit polyclonal antiserum (#9325) raised against a TrpE-Kss1 fusion protein, as described in MATERIALS AND METHODS. (B) Clarified total cell lysates (unlabeled lanes) were prepared from YPH499 transformed with either YE-*KSS1* or YEp-*mycKSS1*, as indicated, and extracts were then fractionated by centrifugation at 100,000 × *g* into a pellet fraction ("P") and a supernatant fraction ("S"). Identical samples of the total lysate, P, and S fractions were resolved on two separate SDS gels, transferred to nitrocellulose, and probed with either antiserum #9325 (top section) or with anti-c-Myc mAb 9E10 (bottom section), as indicated.

lecular mass of the Kss1 polypeptide (42.7 kDa) (Courchesne *et al.*, 1989), as determined by the mobility of the immunoreactive band with respect to the migration of marker proteins of known molecular mass. This antiserum appeared to be directed primarily against antigenic determinants at the carboxyl-terminus of Kss1 because a trunction mutant lacking the 51 most C-terminal residues was not recognized by this antiserum (Figure 1A), but was recognized by other antisera we raised against different portions of Kss1 (our unpublished results).

As a second method for monitoring Kss1, a derivative was constructed that contained a 17-residue c-Myc epitope inserted between residues 8 and 9 of the *KSS1* coding sequence. As expected, this epitope-tagged version of Kss1 displayed a slightly slower electrophoretic mobility than authentic Kss1 on SDS-PAGE and was detected in a highly specific manner by the 9E10 anti-c-Myc mAb (Figure 1B). The tagged protein retained readily detectable *KSS1* function, as judged by complementation tests (see below).

Phosphorylation State of Kss1 Kinase Is Modulated in Response to Pheromone

To determine if the sequence homology between Kss1 and other known ERKs and MAPKs reflects functional similarity, the state of phosphorylation of Kss1 in response to stimulation of the pheromone signal transduction pathway was examined. To facilitate detection and analysis of Kss1 protein, wild-type *MAT***a** cells overexpressing *KSS1* about 30-fold from a multi-copy plasmid (Figure 1) were labeled with $^{32}PO_4^{3-}$ to steady-state during balanced growth, pre-treated with the protein synthesis inhibitor cycloheximide to ensure that any changes observed were due to primary effects of pheromone action on pre-existing protein, and then exposed to α-factor. At various times thereafter, samples were withdrawn, extracts were prepared, and Kss1 was recovered by immunoprecipitation with the rabbit polyclonal anti-Kss1 antibodies from both the cytosolic and particulate fractions that had been solubilized with SDS. Somewhat unexpectedly, based on the reported fractionation properties of mammalian Erk1 and Erk2 (see, for example, Ahn *et al.*, 1990; Boulton *et al.*, 1991a), the majority of the total phosphorylated Kss1 was found in the particulate fraction (Figure 2A). Kss1 was clearly a phosphoprotein even in naive cells unchallenged with pheromone, and this level of phosphorylation did not change appreciably over the course of 90 min. Basal phosphorylation of endogenous Kss1 was also detectable in the absence of overexpression (see Figure 5, A). In contrast, exposure of haploid cells to α-factor caused a rapid and transient phosphorylation of Kss1, which peaked about 5 min after exposure to pheromone (Figure 2A). As judged by quantitation using a Phos-

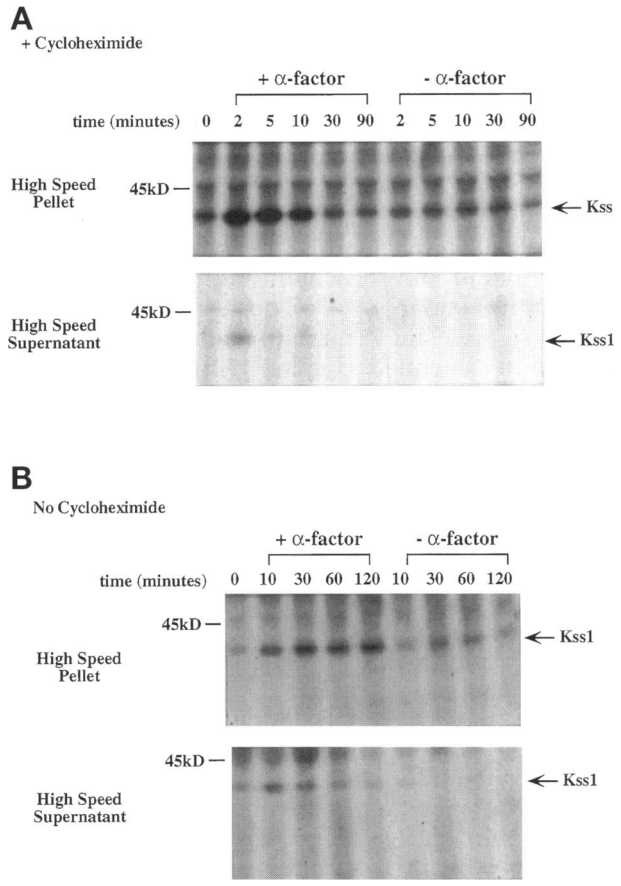

Figure 2. Pheromone-induced phosphorylation of Kss1. A culture (50 ml) of exponentially-growing cells of strain YPH499 carrying YEp-*KSS1* was labeled with 10 mCi of $^{32}PO_4^{3-}$ in low sulfate-low phosphate medium for 1 h at 30°C. The culture was then divided into two equal portions. One sample was treated with 10 μg/ml cycloheximide (A), and the other was left untreated (B). Each sample was then split again and adjusted to pH 3.5, as described in MATERIALS AND METHODS. One portion received α-factor at a final concentration of 12 μM (+ α-factor); the other portion was left untreated (− α-factor). At the indicated times after α-factor addition, aliquots of each culture (equivalent to 2 $A_{600\ nm}$ units) were withdrawn and quenched on ice. The cells were collected by centrifugation, washed, and lysed. Clarified extracts were then fractionated by centrifugation at 100,000 × *g* into a high speed pellet (upper panels in A and B) and the corresponding supernatant fraction (bottom panels in A and B), subjected to immunoprecipitation with rabbit polyclonal anti-Kss1 antibodies (serum #9325), and analyzed by SDS-PAGE and fluorography as described in MATERIALS AND METHODS. The migration position of a ^{14}C-labeled 45-kDa marker protein is indicated on the left.

phorImager, the level of phosphorylation at 5 min in the pheromone-treated cells was at least fivefold higher than the corresponding time point from the control culture, but returned to the basal level by 30 min after exposure to α-factor. Because the cells were pre-treated with cycloheximide before pheromone addition, the increased incorporation of ^{32}P clearly represented a stimulation of the phosphorylation of pre-existing Kss1 molecules and not synthesis of more Kss1 protein. On the other hand, the relatively rapid decrease in phosphorylated Kss1 after its initial elevation might have been due to pheromone-induced degradation of the protein. Immunoblot analysis revealed, however, that the cellular content of Kss1 remained essentially constant in both the particulate and soluble fractions of cycloheximide-treated cells in the absence or presence of pheromone over the next 2 h (our unpublished results).

De Novo Protein Synthesis Is Required for Sustained Pheromone-induced Phosphorylation of Kss1

The kinetics of Kss1 modification in the presence of cycloheximide demonstrated that α-factor–induced phosphorylation was a primary response to pheromone, but it was also important to determine the level of Kss1 phosphorylation after α-factor stimulation in growing cells capable of subsequent mating. For this purpose, the state of Kss1 phosphorylation was followed by radiolabeling and immunoprecipitation from cells in which protein synthesis was not inhibited. Under these conditions, an even more pronounced and sustained level of pheromone-induced incorporation was observed; even 2 h after exposure to α-factor, Kss1 phosphorylation remained markedly elevated compared with the control (Figure 2B). Immunoblot analysis (Figure 3A), as well as immunoprecipitation of Kss1 from cells labeled with $^{35}SO_4^{2-}$ (our unpublished results), demonstrated that there was no significant increase in the cellular content of Kss1 protein over this same time period. Thus, the prolongation of induced Kss1 phosphorylation was not attributable to an increased amount of Kss1 protein.

Phosphorylation of both Thr183 and Tyr185 Are Essential for KSS1 Function

After establishing that phosphorylation of Kss1 was markedly stimulated upon exposure to pheromone, the sites of Kss1 phosphorylation were determined by a combination of chemical and genetic methods. To determine the nature of the pheromone-induced modifications, phosphoamino acid analysis was performed on samples of phosphorylated Kss1 purified by immunoprecipitation and gel electrophoresis (see MATERIALS AND METHODS). Even in the absence of pheromone treatment, the majority of the label was found in phosphotyrosine, but there were also readily detectable amounts of phosphoserine and phosphothreonine (Figure 3B). Reproducibly, in five independent analyses, the qualitative changes upon pheromone stimulation were increases in the relative amounts of phosphotyrosine and phosphothreonine, and a decrease in the relative amount of phosphoserine (Figure 3B). None of these phosphorylation

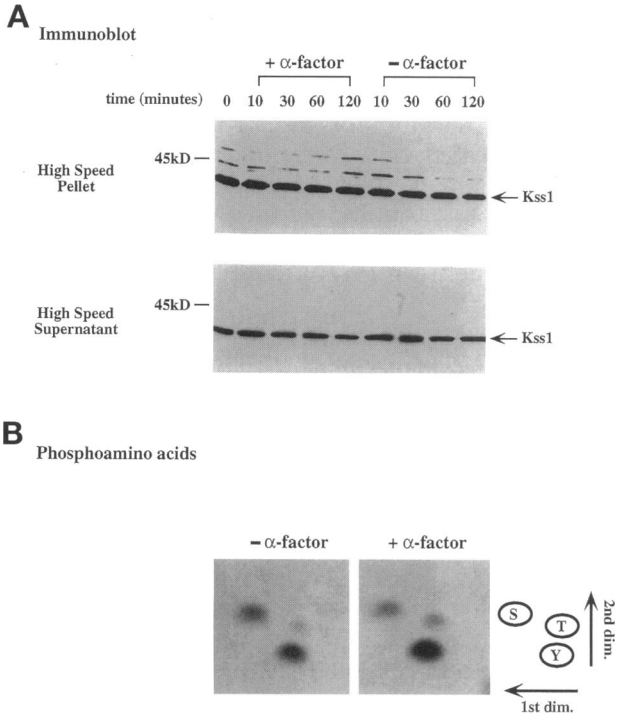

Figure 3. Analysis of the steady-state level and phosphoamino acid content of Kss1. (A) Kss1 protein level remains constant after exposure of cells to α-factor. A culture of strain YPH499 carrying YEp-*KSS1* was grown to mid-exponential phase and then divided into two equal portions. One portion was treated with 12 μM α-factor (+ α-factor); the other portion was left untreated (− α-factor). Samples of equal volume were withdrawn at the indicated times and quenched on ice. The cells were collected by centrifugation, washed, and lysed. Clarified extracts were then fractionated by centrifugation at 100,000 × *g* into a high speed pellet (upper panel) and the corresponding supernatant fraction (bottom panel), and then analyzed by SDS-PAGE and immunoblotting with polyclonal anti-Kss1 antibodies and a chemiluminescence detection system, as described in MATERIALS AND METHODS. (B) Pheromone treatment enhances Kss1 phosphorylation on Tyr and Thr. A culture of strain YPH499 harboring YEp-*KSS1* was labeled with $^{32}PO_4^{3-}$, split into two equal portions, one of which was treated with α-factor for 5 min in the absence of cycloheximide, essentially as described in the legend to Figure 2. Total extracts of both the pheromone-treated and untreated cells were solubilized in SDS and subjected to immunoprecipitation with rabbit polyclonal anti-Kss1 antibodies. The resulting immunoprecipitates were fractionated by SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography to locate the bands corresponding to Kss1. The appropriate portions of the PVDF filter were excised and the protein was hydrolyzed to its constituent amino acids using HCl vapor. Authentic phosphotyrosine, phosphothreonine, and phosphoserine were added (to serve as both carrier and internal standards) to the resulting hydrolysates (~2,000 cpm each), which were then analyzed by two-dimensional thin layer electrophoresis and autoradiography, as described in MATERIALS AND METHODS. Kss1 from naive cells (left); Kss1 from pheromone-treated cells (right). Positions of the phosphoamino acid markers revealed by staining with ninhydrin (inset).

events appears to be the result of autophosphorylation because phosphoamino acid analysis of a catalytically inactive mutant of Kss1 (described below) produced identical results (our unpublished results).

To pinpoint the residues that undergo modification, we used site-directed mutagenesis of the *KSS1* gene and examined the state of phosphorylation of the mutant gene products before and after pheromone treatment. In addition, we also evaluated the functional status of these Kss1 variants by examining their ability to complement the mating defect of a *kss1*Δ *fus3*Δ double mutant and their ability to promote recovery from pheromone-imposed G_1 arrest (see below). For this study, five single mutants and two double mutants were generated (Figure 4A). Like Cdc28 and other Cdks required for cell cycle progression (De Bondt *et al.*, 1993), and like mammalian Erk2 (Zhang *et al.*, 1994), Kss1 possesses a Tyr residue in the Gly-rich loop that anchors the γ-phosphate of the ATP in the catalytic site, which corresponds to domain I conserved in all protein kinases (Hanks and Quinn, 1991; Knighton *et al.*, 1991b). In *Schizosaccharomyces pombe* Cdc2 (Gould and Nurse, 1989) and its human homologue (Morla *et al.*, 1989), phosphorylation and dephosphorylation of the Tyr at this position is a critical aspect of the control of the activity of this kinase. Therefore, the Tyr codon at the analogous position (GEGAYG) in the *KSS1* coding sequence was changed to a Phe codon (mutant Y24F). Another feature shared by the Cdks and the MAPKs, but found in very few other protein kinases, is a pair of Lys residues in conserved domain II. In these enzymes, and in the corresponding sequence of Kss1 (VAIKKI), the first Lys aligns with an invariant Lys found in all other protein kinases (Hanks and Quinn, 1991). In nearly every protein kinase examined to date, including a murine MAPK (Wu *et al.*, 1991), mutation of this Lys to any other residue, even to Arg (Bossemeyer, 1993), abolishes detectable kinase activity in vitro and biological function in vivo (reviewed in Hanks *et al.*, 1988). There are a few examples, however, of protein kinases in which alteration of the conserved Lys drastically reduces (>95%), but does not totally abolish catalytic activity (Robbins *et al.*, 1993; Brill *et al.*, 1994). Therefore, in the absence of the conserved Lys42, it was possible that Lys43 might allow for some residual Kss1 activity in vivo. As a means to examine the requirement for the intrinsic kinase activity of Kss1 in its basal and pheromone-induced phosphorylation, mutations that eliminated these Lys residues in Kss1 singly (mutants K42R and K43R), and in combination (mutant K42R K43R), were generated (Figure 4A). Finally, the codons specifying the residues of Kss1 (FMTEYVA) that correspond to the sites of tyrosine and threonine phosphorylation required for activation of mammalian MAPKs (Anderson *et al.*, 1990; Payne *et al.*, 1991) were changed to their nonphosphorylatable counterparts, singly (mutants T183A and Y185F) and in combination (mutant T183A Y185F) (Figure 4A).

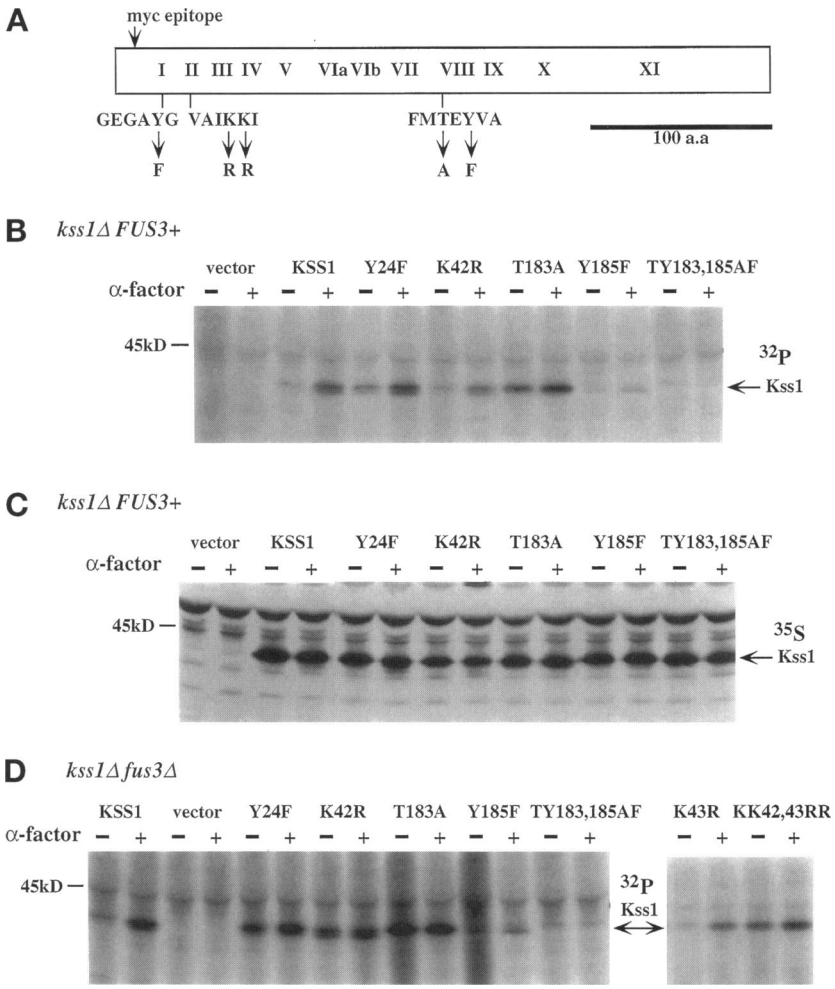

Figure 4. Phosphorylation of site-specific mutants of Kss1. (A) Location of site-directed mutations within the primary sequence of Kss1. The open bar represents the Kss1 polypeptide. The Roman numerals within the bar denote the relative positions of the eleven sequence domains highly conserved in protein kinases (Hanks *et al.*, 1988; Hanks and Quinn, 1991). The locations of five single mutations (Y24F, K42R, K43R, T183A, and Y185F), two double mutations (K42R K43R and T183A Y185F), and the site of insertion of a c-Myc epitope tag are indicated by arrows. The K42R mutation also inadvertently contained another substitution, Q45P. (B) Phosphorylation of the site-specific Kss1 mutants in a *kss1Δ FUS3*$^+$ strain. Exponentially growing cells of strain YDM100 carrying either vector alone (YEp352), plasmid YEp-*KSS1*, or multicopy plasmids expressing the various Kss1 mutants indicated, were labeled with $^{32}PO_4^{3-}$ for 1 h at 30°C. Each culture was then treated with 10 μg/ml cycloheximide for 15 min, then divided into two equal portions, one of which was treated with 12 μM α-factor for 5 min (+) and the other of which was left untreated (−). Total extracts of samples of each culture (equivalent to 2 A_{600} units) were prepared, solubilized in SDS, and subjected to immunoprecipitation with rabbit polyclonal anti-Kss1 antibodies and analyzed as described in the legend to Figure 2. (C) Steady-state level of the site-specific Kss1 mutants in a *kss1Δ FUS3*$^+$ strain. Radiolabeling, lysis, immunoprecipitation, and analysis was conducted exactly as described in part B, except that the cells were labeled with a mixture of [^{35}S]Met and [^{35}S]Cys. (D) Phosphorylation of the site-specific Kss1 mutants in a *kss1Δ fus3Δ* strain. Exponentially growing cells of strain YDM300 carrying either vector alone (YEp352), plasmid YEp-KSS1, or multicopy plasmids expressing the various Kss1 mutants indicated, were labeled with $^{32}PO_4^{3-}$, and the Kss1 polypeptides were recovered and analyzed, exactly as described in part B.

To examine the effect of these mutations on the steady-state level and phosphorylation state of Kss1, *MAT***a** *kss1Δ* cells (YDM100) expressing from a multicopy plasmid either the normal protein or each of the mutants were pre-labeled for 1 h with either [^{35}S]Cys and [^{35}S]Met, or with $^{32}PO_4^{3-}$, treated with cycloheximide for 15 min and then with α-factor for 5 min. Extracts of the cells were analyzed by immunoprecipitation. In *kss1Δ* cells carrying the vector alone, no 43-kDa species was immunoprecipitable, as expected (Figure 4B). All of the mutant proteins were expressed at a level similar to wild-type Kss1, except perhaps the K42R mutant, based on the amount of ^{35}S-labeled protein recovered (Figure 4C) and by immunoblot analysis (our unpublished results). However, the various mutants differed strikingly in their state of phosphorylation (Figure 4B). We noted that, as in the initial experiments (Figure 2), normal Kss1 protein was resolved into a doublet of ^{32}P-labeled species, and that the amount of label incorporated into both species was markedly enhanced in response to pheromone. For the Y24F mutant, the pattern of basal and pheromone-induced phosphorylation was not detectably altered, suggesting that, unlike Cdc2, the Tyr in the nucleotide-binding loop of Kss1 is not a major site for phosphorylation (Figure 4B). In contrast, for the Y185F mutant, the amount of incorporation was greatly reduced and the upper band of the doublet was eliminated, indicating that the Tyr in the TEY motif is a major phosphorylation site (Figure 4B). In the T183A mutant, the lower band of the doublet was eliminated, but the total amount of incorporation was not dramatically reduced, suggesting that Thr183 of the TEY motif is a less prominent phosphorylation site than Tyr185, in agreement with the results of the phosphoamino acid analysis. As expected if Thr183 and Tyr185 are the major sites of phosphorylation, the T183A Y185F double mutant showed little or no ^{32}P incorporation in either the absence or presence of pheromone (Figure 4B), despite the fact that phosphoamino acid analysis

detected significant amounts of phosphoserine in wild-type Kss1. It is possible that phosphorylation at Thr183 and/or Tyr185 is normally a prerequisite for Kss1 phosphorylation at one or more Ser residues.

Pheromone-stimulated Phosphorylation of Kss1 Does Not Require Autophosphorylation

MAPKs are capable of undergoing autocatalytic activation in vitro, albeit at a slow rate and to a low extent (Robbins and Cobb, 1992; Seger *et al.*, 1991). To examine whether autophosphorylation is obligatory for either basal or pheromone-induced phosphorylation of Kss1, the K42R mutant was examined. We found that, when normalized for the somewhat lower recovery of this variant upon immunoprecipitation (Figure 4C), the pattern of both its basal and pheromone-stimulated phosphorylation was essentially indistinguishable from wild-type Kss1 (Figure 4B). Because this mutation completely abolished *KSS1* function in vivo (see Table 2), presumably because catalytic activity had been totally destroyed, these results indicated that the intrinsic kinase activity of Kss1 is not required for its phosphorylation in vivo. To eliminate the possibility that, in the K42R mutant, Lys43 was capable of functionally substituting for the altered residue, the phosphorylation state of a K43R and a K42R K43R double mutant was also examined (Figure 4D). Like the K42R mutant, the K43R mutant and even the K42R K43R double mutant displayed both basal and pheromone-induced phosphorylation and migrated as a doublet identical to that found for wild-type Kss1. These results confirmed that both the basal and the pheromone-induced phosphorylation of Kss1 must be mediated by another cellular protein kinase(s).

Fus3 Is Not the Kinase Responsible for Phosphorylation of Kss1

Kss1 and Fus3 are homologous in sequence and partially overlapping in function (Elion *et al.*, 1991ab; Ma, Cook, Hasson and Thorner, unpublished data). Furthermore, the *FUS3* gene is pheromone-inducible (Elion *et al.*, 1990); and, as shown here for Kss1, the Fus3 enzyme is phosphorylated in response to pheromone (Gartner *et al.*, 1992; Doi *et al.*, 1994). It was possible, therefore, that Fus3 might be the cellular kinase responsible for the basal and/or the pheromone-induced phosphorylation of Kss1. To examine this possibility, we determined the phosphorylation state of normal Kss1 and the site-directed mutants in a *MAT***a** *kss1*Δ *fus3*Δ double mutant (YDM300). The pattern of phosphorylation observed was similar to that found in the MAT**a** *kss1*Δ strain, except that the basal level of phosphorylation of all of the inactive, but phosphorylatable, variants was distinctly higher (Figure 4D). Taken together, these findings indicate, first, that Fus3 is clearly not required for either the basal or pheromone-stimulated modification of Kss1 and, second, that the presence of either a functional Kss1 or a functional Fus3 actually suppresses basal phosphorylation.

Table 2. Catalytic function of Kss1 kinase is required for the mating process

MAT[a]	*KSS1*	*FUS3*	Plasmid[b]	Input haploids	Diploids formed	Mating efficiency[c]
				($\times 10^{-7}$)	($\times 10^{-7}$)	(% wild type)[d]
a	+	+	None	2.8–3.5	1.7–7.0	[100]
	Δ	Δ	YEp352	1.4–2.1	0	0
	Δ	Δ	YEp*FUS3*	1.6–1.9	0.4–4.0	35–132
	Δ	Δ	YEp*KSS1*	1.3–1.6	0.01–0.08	1–2.6
	Δ	Δ	YEp-Y24F	1.1–2.0	0	0
	Δ	Δ	YEp-K42R	0.8–1.9	0	0
	Δ	Δ	YEp-K43R	1.6–2.0	0.04–0.05	1.3–3.2
	Δ	Δ	YEp-RR	1.6–1.7	0	0
	Δ	Δ	YEp-T183A	1.1–2.1	0	0
	Δ	Δ	YEp-Y185F	0.8–2.2	0	0
	Δ	Δ	YEp-AF	0.4–1.9	0	0

[a] The "+" denotes a strain (YPH499) carrying wild-type *KSS1* and *FUS3* loci; whereas the "Δ" denotes an otherwise isogenic strain (YDM230; Table 1) carrying *kss1* and *fus3* null alleles.

[b] All plasmids are derivatives of YEp352, constructed as described in MATERIALS AND METHODS. YEp-RR = YEp-K42R, K43R; YEp-AF = YEp-T183A, Y185F.

[c] Values given represent the range observed in four independent trials, each conducted in duplicate, using DC17 as the *MAT*α partner.

[d] For the purposes of comparison, mating efficiency was calculated in each experiment as (number of diploids formed per number of input haploids of the indicated genotype) × 100, and then normalized to the value observed in the same experiment for the wild-type control, which was set at 100%.

Ste11 and Ste7 Act Upstream of Kss1

Recent genetic and biochemical data demonstrate that the Ste11 and Ste7 protein kinases act upstream of Fus3 (Cairns *et al.*, 1992; Gartner *et al.*, 1992; Stevenson *et al.*, 1992) and that Fus3 is the direct substrate of Ste7 (Elion *et al.*, 1993; Errede *et al.*, 1993; Zhou *et al.*, 1993; Neiman and Herskowitz, 1994). To determine if the phosphorylation of Kss1 was also dependent on the activity of these kinases, *KSS1* was overexpressed from a multi-copy plasmid in strains harboring temperature-sensitive alleles of either *STE7* or *STE11* (Hartwell, 1980), and the phosphorylation state of Kss1 in these cells was examined at both the permissive and restrictive temperatures in the absence or presence of α-factor. Temperature-sensitive alleles were chosen (instead of null alleles) because, in the absence of a functional pheromone response pathway, a number of components of the pathway, including the α-factor receptor (Ste2), are poorly expressed (reviewed in Sprague and Thorner, 1992). In cells containing a temperature-sensitive Ste7, the basal and pheromone-dependent phosphorylation of Kss1 were barely detectable even at the permissive temperature (Figure 5A). This defect in phosphorylation correlated with the weak mating supported by this particular *ste7*^{ts} allele even at the permissive temperature (Cook, unpublished observations). At the restrictive temperature, phosphorylation of Kss1 in both the presence and absence of pheromone was abolished in the *ste7*^{ts} cells, indicating that phosphorylation of Kss1 absolutely requires the function of Ste7. In cells harboring a temperature-sensitive *ste11* allele, Kss1 was phosphorylated essentially as in wild-type strains at the permissive temperature; but, at the restrictive temperature, Kss1 was only very poorly phosphorylated, even upon pheromone stimulation (Figure 5A). Thus, Kss1 phosphorylation also requires a functional Ste11 enzyme.

Ste11 can be constitutively activated in the absence of pheromone treatment by mutations that delete its N-terminus, which is thought, therefore, to constitute a negative regulatory domain (Cairns *et al.*, 1992; Ramer *et al.*, 1992). Transformants carrying both multicopy *KSS1* and a plasmid (pYGU-*STE11ΔN*) expressing such an N-terminally truncated allele of *STE11* under control of the *GAL1* promoter were examined for the ability to phosphorylate Kss1 in the absence or presence of α-factor. When these cells were grown and labeled in glucose-containing medium, where the *GAL1* promoter is repressed, Kss1 was phosphory-

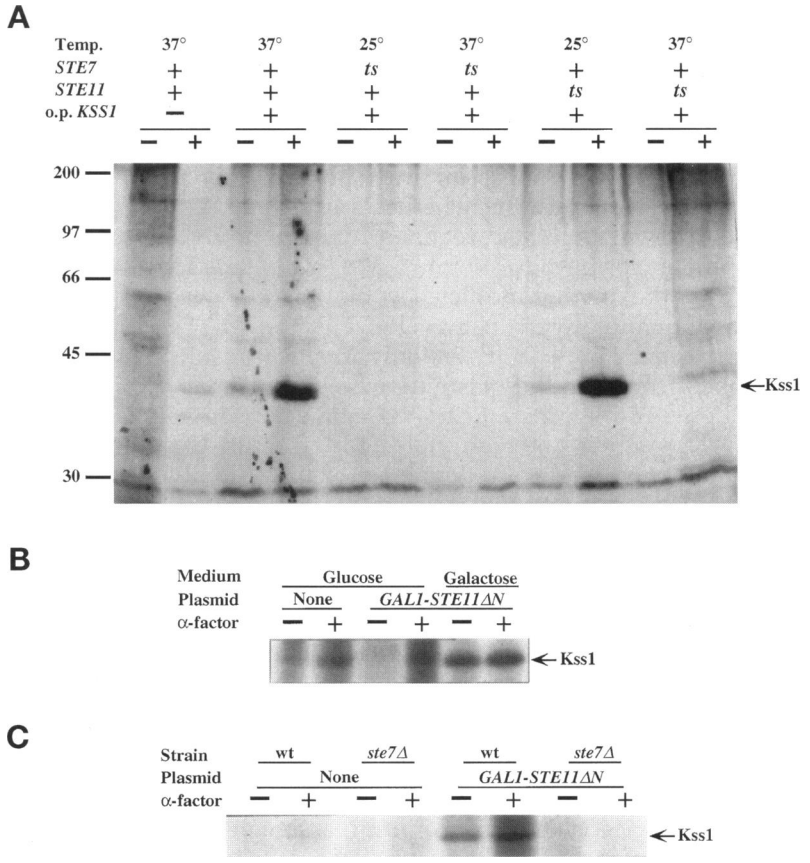

Figure 5. Ste11 and Ste7 are required for pheromone-induced phosphorylation of Kss1. (A) Pheromone-induced phosphorylation of Kss1 requires functional Ste7 and Ste11. Cultures of strain YPH499, and its congenic derivatives JGY7 and JGY11, each carrying YEp-*KSS1* were labeled with $^{32}PO_4^{3-}$ in low-phosphate medium at 25°C for 1 h in a waterbath shaker, then split into two equal samples. One sample was shifted to a 37°C waterbath shaker; the other remained at 25°C. After 20 min, each culture was split again, and one portion was treated with 12 μM α-factor for 5 min (+) and the other portion was left untreated (−). The cells in each sample were harvested, lysed, subjected to immunoprecipitation and analyzed as described in the legend to Figure 2. (B) Expression of hyperactive Ste11 causes pheromone-independent phosphorylation of Kss1. Cultures of YPH499 carrying YEp-*KSS1*, or both YEp-*KSS1* and pYGU-*STE11ΔN*, as indicated, were grown in sucrose-containing low-phosphate medium and split into two equal portions. Cells were collected by centrifugation and resuspended in either glucose- or galactose-containing low-phosphate medium, as indicated, and incubated with shaking for an additional 3 h at 30°C. Radiolabeling, pheromone-treatment, lysis, immunoprecipitation, and analysis were performed as above. (C) Pheromone-independent phosphorylation of Kss1 by hyperactive Ste11 requires Ste7. Cultures of either YPH499 carrying both YEp-*KSS1* and pYGU-*STE11ΔN* or strain E929-6C-1 (*ste7Δ*) carrying both YEp-*KSS1* and pYGU-*STE11ΔN* were induced with galactose, labeled, treated with pheromone, and analyzed as described in part B.

lated only in a pheromone-dependent manner, as before. However, when these cells were grown and labeled in galactose-containing medium, where the constitutively active Ste11 is expressed, Kss1 became heavily phosphorylated even in the absence of α-factor (Figure 5B).

To determine whether phosphorylation of Kss1 stimulated by the hyperactive Ste11 enzyme required the Ste7 protein kinase, both the multicopy *KSS1* plasmid and pYGU-*STE11ΔN* were introduced into a *MAT***a** strain (E969–6C-1) carrying a deletion of the chromosomal *STE7* gene. Kss1 was not detectably phosphorylated in this strain, regardless of whether the cells were grown on glucose or galactose and regardless of whether the cells were treated with α-factor (Figure 5C). The absence of Kss1 phosphorylation rules out the possibility that the *STE11ΔN* allele is a bypass mutation producing a Ste11 protein kinase with an altered substrate specificity that permits the enzyme to phosphorylate Kss1 directly. Collectively, our results support the conclusion that Ste7 is required for Kss1 phosphorylation, that Ste11 is necessary but not sufficient for Kss1 phosphorylation, and furthermore, that their order of function is: Ste11 → Ste7 → Kss1.

Kinase Activity of Kss1 Is Required for Its Role in Signal Transmission

A *MAT***a** *kss1Δ fus3Δ* strain is sterile, but reintroduction of either *KSS1* or *FUS3* on a multi-copy plasmid restores a readily detectable degree of mating (Elion *et al.*, 1991a,b; Ma, Cook, Hasson and Thorner, unpublished data). To determine whether any of the Kss1 mutants that we generated retained function in vivo, each of them was introduced into a *MAT***a** *kss1Δ fus3Δ* strain and the mating proficiency of the cells was examined quantitatively using DC17 as the MATα mating partner (Table 2). Essentially identical results were obtained when the plasmids were tested in appropriate *MATα* strains and mated against DC14 as the *MAT***a** partner (our unpublished results). The K43R mutant conferred a degree of mating ability indistinguishable from that provided by wild-type Kss1, confirming that this substitution is a silent mutation. In contrast, as expected for alteration of the conserved Lys critical for catalytic activity, the K42R mutant, as well as the K42R K43R mutant, was totally unable to complement the mating deficiency of the *MAT***a** *kss1Δ fus3Δ* strain. Similarly, all three alterations of the TEY motif (T183A, Y185F, and T183A Y185F) were nonfunctional, suggesting that T183 and Tyr185 are required for catalytic activity in vivo. Apparently, in the absence of phosphorylation of these sites, Kss1 is incapable of transmitting a signal to its appropriate downstream effector molecules.

The effects of the Y24F mutation were somewhat surprising. Clearly, Tyr24 is not a significant phosphorylation site (Figure 4). However, the analogous site in *S. pombe* Cdc2 (Tyr15) is a site for phosphorylation, and a Y15F mutation permits that kinase to act constitutively, leading to mitotic catastrophe (Gould and Nurse, 1989; Morla *et al.*, 1989). If the Y24F mutation similarly caused the constitutive activation of Kss1, the mating pathway would be turned on even in the absence of pheromone, possibly leading to G_1 arrest via production and phosphorylation of pheromone-responsive gene products, such as the cell cycle inhibitor, Far1 (Peter *et al.*, 1993; Tyers and Futcher, 1993). If this mutation activates Kss1, it is possible that all viable transformants containing the Y24F mutant have acquired a secondary mutation, either in the *KSS1* sequence itself, thereby inactivating its catalytic activity, or in a gene required for stages of the pheromone response pathway downstream of Kss1 action. However, we constructed the Y24F mutation three independent times, and preservation of the region immediately surrounding it (~40 amino acids on either side) was confirmed by direct nucleotide sequencing. Most significantly, haploid transformants containing the three independently generated Y24F mutants were obtained at the same frequency as normal Kss1 and all of the other variants, suggesting that expression of the Y24F variant was not deleterious to growth or viability.

Evidence that Kss1-promoted Recovery Is Due to Interference with Fus3 Action

Both *KSS1* and *FUS3* are required for optimal pheromone response and mating efficiency. However, Fus3 seems to play a critical role in the imposition of G_1 arrest (Elion *et al.*, 1990, 1991a), whereas Kss1 seems to be more important for maximal pheromone-induced transcription of genes required for mating (Elion *et al.*, 1991b; Ma, Cook, Hasson and Thorner, unpublished data). Nevertheless, we have shown previously that when *KSS1* is overexpressed, resumption of cell cycling is dramatically promoted (Courchesne *et al.*, 1989). Given the requirement for Fus3 activity to efficiently inhibit cell cycle progression (Peter *et al.*, 1993; Tyers and Futcher, 1993), the apparent effect of Kss1 overproduction in stimulating recovery from pheromone-induced G1 arrest could be due to the phosphorylating activity of Kss1 somehow counteracting the action of Fus3, or it could be due to steric interference with Fus3 action by excess Kss1 protein (e.g. by inappropriate binding of Kss1 to a critical Fus3 activator or target substrate). To help distinguish between these possibilities, we examined the efficacy of each of the mutant proteins in promoting recovery when overproduced. To enhance the sensitivity of the assay, a *MAT***a** *sst2Δ* strain (YDM400) that is hypersensitive

to the growth-arresting effect of α-factor (Reneke *et al.*, 1988) was used as the recipient for transformation with multi-copy plasmids that expressed from the *GAL1* promoter either the normal *KSS1* gene or each of the various mutations we constructed. When the *GAL1* promoter was induced, Kss1 was overproduced 50- to 100-fold, as judged by immunoblotting (our unpublished results). To measure pheromone-induced G_1 arrest and recovery, an agar diffusion bioassay was used in which a disk containing α-factor is placed on an incipient lawn of the cells to be tested and examined after 2 days. A clear zone of growth inhibition ("halo") around the disk indicates that division of the surrounding cells has been held in check efficiently, whereas a turbid zone indicates that the cells have adapted and resumed growth during this time period. On glucose medium, where the *GAL1* promoter was repressed, all of the recipient cells arrested growth in response to pheromone, regardless of whether they carried the vector alone, a plasmid containing the normal *KSS1* gene, or any of the mutant versions (Figure 6). In marked contrast, on galactose medium, all of the cells (except the vector only control) yielded turbid haloes indicative of recovery from G_1 arrest. We noted that the halo-filling process was distinctly more rapid for cells expressing any of the nonfunctional Kss1 mutants than it was for cells expressing normal Kss1 or the functional K43R mutant (Figure 6). Thus, mutants incapable of restoring mating, as judged by complementation (Table 2), were nonetheless extremely efficacious in promoting recovery from α-factor–induced G_1 arrest. As judged by subcellular fractionation and immunoblotting, none of the mutant proteins was mislocalized or distributed differently than normal Kss1 (our unpublished results). Because biologically nonfunctional and presumably catalytically inactive mutants were nonetheless fully capable of stimulating resumption of cell cycling, overproduction of Kss1 cannot promote recovery by phosphorylating Fus3 or any other cellular protein.

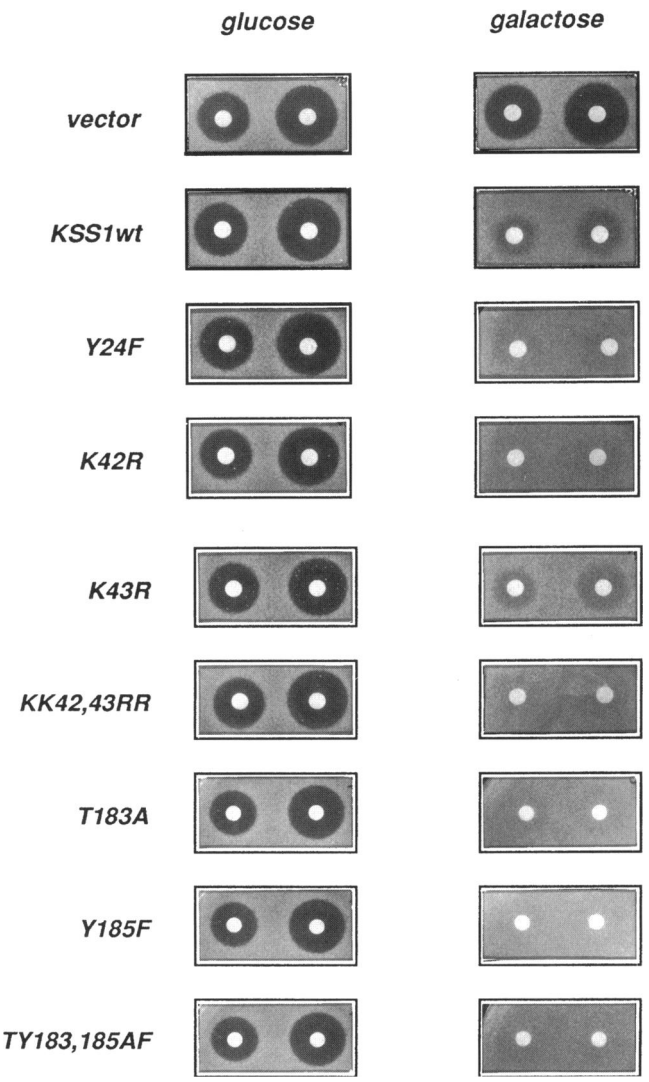

Figure 6. The recovery-promoting effect of Kss1 overproduction does not require the catalytic function of the Kss1 kinase. Cells of strain YDM400 (*MAT***a** *sst2*Δ*::ura3*) carrying either vector alone (YEp352GAL), or the same plasmid expressing either wild-type Kss1 or each of the various Kss1 mutants described in Figure 4 as indicated, were plated in soft agar on -Ura minimal plates selective for maintenance of the plasmids and containing either glucose (left column) or galactose (right column) as the carbon source. Sterile cellulose filter discs spotted with water containing either 0.5 μg (left) or 1 μg (right) of α-factor were then placed on the nascent lawn using sterile forceps, and the plates were incubated for 40 h at 30°C.

Subcellular Distribution of Kss1 Is Unaltered by Pheromone Action

Given that the majority of Kss1 is associated with the particulate fraction, one consequence of pheromone-induced phosphorylation might be to promote release of Kss1 into a soluble form. Such a change in subcellular distribution is a potential regulatory mechanism. However, the ratio of the amount of Kss1 in the soluble fraction to that in the particulate fraction did not change significantly after pheromone administration for either the phosphorylated species (Figure 2) or the total Kss1 protein (Figure 3). On the other hand, when the *KSS1* gene is overexpressed in cells, the proportion of Kss1 found in the soluble fraction can reach 30% of the total (Figure 1B), whereas in cells expressing *KSS1* from only its normal chromosomal locus nearly all of the Kss1 is found in the particulate fraction. Thus, it was possible that overexpression could have obscured a significant shift in the cellular distribution of Kss1 after pheromone treatment. Nonetheless, when cells expressing *KSS1* at its normal chromosomal level were examined in the same way, again there was essentially no change in the fractionation behavior of the phos-

phorylated Kss1 species after pheromone treatment (our unpublished results).

Kss1 Is Concentrated in the Nucleus

Immunocytological studies of mammalian MAPK isoforms have shown that, in some cell types, the activated enzymes translocate from the cytoplasm to the nucleus in response to an appropriate stimulus (Chen *et al.*, 1992; Lenormand *et al.*, 1993; Hsiao *et al.*, 1994). However, based on the physiology of the yeast mating response, Kss1 and Fus3 are predicted to have both cytoplasmic and nuclear targets. Therefore, it was of interest to determine whether the observed fractionation of Kss1 with the particulate material reflected its association with a specific subcellular compartment and, if so, whether the pattern of Kss1 localization changed in response to pheromone. To achieve the greatest degree of specificity for detecting Kss1 by indirect immunofluorescence, a version of Kss1 tagged with a c-Myc epitope situated between residues 8 and 9 was constructed (Figure 4A). The antibody used to recognize this epitope (mAb 9E10) displays very low, nonspecific background staining of yeast cells (see, for example, Kuchler *et al.*, 1993). Indeed, mycKss1 was recognized by mAb 9E10 with high specificity on immunoblots (Figure 1B); in the absence of any c-Myc-tagged protein in the cell, the 9E10 mAb gave very low background staining when used for indirect immunofluorescence (see Figure 7).

To determine whether the epitope insertion affected the function of Kss1, the ability of mycKss1 to restore mating to a *kss1*Δ *fus3*Δ strain and its ability to promote recovery of an *sst2*Δ mutant from pheromone-induced G1 arrest were examined. The mycKss1 derivative, even when expressed from a low-copy (CEN) vector, was capable of complementing the sterility of *kss1*Δ *fus3*Δ cells, stimulating mating 1000-fold above that observed for the vector-only control cells (our unpublished results); however, mycKss1 did not increase mating frequency to the same extent as that observed with untagged Kss1 (10^5-fold) (see Table 2). Likewise, when overexpressed, mycKss1 markedly enhanced the rate of recovery of *sst2*Δ mutants from pheromone-induced growth arrest compared with the control cells carrying vector alone (our unpublished results); however, again untagged Kss1 stimulated recovery of the same cells even more efficiently, as judged by the rate and extent with which the zone of growth inhibition (halo) filled in (see Figure 6). The somewhat reduced efficacy of the mycKss1 derivative compared with untagged Kss1 in the two different in vivo assays was not due to mislocalization of mycKss1 because the distribution of the tagged protein, based on its biochemical fractionation properties, was indistinguishable from that of Kss1 itself (for example, Figure 1). In some experiments, it was noted that the steady-state level of mycKss1 was significantly lower than that of normal Kss1 (our unpublished results), which may explain, at least in part, the somewhat reduced biological potency of the tagged protein.

The subcellular distribution of mycKss1 expressed from its own promoter on a multi-copy plasmid was analyzed by indirect immunofluorescence in *MAT***a**/*MAT***a** homozygous diploids (Figure 7). The ability to discriminate immunofluorescent signals from different subcellular compartments is often enhanced by the use of diploid cells because of their larger cell size. The mycKss1 was found predominantly in the nucleus, as indicated by the intense staining in the center of the cell that was completely congruent with the DNA counter-stain (DAPI). In addition, Kss1-specific fluorescence was consistently observed throughout the cytoplasm, but excluded from the vacuole (Figure 7). In the *MAT***a**/*MAT***a** cells expressing mycKss1 from its own promoter on a low-copy (*CEN*) plasmid, or in *MAT***a** *kss1*Δ haploid cells expressing mycKss1 from the same vector, a very similar pattern of strong nuclear staining and weak diffuse cytosolic staining was observed (although, as expected, the overall signals were less intense) (our unpublished results).

In contrast to the nuclear translocation reported for mammalian MAPKs in response to mitogens, the apparent distribution of mycKss1 did not change perceptibly in the MAT**a**/*MAT***a** cells at any time within the first hour after exposure to pheromone (our unpublished results). Even when pheromone treatment was extended for several hours, to the point where the changes in cell shape characteristic of mating cells were observed, mycKss1 was still found primarily in the nucleus with diffuse staining in the cytoplasm (Figure 7). Hence, it appears that Kss1 is already concentrated in the nucleus before its pheromone-induced activation. Furthermore, a significant proportion of the population of Kss1 molecules remains in the cytoplasm after pheromone-induced activation.

DISCUSSION

We have shown here that Kss1 becomes rapidly phosphorylated in response to a pheromone signal. Because Kss1 appears to be a relatively stable protein, the reduction in phosphorylation observed after the initial burst in cycloheximide-treated cells is almost certainly due to dephosphorylation (Clarke, 1994; Nebreda, 1994). A dual-specificity phosphatase, Msg5, implicated in the process of adaptation and recovery, is capable of dephosphorylating Fus3 and becomes highly induced within 15 min after cells are exposed to pheromone (Doi *et al.*, 1994). The role of Msg5 in mediating Kss1 dephosphorylation is unclear because Kss1 was efficiently dephosphorylated in cycloheximide-treated cells (which should have blocked phero-

mone-induced synthesis of Msg5) and because, in the absence of protein synthesis inhibitor, elevated phosphorylation of Kss1 persisted for at least 2 h after pheromone administration (even though Msg5 induction was free to occur under these conditions). Perhaps calcineurin (phosphoprotein phosphatase 2B), which has also been implicated in recovery (Cyert *et al.* 1991; Cyert and Thorner, 1992), participates in the deactivation of Kss1.

The modification of Kss1 reflects the balance between its rate of phosphorylation and its rate of dephosphorylation. Hence, the requirement for de novo protein synthesis to sustain pheromone-induced phosphorylation of Kss1 could reflect that one or more of the kinases (or other cellular components) necessary for Kss1 phosphorylation are unstable. Thus, rapid decay of the amount (or activity) of one or more of the upstream kinases could also contribute to the transient nature of the modification seen in cycloheximide-treated cells. Alternatively, as yet unidentified protein factors that inhibit the phosphatases that dephosphorylate Kss1 may be highly unstable.

Biochemical and genetic analysis of the requirements for the pheromone-induced phosphorylation of Kss1 indicated that it becomes phosphorylated almost exclusively on Thr183 and Tyr185, sites analogous to those modified in response to extracellular signals in mammalian MAPKs. In contrast to what has been reported recently for Fus3 (Brill *et al.*, 1994), our results using variants (K42R and K42R K43R) that should be catalytically inactive indicate that autophosphorylation plays no obligatory role in either the basal or the pheromone-stimulated phosphorylation of Kss1. Moreover, because inactive variants of Kss1 displayed an elevation in the level of both their basal and pheromone-induced phosphorylation in *kss1*Δ *fus3*Δ double mutants, cross-phosphorylation by Fus3 is also not obligatory for either basal or pheromone-stimulated phosphorylation of Kss1. Furthermore, this observation indicates that when either Kss1 or Fus3 is functional, they exert some form of negative feedback control on the extent of their own activation. Perhaps Kss1 and Fus3 are able to phosphorylate and thereby activate one or more of the phosphatases that can dephosphorylate Thr183 and/or Tyr185 (Doi *et al.*, 1994). Alternatively, there is some evidence that Kss1 and Fus3 may negatively regulate the activity of one or more of the upstream kinases in the pathway as a mechanism to dampen signal propagation (Cairns *et al.*, 1992; Stevenson *et al.*, 1992). Indeed, it has been shown that Ste7 undergoes a pheromone-induced increase in its level of phosphorylation (with an accompanying electrophoretic mobility shift) and that this phosphorylation depends not only on its upstream activator, Ste11, but also on Kss1 and Fus3 (Zhou *et al.*, 1993; Bardwell, Chang, and Thorner, unpublished observations). Even though at least Fus3 can phosphorylate Ste7 directly in vitro (Errede *et al.*, 1993), the effect of this in vitro phosphorylation on the activity of Ste7 is not yet known.

When both Thr183 and Tyr185 were substituted with residues that are incapable of being phosphorylated, there was no detectable modification of Kss1, as judged by ^{32}P incorporation, and the enzyme was nonfunctional in vivo, as judged by its inability to complement the mating defect of a *kss1*Δ *fus3*Δ mutant. Phosphorylation of both residues is required for function as evidenced by the fact that for the T183A mutant, the level of basal and pheromone-induced phosphorylation (presumably at Tyr185) was just as high, if not higher, than in normal Kss1, yet the mutant enzyme was nonfunctional. Similarly, for the Y185F mutant, basal and pheromone-stimulated incorporation (presumably at Thr183) was still observed, yet the mutant enzyme was nonfunctional.

While our work was in progress, a publication on the pheromone-induced phosphorylation of Fus3 appeared (Gartner *et al.*, 1992), which also presented a few preliminary results on Kss1. Although some of the findings presented and conclusions reached are similar to our own, there were some significant differences and discrepancies with regard to Kss1. First, Gartner *et al.* (1992) reported that, in the absence of pheromone, phosphorylation of Kss1 was undetectable and that only a single ^{32}P-labeled species was observed after pheromone stimulation. In contrast, we found that Kss1 is a phosphoprotein even in naive cells and is resolvable into two ^{32}P-labeled species. Considerable evidence indicates that the pheromone response pathway is active at a basal level even in the absence of pheromone (see Sprague and Thorner, 1992), which presumably accounts for the P-Tyr and P-Thr in Kss1 that we observed even in naive cells. Presumably, sensitivity of detection was compromised in the study of Gartner *et al.* (1992) by the lower culture density ($A_{600\ nm} = 0.4$), the lower specific activity label (25–75 μCi $^{32}PO_4^{3-}$/ml), and the lower affinity antibody used, compared with our conditions (see MATERIALS AND METHODS).

Second, and more importantly, Gartner *et al.* (1992) reported that phosphorylated Kss1 contains essentially equimolar amounts of P-Thr and P-Tyr, as judged by phosphoamino acid analysis. In contrast, in our phosphoamino acid analyses, we found that phosphorylation on Tyr was significantly more prominent than on Thr. This conclusion was further corroborated by the extent of labeling observed for our individual site-directed mutants; T183A still displayed prominent labeling (on Tyr185), whereas Y185F was labeled much more weakly (on Thr183). In this regard, it should be noted that the mobility of the ^{32}P-labeled Y185F mutant was actually intermediate between the two bands observed for normal Kss1, whereas the ^{32}P-labeled T183A mutant co-migrated with the upper

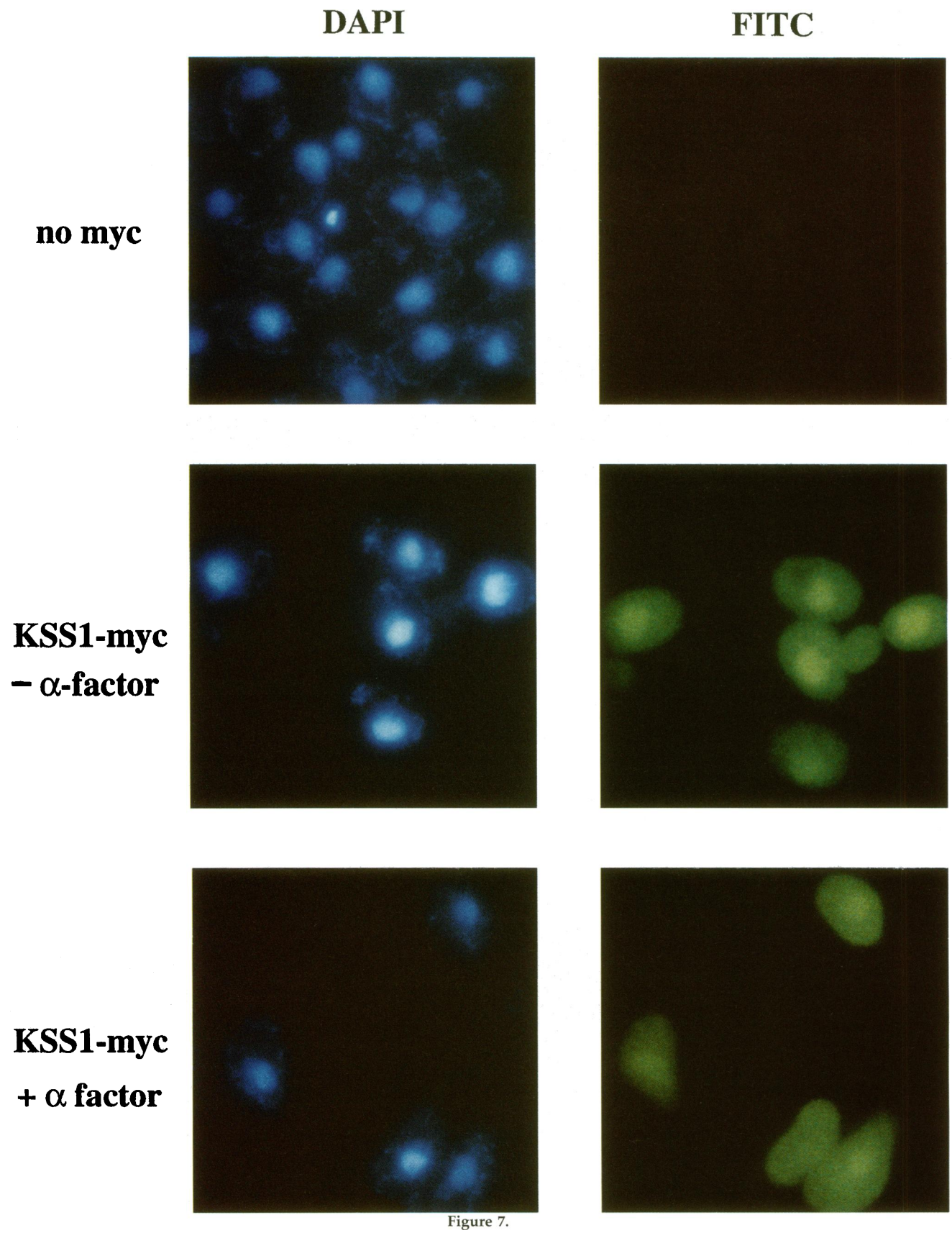

Figure 7.

band. These considerations suggest that the upper band represents Kss1 modified solely on Tyr185, whereas the lower band represents Kss1 modified at both Thr183 and Tyr185.

Third, Gartner *et al.* (1992) found no P-Ser associated with Kss1. In contrast, we found that Kss1 contains P-Ser. Mammalian MAPKs also have been reported to contain P-Ser (Chen *et al.*, 1992; Robbins and Cobb, 1992; Robbins *et al.*, 1993). The P-Ser that we detected is unlikely to be derived from a contaminant because Kss1 was overproduced, and the ^{32}P-labeled molecules we analyzed were purified by immunoprecipitation under denaturing conditions and then further resolved by SDS-PAGE. Furthermore, when these manipulations were performed on cells lacking a *KSS1* gene, the same region of the gel was completely devoid of radioactivity (see control lanes of Figure 4, B and D). The fact that the T183A Y185F mutant showed no detectable ^{32}P incorporation suggests that activation of the enzyme is a prerequisite to its phosphorylation on Ser. Phosphorylation of Kss1 on Ser cannot be the result of autophosphorylation because phosphoamino acid analysis of the catalytically inactive K42R mutant produced results that were identical to wild-type Kss1 (Cook, unpublished results). The fact that the amount of phosphoserine in Kss1 decreased upon pheromone stimulation suggests that phosphorylation at Ser is associated primarily with inactive Kss1 and, thus, may reflect a mechanism of negative regulation of Kss1 activity.

A tyrosine residue in the "phosphate anchor loop" (Knighton *et al.*, 1991a) is a feature that is highly conserved among MAPKs and in the Cdk family of cell cycle kinases, but absent in almost all other classes of protein kinases (Hanks and Quinn, 1991). Our data suggest that, unlike Cdc2, this residue (Tyr24 in Kss1) is not a site for phosphorylation in the MAPKs. Nonetheless, this residue appears to be critical for some other aspect of MAPK function in vivo because the Y24F mutant was unable to complement a *kss1Δ fus3Δ* mutant. The Y24 in Kss1, and the Tyr conserved at the analogous position in other MAPKs, may serve a role in formation of the proper secondary and tertiary structure required for the catalytic function of the activated form of these enzymes. This structural role has not yet been revealed because the three-dimensional structure of a MAPK has only been solved for the unphosphorylated (inactive) state of the enzyme (Zhang *et al.*, 1994).

The fact that Fus3 and Kss1 serve at least partially redundant functions in the pheromone signaling pathway indicates that, to some degree, they must possess common upstream activators and downstream substrates. Indeed, as we have demonstrated here, pheromone-induced phosphorylation of Kss1 requires the same kinases (and in the same order) as pheromone-stimulated phosphorylation of Fus3. Despite their sequence homology, however, the roles of Fus3 and Kss1 in pheromone signal transduction are not identical. For example, as judged by the halo bioassay, YPH499 derivatives that lack Fus3 are still nearly half as sensitive to α-factor–induced cell cycle arrest as wild-type cells, whereas strains lacking both Fus3 and Kss1 are completely resistant to the growth inhibitory action of pheromone (Cook, unpublished observations), suggesting that Kss1 contributes to transmission of the signal for G1 arrest, but does so less efficiently than Fus3. Another difference between Fus3 and Kss1 is highlighted by the effects of overproduction of these proteins. Overexpression of *KSS1* promotes recovery from pheromone-induced growth arrest, whereas overexpression of *FUS3* delays this recovery and increases sensitivity of cells to pheromone (Courchesne *et al.*, 1989). As we have shown here, catalytically and functionally inactive Kss1 mutants nonetheless promote recovery when overexpressed. The fact that normal Kss1 can mediate G_1 arrest to some degree may explain why overproduction of the catalytically inactive variants promoted recovery more efficiently than did overproduction of functional Kss1. Because catalytic activity is not required, it appears that Kss1 overproduction promotes recovery by titrating out some cellular substrate that is normally a target for Fus3 and is required for imposing cell cycle arrest. Alternatively, high levels of Kss1 may simply sequester the Ste7 kinase, so that it is unavailable to maintain the active (phosphorylated) state of Fus3.

Kss1 resides primarily in the nucleus and is firmly associated with particulate subcellular elements, both before and after pheromone treatment. Therefore, it appears that the pheromone signal does not result in a significant redistribution of Kss1. These results contrast with some studies in mammalian cells, which indicate that, once activated, MAPKs translocate to the nucleus (Chen *et al.*, 1992; Lenormand *et al.*, 1993; Hsiao *et al.*, 1994). However, even in mammalian systems, MAPK activation is not always associated with

Figure 7 cont. Subcellular localization of Kss1 by indirect immunofluorescence. Strain FC180 was transformed with either YEp-*KSS1* (upper panels) or with YEp-*mycKSS1* (middle and lower panels) and grown to mid-exponential phase. A sample of the cells carrying YEp-*mycKSS1* was then treated with 0.2 μM α-factor over the course of 3 h at 30°C until morphological elongation of the cells became obvious in the phase contrast microscope (lower panels). Portions of all three cultures were then fixed, prepared for analysis by indirect immunofluorescence, stained with a DNA dye (DAPI), and decorated with the anti-c-Myc mAb 9E10 followed by a fluorescein-conjugated secondary antibody (FITC).

translocation. Certain stimuli, especially those that elicit extremely rapid activation of MAPKs, like the rapid kinetics observed here for Kss1 phosphorylation, do not appear to result in redistribution of MAPK (Lenormand *et al.*, 1993; Nguyen *et al.*, 1993). Our results on Kss1 localization suggest that, during signal transmission from the pheromone receptors at the plasma membrane, some other component of the signaling network in the cytosol must translocate to the nucleus to activate Kss1. To date, the intracellular locations of Ste20, Ste11, and Ste7 before and after pheromone addition have not been reported. Like other MAPKs, Kss1 has no identifiable nuclear localization signal in its primary structure. Hence, Kss1 may be retained in the nucleus by virtue of its association with one or more proteins that do contain nuclear targeting information. The only other component of the mating signal transduction pathway known to be concentrated in the nucleus is the transcription factor, Ste12, due to its constitutive DNA-binding activity (Song *et al.*, 1991). Perhaps Kss1 localizes to the nucleus through its association with Ste12 because interaction between Kss1 and Ste12 can be detected by the two-hybrid method (Fields, 1993), both before and after pheromone stimulation (Cook, unpublished observations). However, localization of Kss1 has not yet been examined by indirect immunofluorescence in *ste12*Δ cells or in cells deficient in any other component of the pheromone response signaling cascade.

The association of Kss1 with particulate cellular components does not appear to be the result of its adsorption to membranes because fractionation in the presence of nondenaturing detergents does not release Kss1 into the soluble fraction (Ma, Cook, Hasson and Thorner, unpublished data). At least one other component of the pheromone response pathway, Ste5, displays the same kind of insolubility as Kss1. Like Kss1, the majority of the cellular pool of Ste5 resides in the nucleus, as determined by subcellular fractionation studies (Hasson, 1992) and analysis by indirect immunofluorescence using anti-Ste5 antibodies (Inouye and Thorner, unpublished results). There is growing evidence from both genetic (Printen and Sprague, 1994) and biochemical (Choi *et al.*, 1994; Kranz et al., 1994) approaches that a number of the components of the pheromone signaling pathway can exist in a large, multi-protein complex. Even if leached from the nucleus, this complex may be readily sedimentable, thereby explaining the appearance of the bulk of the Kss1 in the particulate fraction of whole cell extracts.

ACKNOWLEDGMENTS

We thank Bret Benton, Bill Courchesne, Elaine Elion, Beverly Errede, Jim Hicks, Phil Hieter, and Sandy Ramer for providing plasmids or strains, Randy Schekman for use of his PhosphorImager, Melanie Cobb for helpful discussions, and Bill Biggs for the communication of unpublished results. This work was supported by Postdoctoral Fellowship J46–89 from the California Division of the American Cancer Society (to D.M.), by National Institutes of Health Predoctoral Traineeship GM-07232 and an Academic Development Program Predoctoral Fellowship from Merck & Co. (to J.G.C.), by facilities provided by the Cancer Research Laboratory of the University of California, Berkeley, CA, and by National Institute of Health Research Grants GM-44173 and GM-21841 (to J.T.).

REFERENCES

Ahn, N.G., Weiel, J.E., Chan, C.P., and Krebs, E.G. (1990). Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. J. Biol. Chem. *265*, 11487–11494.

Alani, E., Cao, L., and Kleckner, N. (1987). A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics *116*, 541–545.

Ammerer, G. (1994). Sex, stress and integrity: the importance of MAPKs in yeast. Curr. Opin. Genetics Dev. *4*, 90–95.

Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAPK. Nature *343*, 651–653.

Bardwell, L., Cook, J.G., Inouye, C.J., and Thorner, J. (1994). Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae*. Dev. Biol. *166*, 363–379.

Becker, D.M., and Guarente, L. (1991). High-efficiency transformation of yeast by electro-poration. Methods Enzymol. *194*, 182–187.

Benton, B.M., Eng, W.-K., Dunn, J.J., Studier, F.W., Sternglanz, R., and Fisher, P.A. (1990). Signal-mediated import of bacteriophage T7 RNA polymerase into the *Saccharomyces cerevisiae* nucleus and specific transcription of target genes. Mol. Cell. Biol. *10*, 353–360.

Blumer, K.J., and Johnson, G.L. (1994). Diversity in function and regulation of MAPK pathways. Trends Biochem. Sci. *19*, 236–240.

Boeke, J., LaCroute, F., and Fink, G.R. (1984). A positive selection for mutants lacking orotidine 5′-phosphate decarboxylase activity in yeast: 5′-fluoro-orotic acid resistance. Mol. Gen. Genet. *197*, 345–346.

Bossemeyer, D. (1993). Loss of kinase activity. Nature *363*, 590.

Boulton, T.G., Gregory, J.S., and Cobb, M.H. (1991a). Purification and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. Biochemistry *30*, 278–286.

Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos, G.D. (1991b). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell *65*, 663–675.

Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science *249*, 64–67.

Boyer, H., and Roilland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. *41*, 459–472.

Brewster, J.L., De Valoir, T., Dwyer, N.D., Winter, E., and Gustin, M.C. (1993). An osmosensing signal transduction pathway in yeast. Science *259*, 1760–1763.

Brill, J.A., Elion, E.A., and Fink, G.R. (1994). A role for autophosphorylation revealed by activated alleles of *FUS3*, the yeast MAPK homolog. Mol. Biol. Cell *5*, 297–312.

Brunner, D., Oellers, N., Szabad, J., Biggs, W.H., Zipursky, S.L., and Hafen, E. (1994). A gain-of-function mutation in *Drosophila* MAPK activates multiple receptor tyrosine kinase signaling pathways. Cell *79*, 875–888.

Cairns, B.R., Ramer, S.W., and Kornberg, R.D. (1992). Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. Genes Dev. *6*, 1305–1318.

Cano, E., and Mahadevan, L.C. (1995). Parallel signal processing among mammalian MAPKs. Trends Biochem. Sci. *20*, 117–122.

Chan, R.K., and Otte, C.A. (1982). Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by **a**-factor and α-factor pheromones. Mol. Cell. Biol. *2*, 21–29.

Chen, R.-H., Sranecki, C., and Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol. Cell. Biol. *12*, 915–927.

Choi, K., Satterberg, B., Lyons, D.M., and Elion, E.A. (1994). Ste5 tethers multiple protein kinases in the MAPK cascade required for mating in *S. cerevisiae*. Cell *78*, 499–512.

Ciejek, E.M., and Thorner, J. (1979). Recovery of *S. cerevisiae* **a** cells from G1 arrest by alpha-factor pheromone requires endopeptidase action. Cell *18*, 623–635.

Clarke, P.R. (1994). Switching off MAPKs. Curr. Biol. *4*, 647–650.

Cobb, M.H., Robbins, D.J., and Boulton, T.G. (1991). ERKs, extracellular signal-regulated MAP-2 kinases. Curr. Opin. Cell Biol. *3*, 1025–1032.

Company, M., Adler, C., and Errede, B. (1988). Identification of a Ty1 regulatory sequence responsive to STE7 and STE12. Mol. Cell. Biol. *8*, 2545–2554.

Cooper, J.A., Sefton, B.M., and Hunter, T. (1983). Detection and quantification of phosphotyrosine in protein. Methods Enzymol. *99*, 387–402.

Courchesne, W.E., Kunisawa, R., and Thorner, J. (1989). A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. Cell *58*, 1107–1119.

Cyert, M.S., Kunisawa, R., Kiam, D., and Thorner, J. (1991). Yeast has homologs (CNA1 and CNA2 gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc. Natl. Acad. Sci. USA *88*, 7376–7380.

Cyert, M.S., and Thorner, J. (1992). Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell. Biol. *12*, 3460–3469.

De Bondt, H.L., Rosenblatt, J., Jancarik, J., Jones, H.D., Morgan, D.O., and Kim, S.H. (1993). Crystal structure of cyclin-dependent kinase 2. Nature *363*, 595–602.

Dietzel, C., and Kurjan, J. (1987). Pheromonal regulation and sequence of the *Saccharomyces cerevisiae SST2* gene: a model for desensitization to pheromone. Mol. Cell. Biol. *7*, 4169–4177.

Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K., and Matsumoto, K. (1994). MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. EMBO J. *13*, 61–70.

Elion, E., Satterberg, B., and Kranz, J. (1993). Fus3 phosphorylates multiple components of the mating signal transduction cascade: evidence for Ste12 and Far1. Mol. Biol. Cell *4*, 495–510.

Elion, E.A., Brill, J.A., and Fink, G.R. (1991a). FUS3 represses CLN1 and CLN2 and in concert with KSS1 promotes signal transduction. Proc. Natl. Acad. Sci. USA *88*, 9392–9396.

Elion, E.A., Brill, J.A., and Fink, G.R. (1991b). Functional redundancy in the yeast cell cycle: FUS3 and KSS1 have both overlapping and unique functions. Cold Spring Harbor Symp. Quant. Biol. *56*, 41–49.

Elion, E.A., Grisafi, P.L., and Fink, G.R. (1990). *FUS3* encodes a cdc^{2+}/CDC28-related kinase required for the transition from mitosis into conjugation. Cell *60*, 649–664.

Errede, B., Gartner, A., Zhou, Z., Nasmyth, K., and Ammerer, G. (1993). MAPK-related Fus3 from *S. cerevisiae* is activated by Ste7 in vitro. Nature *362*, 261–264.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for the human c-*myc* proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Fields, S. (1993). The two-hybrid system to detect protein-protein interactions. Methods Enzymol. *5*, 116–124.

Gartner, A., Nasmyth, K., and Ammerer, G. (1992). Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Genes Dev. *6*, 1280–1292.

Gould, K.L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast *cdc2+* protein kinase regulates entry into mitosis. Nature *342*, 39–45.

Haccard, O., Sarcevic, B., Lewellyn, A., Hartley, R., Roy, L., Izumi, T., Erikson, E., and Maller, J.L. (1993). Induction of metaphase arrest in cleaving *Xenopus* embryos by MAPK. Science *262*, 1262–1265.

Hanks, S.K., and Quinn, A.M. (1991). Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol. *200*, 38–62.

Hanks, S.K., Quinn, A.M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42–52.

Harlow, E., and Lane D. (1988). Antibodies: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hartwell, L.H. (1980). Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by peptide mating pheromone. J. Cell Biol. *85*, 811–822.

Hasson, M.S. (1992). Analysis of *Saccharomyces cerevisiae* pheromone response: biochemical and genetic characterization of the Ste5 protein. Doctoral Dissertation. Berkeley, CA: University of California, 1–186.

Herskowitz, I. (1995). MAPK pathways in yeast: for mating and more. Cell *80*, 187–197.

Hildebrandt, E., and Fried, V.A. (1989). Phosphoamino acid analysis of protein immobilized on polyvinylidene difluoride membrane. Anal. Biochem. *177*, 407–412.

Hill, J.E., Myers, A.M., Koerner, T.J., and Tzagoloff, A. (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast *2*, 163–167.

Hoffman, C.S., and Winston, F. (1987). A ten minute DNA preparation from yeast efficiently releases autonomous for transformation of *E. coli*. Gene *57*, 265–272.

Hsiao, K.M., Chou, S.Y., Shih, S.J., and Ferrell, J.E., Jr. (1994). Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer in vivo. Proc. Natl. Acad. Sci. USA *91*, 5480–5484.

Johnson, G.L., and Vaillancourt, R.R. (1994). Sequential protein kinase reactions controlling cell growth and differentiation. Curr. Biol. *6*, 230–238.

Julius, D., Blair, L., Brake, A., Sprague, G., and Thorner, J. (1983). Yeast α-factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. Cell *32*, 839–852.

Julius, D., Schekman, R., and Thorner, J. (1984). Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell *36*, 309–318.

Knighton, D.R., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N.-H., Taylor, S.S., and Sowadski, J.M. (1991a). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science *253*, 407–414.

Knighton, D.R., Zheng, J., Ten Eyck, L.F., Xuong, N.-H., Taylor, S.S., and Sowadski, J.M. (1991b). Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science *253*, 414–420.

Koerner, T.J., Hill, J.E., Myers, A.M., and Tzagoloff, A. (1991). High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. Methods Enzymol. *194*, 477–490.

Kranz, J.E., Satterberg, B., and Elion, E.A. (1994). The MAPK Fus3 associates with and phosphorylates the upstream signaling component Ste5. Genes Dev. *8*, 313–327.

Krisak, L., Strich, R., Winters, R.S., Hall, P., Mallory, M.J., Kreitzer, D., Tuan, R.S., and Winter, E. (1994). SMK1, a developmentally regulated MAPK, is required for spore wall assembly in *Saccharomyces cerevisiae*. Genes Dev. *8*, 2151–2161.

Kuchler, K., Dohlman, H.G., and Thorner, J. (1993). The **a**-factor transporter (*STE6* gene product) and cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. *120*, 1203–1215.

Lackner, M.R., Kornfeld, K., Miller, L.M., Horvitz, H.R., and Kim, S.K. (1994). A MAPK homolog, mpk-1, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. Genes Dev. *8*, 160–173.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature *227*, 680–685.

Leberer, E., Dignard, D., Harcus, D., Thomas, D.Y., and Whiteway, M. (1992). The protein kinase homologue Ste20 is required to link the yeast pheromone response G-protein beta-gamma subunits to downstream signalling components. EMBO J. *11*, 4815–4824.

Lee, K.S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin, D.E. (1993). A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol. Cell. Biol. *13*, 3067–3075.

Lenormand, P., Sardet, C., Pagés, G., L'Allemain, G., Brunet, A., and Pouysségur, J. (1993). Growth factors induce nuclear translocation of MAPKs ($p42^{mapk}$ and $p44^{mapk}$) but not of their activator MAPK kinase ($p45^{mapkk}$) in fibroblasts. J. Cell Biol. *122*, 1079–1088.

Marshall, C.J. (1994). MAPK kinase kinase, MAPK kinase, and MAPK. Curr. Opin. Genet. Dev. *4*, 82–89.

Morla, A.O., Draetta, G., Beach, D., and Wang, J.Y.J. (1989). Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. Cell *58*, 193–203.

Nakamaye, K.L., and Eckstein, F. (1986). Inhibition of restriction endonuclease *Nci*I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. Nucleic Acids Res. *14*, 9679–9698.

Nebreda, A.R. (1994). Inactivation of MAPKs. Trends Biochem. Sci. *19*, 1–2.

Neiman, A.M., and Herskowitz, I. (1994). Reconstitution of a yeast protein kinase cascade in vitro: activation of the yeast MEK homologue STE7 by STE11. Proc. Natl. Acad. Sci. USA *91*, 3398–3402.

Nguyen, T.T., Scimeca, J., Filloux, C., Peraldi, P., Carpentier, J., and Van Obberghen, E. (1993). Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1 and the 90-kDa ribosomal s6 kinase in PC12 cells. J. Biol. Chem. *268*, 9803–9810.

Oehlen, B., and Cross, F.R. (1994). Signal transduction in the budding yeast, *Saccharomyces cerevisiae*. Curr. Opin. Cell Biol. *6*, 836–841.

Payne, D.M., Rossomando, A.J., Martino, P., Erikson, A.K., Her, J.-H., Shabanowitz, J., Hunt, D.F., Weber, M.J., and Sturgill, T.W. (1991). Identification of the regulatory phosphorylation sites in Pp 42/mitogen-activated protein kinase (MAPK). EMBO J. *10*, 885–892.

Pelech, S.L., and Sanghera, J.S. (1992). Mitogen-activated protein kinases: versatile transducers for cell signaling. Trends Biochem. Sci. *17*, 233–238.

Peter, M., Gartner, A., Horecka, J., Ammerer, G., and Herskowitz, I. (1993). FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell *73*, 747–760.

Pringle, J.R., Adams, A.E.M., Drubin, D.G., and Haarer, B.K. (1991). Immunofluorescence methods for yease. Methods Enzymol. *194*, 565–602.

Printen, J.A., and Sprague, G.F., Jr. (1994). Protein-protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAPK cascade. Genetics *138*, 609–619.

Qui, M.S., and Green, S.H. (1992). PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. Neuron *9*, 705–717.

Ramer, S., and Davis, R.W. (1992). A dominant truncation allele identifies a gene, STE20, that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *90*, 452–456.

Ramer, S., Elledge, S., and Davis, R.W. (1992). Dominant genetics using a yeast genomic library under the control of a strong inducible promoter. Proc. Natl. Acad. Sci. USA *89*, 11589–11593.

Ray, L.B., and Sturgill, T.W. (1988). Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. Proc. Natl. Acad. Sci. USA *85*, 3753–3757.

Reneke, J.E., Blumer, K.J., Courchesne, W.E., and Thorner, J. (1988). The carboxy-terminal segment of the yeast alpha-factor receptor is a regulatory domain. Cell *55*, 221–234.

Rhodes, N., Connell, L., and Errede, B. (1990). *STE11* is a protein kinase required for cell-type–specific transcription and signal transduction in yeast. Genes Dev. *4*, 1862–1874.

Robbins, D.J., and Cobb, M.H. (1992). Extracellular signal-regulated kinases 2 autophosphorylates on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: analysis by peptide mapping. Mol. Biol. Cell *3*, 299–308.

Robbins, D.J., Zhen, E., Owaki, H., Vanderbilt, C.A., Ebert, D., Geppert, T.D., and Cobb, M.H. (1993). Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J. Biol. Chem. *268*, 5097–5106.

Roberts, R.L., and Fink, G.R. (1994). Elements of a single MAPK cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev. *8*, 2974–2985.

Rossomando, A.J., Sanghera, J.S., Marsden, L.A., Weber, M.J., Pelech, S.L., and Sturgill, T.W. (1991). Biochemical characterization of a family of serine/threonine protein kinases regulated by tyrosine and serine/threonine phosphorylations. J. Biol. Chem. *266*, 20270–20275.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Scherer, S., and Davis, R.W. (1979). Replacement of chromosome segments with altered DNA sequences constructed in vitro Proc. Natl. Acad. Sci. USA *76*, 4951–4955.

Seger, R., Ahn, N.G., Boulton, T.G., Yancopoulos, G.D., Panayotatos, N., Radziejewska, E., Ericson, L., Bratlien, R.L., Cobb, M.H., and Krebs, E.G. (1991). Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: implications for their mechanism of activation. Proc. Natl. Acad. Sci. USA *88*, 6142–6146.

Sherman, F., Fink, G.R., and Hicks, J.B. (1986). Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19–27.

Song, O., Dolan, J.W., Yuan, Y.O., and Fields, S. (1991). Pheromone-dependent phosphorylation of the yeast Ste12 protein correlates with transcriptional activation. Genes Dev. *5*, 741–750.

Southern, E.M. (1975). Detecting specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503–517.

Sprague, G.F., Jr., and Thorner, J. (1992). Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression, ed. E.W. Jones, J.R. Pringle, and J.R. Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 657–744.

Sprague, G.F.J. (1991). Assay of yeast mating reaction. Methods Enzymol. *194*, 77–93.

Stevenson, B.J., Rhodes, N., Errede, B., and Sprague, G.F., Jr. (1992). Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein. Genes Dev. *6*, 1293–1304.

Struhl, K., Stinchcomb, D.T., Scherer, S., and Davis, R.W. (1979). High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA *76*, 1035–1039.

Teague, M.A., Chaleff, D.T., and Errede, B. (1986). Nucleotide sequence of the yeast regulatory gene *STE7* predicts a protein homologous to protein kinases. Proc. Natl. Acad. Sci. USA *83*, 7371–7375.

Tobe, K., Kadowaki, T., Tamemoto, H., Ueki, K., Hara, K., Koshio, O., Momomura, K., Gotoh, Y., Nishida, E., Akanuma, Y., Yazaki, Y., and Kasuga, M. (1991). Insulin and 12-*O*-tetradecanoylphorbol-13-acetate activation of two immunologically distinct myelin basic protein/microtubule-associated protein 2 (MBP/MAP) kinases via de novo phosphorylation of threonine and tyrosine residues. J. Biol. Chem. *266*, 24793–24803.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells: comparison of the effects of nerve growth factor and epidermal growth factor. Biochem. J. *288*, 351–355.

Tyers, M., and Futcher, B. (1993). Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase cdc28 kinase complexes. Mol. Cell. Biol. *13*, 5659–5669.

Waskiewicz, A.J., and Cooper, J.A. (1993). Evolutionary conservation of *Xenopus laevis* mitogen-activated protein kinase activation and function. Cell Growth Differ. *4*, 965–973.

Wu, J., Rossomando, A.J., Her, J.-H., Del Vecchio, R., Weber, M.J., and Sturgill, T.W. (1991). Autophosphorylation in vitro of recombinant 42-kilodalton mitogen-activated protein kinase on tyrosine. Proc. Natl. Acad. Sci. USA *88*, 9508–9512.

Zhang, F., Strand, A., Robbins, D., Cobb, M.H., and Goldsmith, E.J. (1994). Atomic structure of the MAPK ERK2 at 2.3 angstrom resolution. Nature *367*, 704–711.

Zhou, Z., Gartner, A., Cade, R., Ammerer, G., and Errede, B. (1993). Pheromone-induced signal transduction in *Saccharomyces cerevisiae* requires the sequential function of three protein kinases. Mol. Cell. Biol. *13*, 2069–2080.