Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK

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The mitogen-activated protein kinase (MAPK) Kss1 has a dual role in regulating filamentous (invasive) growth of the yeast Saccharomyces cerevisiae. The stimulatory function of Kss1 requires both its catalytic activity and its activation by the MAPK/ERK kinase (MEK) Ste7; in contrast, the inhibitory function of Kss1 requires neither. This study examines the mechanism by which Kss1 inhibits invasive growth, and how Ste7 action overcomes this inhibition. We found that unphosphorylated Kss1 binds directly to the transcription factor Ste12, that this binding is necessary for Kss1-mediated repression of Ste12, and that Ste7-mediated phosphorylation of Kss1 weakens Kss1–Ste12 interaction and relieves Kss1-mediated repression. Relative to Kss1, the MAPK Fus3 binds less strongly to Ste12 and is correspondingly a weaker inhibitor of invasive growth. Analysis of Kss1 mutants indicated that the activation loop of Kss1 controls binding to Ste12. Potent repression of a transcription factor by its physical interaction with the unactivated isoform of a protein kinase, and relief of this repression by activation of the kinase, is a novel mechanism for signal-dependent regulation of gene expression.

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way, pheromone-receptor binding leads to activation of a MAPK module. Ste11 (a MAPK kinase kinase or MEKK) phosphorylates and activates Ste7 (a MAPK kinase or MEK); Ste7, in turn, phosphorylates and activates two MAPKs, Kss1 and Fus3 (Errede et al. 1993; Neiman and Herskowitz 1994; Bardwell et al. 1996). Kss1 and Fus3 then stimulate, directly or indirectly, the transcription factor, Ste12, permitting expression of a battery of genes involved in the mating process (Erdman et al. 1998). Deletion (or mutation that destroys the catalytic activity) of any tier of this MAPK cascade—STE11, STE7, or both KSS1 and FUS3—abolishes pheromone-induced transcription and results in sterility (Rhodes et al. 1990; Elion et al. 1991; Cairns et al. 1992; Ma et al. 1995).

Invasive (filamentous) growth is a distinct developmental option available to both haploids and diploids. Cells undergoing this process become elongated; alter their cell cycle, budding pattern, and adhesiveness; and acquire the capacity to penetrate beneath the surface of an agar plate (Gimeno et al. 1992; Kron et al. 1994; Roberts and Fink 1994). Invasive growth is regulated by a signaling network containing at least two parallel branches (Cook et al. 1997; Lo et al. 1997; Chandarlapaty and Errede 1998). It has been shown (Liu et al. 1993; Roberts and Fink 1994) that one branch utilizes proteins that are also components of the pheromone response pathway, including the protein kinases, Ste20 [a p21-activated kinase (PAK) homolog], Ste11, Ste7, and the Ste12 transcription factor. Ste12 cooperates with the Tec1 transcription factor to regulate genes specific for invasive growth (Laloux et al. 1994; Gavrias et al. 1996; Baur et al. 1997; Madhani and Fink 1997). Upstream components of the invasive growth signaling network, including Mep2 (an ammonium permease; Lorenz and Heitman 1998), Gpa2 (a Gx subunit; Kübler et al. 1997), Bmh1 and Bmh2 (14-3-3 proteins; Roberts et al. 1997), and Ras2 and Cdc42 (two small GTPases; Gimeno et al. 1992; Mösch et al. 1996), are coupled to downstream branches by mechanisms that are not completely understood.

Until recently, the MAPK target of Ste7 in invasive growth was unknown. Because deletion of either KSS1 or FUS3 (or both) only moderately affected invasive growth, whereas deletion of STE7 caused a substantial reduction in invasiveness (Liu et al. 1993; Roberts and Fink 1994), it was proposed that Ste7 has substrates other than Kss1 and Fus3, perhaps even non-MAPK targets (for review, see Hunter and Plowman 1997). This conundrum was resolved by experiments showing that invasive growth could be restored to a strain lacking STE7 by also deleting KSS1 (Cook et al. 1997; Madhani et al. 1997). This result established that Kss1 acts to inhibit invasive growth, and suggested that Ste7 functions, in response to appropriate upstream signals, to alleviate Kss1-mediated inhibition. Fus3 also inhibits invasive growth, but is less potent, and only functions in haploid cells (Cook et al. 1997). Kss1 also has a stimulatory role in invasive growth that (in contrast to its inhibitory role) requires its protein kinase activity (Cook et al. 1997; Madhani et al. 1997).

Here, we demonstrate that Kss1-mediated inhibition of invasive growth operates at the level of transcription, characterize the mechanism of this transcriptional repression, and examine its regulation by upstream signals.

Results

Ste7-mediated phosphorylation is necessary and sufficient to relieve Kss1-mediated inhibition of invasive growth

Deletion of STE7 substantially reduces invasive growth (Liu et al. 1993; Roberts and Fink 1994). We found that neither an unactivatable ste7 allele (T363V; Neiman and Herskowitz 1994) nor a catalytically inactive allele (K220R; Cairns et al. 1992) could restore invasive growth to ste7a cells (data not shown). Thus, both phosphorylation and activation of Ste7 (presumably by Ste11) and the subsequent phosphorylation of downstream substrates by Ste7 are required for invasive growth. Ste7 is required for invasive growth only if Kss1 is also present, implicating Kss1 as the principal target of Ste7 in this process (Cook et al. 1997; Madhani et al. 1997). To examine how Kss1-mediated inhibition of invasive growth is relieved by Ste7 function, a series of kss1 alleles (Fig. 1A, a) was introduced, on low-copy (CEN) vectors, into a derivative of 11278b lacking both Kss1 and Fus3, strain JCY130 (MATa STE7+ kss1a fus3a). The biochemical and genetic properties of these Kss1 mutants with respect to pheromone response have been extensively characterized (Ma et al. 1995; Bardwell et al. 1996). Two of these mutants, Kss1T24F and Kss1K42R Q45P, contain substitutions in conserved subdomains involved in ATP binding and are catalytically inactive, but are phosphorylated by Ste7 to the same extent as wild-type Kss1 (Ma et al. 1995; Bardwell et al. 1996; see also Fig. 1C). The other three mutants are altered in the target site (I83-Thr-Glu-Tyr-185) for Ste7-mediated phosphorylation: Kss1T183A, which can be phosphorylated only on Y185; Kss1Y185F, which can be phosphorylated only on T183; and Kss1A1E (containing both substitutions), which cannot be phosphorylated by Ste7 (Ma et al. 1995). These three mutants are unactivatable, and consequently lack detectable protein kinase activity (Ma et al. 1995; Bardwell et al. 1996). Nevertheless, both classes of inactive mutants repress invasive growth when Ste7 is absent (Cook et al. 1997).

Strain JCY130 expressing each mutant, normal Kss1, or no Kss1 at all, grew equally vigorously (Fig. 1A, b). Only wild-type Kss1 supported detectable mating (Fig. 1A, c), as observed previously in kss1a fus3a strains of the S288C and W303 lineages (Elion et al. 1991; Ma et al. 1995). In the standard bioassay for invasive growth (Fig. 1A, d), neither the absence of Kss1 nor the presence of wild-type Kss1 prevented invasive growth. In contrast, none of the three unactivatable mutants (Kss1T183A, Kss1Y185F, and Kss1A1E) permitted normal invasive growth (Fig. 1A, d). This reduced level of invasiveness was quite comparable with that of an otherwise isogenic ste7a kss1+ strain (data not shown). Thus, phosphorylation of Kss1 on both T183 and Y185 is required to relieve
Phosphorylation of Kss1 is necessary and sufficient to permit haploid invasive growth. (A) Strain JCY130 (MATa STE7 yap1a kss1a his1) was transformed with centromeric (low-copy) plasmids YCpU (empty vector), YCpU-KSS1 (W.T.), YCpU–kss1(F42R Q45P), YCpU–kss1(Y24F), YCpU–kss1(T183A), YCpU–kss1(Y185F), or YCpU–kss1(AEF) (a). The resulting transformants were streaked onto a plate selective for plasmid maintenance. After 2 days at 30°C, plates were replica-plated to rich medium (YPD) plates and scored for surface growth. The resulting transformants were streaked onto a plate selective for plasmid maintenance. After 2 days at 30°C, plates were replica-plated to rich medium (YPD) plates and scored for surface growth. (b) Expression of the FRE(Y1)-lacZ reporter gene. The strains described in A were transformed with plasmid YEpL–FTyZ, grown to mid-log phase in liquid medium, and subjected to β-galactosidase-specific activity measurement. Values are normalized to that observed for JCY130 carrying YCpU (3936 nmoles/min per milligram of protein) and represent the average of measurements, made in duplicate, on protein extracts prepared from at least three independent transformants of each strain (error bars indicate standard deviation). (C) Portions (20 μg) of the protein extracts used in B, plus an additional extract (lane 1) prepared from strain JCY100 (MATa kss1a), were resolved on a 10% SDS-polyacrylamide gel and analyzed by immunoblotting with either anti-Kss1 (bottom) or anti-phospho-MAPK (top) antiserum.

Kss1-mediated inhibition of invasive growth. Revealingly, both of the catalytically inactive (yet phosphorylatable) mutants Kss1Y24F and Kss1K42R Q45P permitted haploid invasive growth (Fig. 1A, d). Hence, phosphorylation of Kss1 by Ste7 suffices to relieve inhibition, even in the absence of the resultant activity of this MAPK. When Fus3 is present, however, there is a stronger requirement for the protein kinase activity of Kss1 (Cook et al. 1997).

Transcription from filamentation response elements (FREs), which contain binding sites for Ste12 and Tec1 (Baur et al. 1997; Madhani and Fink 1997), correlates with MAPK cascade signaling in the invasive growth response (Mösch et al. 1996; Madhani and Fink 1997) and reflects the degree of MAPK-mediated inhibition of invasive growth (Cook et al. 1997). Quantitative measurement of the expression of an FRE-lacZ reporter gene confirmed the results obtained by use of the bioassay. The three unactivatable alleles permitted only minimal FRE-dependent transcription, whereas the two catalytically inactive alleles allowed near wild-type levels (Fig. 1B). Immunoblot analysis of protein extracts from these strains (Fig. 1C, bottom) revealed that each Kss1 mutant (Fig. 1C, lanes 3–8) was expressed comparably with endogenous Kss1 (Fig. 1C, lane 1). Staining of these same extracts with an antibody that preferentially recognizes the MEK-phosphorylated isoforms of MAPKs of the ERK (extracellular signal-regulated kinase) family, including Kss1 (see Materials and Methods), confirmed phosphorylation of wild-type Kss1 and the catalytically inactive derivatives, and the lack (or reduction) of phosphorylation on the unactivatable Kss1 mutants (Fig. 1C, top). Kss1 binds directly to Ste12

Because inhibition of invasive growth by unphosphorylated Kss1 is accompanied by repression of FRE-mediated transcription (Fig. 1B), and because Kss1 is concentrated in the nucleus (Ma et al. 1995), Kss1 has an opportunity to associate physically with and inhibit Ste12 and/or Tec1. Indeed, Kss1 interacts with Ste12 in the two-hybrid assay (Printen and Sprague 1994; Cook et al. 1996). Moreover, Kss1- and Ste12-containing complexes (Madhani et al. 1997; L. Bardwell, unpubl.), and Fus3- and Ste12-containing complexes (Elion et al. 1993; Tedford et al. 1997), have been recovered from cell extracts. However, neither of these assays demonstrates that the interaction between a MAPK and Ste12 is direct. It has been proposed (Tedford et al. 1997) that the Dig1/Rst1 and Dig2/Rst2 proteins, which bind directly to both Ste12 and Kss1 (Cook et al. 1996), may bridge the interaction between the MAPKs and Ste12. To determine whether Kss1 can bind directly to full-length Ste12 in the absence of any other yeast protein, radiolabeled versions of these proteins were prepared by in vitro translation in rabbit reticulocyte lysates, and their interactions were assessed by coimmunoprecipitation (Fig. 2). For this purpose, Ste12 was fused to an amino-terminal c-Myc epitope tag (see Material and Methods for details). Kss1 and Ste12 were efficiently produced in radiolabeled form under the conditions used (Fig. 2, Input). As expected, tagged Ste12 was immunoprecipitated by the monoclonal antibody recognizing the c-Myc epitope (Fig. 2, lane 8). When Kss1 and Ste12 were present in the same
Kss1 mutants specifically defective in Ste12 binding

To provide a critical in vivo test of the importance of Kss1 association with Ste12 in the mechanism of Kss1-mediated inhibition of invasive growth, we sought to generate Kss1 derivatives specifically defective in binding to Ste12. Two such mutants were identified. The first derivative, Kss1_{\text{Dloop}} (Fig. 3), was constructed by site-directed mutagenesis (Bardwell et al. 1996). In Kss1_{\text{Dloop}}, a sequence designated the activation loop—also called the activation segment (Johnson et al. 1996), phosphorylation lip (Cobb and Goldsmith 1995), or T-loop (Morgan and De Bondt 1994)—has been deleted and replaced with a shorter segment from an unrelated protein kinase (yeast Tpk3). The activation loop lies along the bottom of the active site cleft in the crystal structure of unphosphorylated Erk2 (Zhang et al. 1994, 1995), and contains the target phosphoacceptor residues for MEK-mediated phosphorylation (Fig. 3). In Erk2, this loop refolds upon phosphorylation, leading to activation of the enzyme (Canagarajah et al. 1997).

We isolated the second Kss1 mutant using a reverse two-hybrid screen (see Materials and Methods for details). This derivative, Kss1_{\text{Y231C}}, contains a single-residue substitution (to cysteine) at a conserved tyrosine that, in unphosphorylated Erk2 (Zhang et al. 1995), forms a hydrogen bond with the glutamate in the TEY phosphoacceptor motif (Fig. 3). Hence, alteration of this residue in Kss1_{\text{Y231C}} may disrupt this hydrogen bond (and/or cause other perturbations resulting from the chemical differences between tyrosine and cysteine), thereby affecting the conformation of the activation loop region. Consistent with this conclusion, when tested in an in vitro protein kinase assay (Bardwell et al. 1996), Kss1_{\text{Y231C}} was a much less efficient phosphoacceptor substrate for Ste7 than Kss1 (data not shown).

The fact that the mutations in Kss1_{\text{Dloop}} and Kss1_{\text{Y231C}} specifically affect the ability of Kss1 to interact with Ste12 was demonstrated in several ways. First, Kss1, Kss1_{\text{AEF}}, Kss1_{\text{Dloop}}, and Kss1_{\text{Y231C}} were expressed as Gal4 DNA-binding domain (GDB) fusions and tested for interaction with either Ste12, Dig1, or Dig2 expressed as Gal4 DNA-binding domain (GDB) fusions and tested for interaction with either Ste12, Dig1, or Dig2 expressed as Gal4 activation domain (GAD) fusions by use of the two-hybrid method in a yeast strain containing a GAL promoter-driven reporter gene (Escherichia coli lacZ). Interaction was measured by determination of β-galactosidase-specific activity (Fig. 4A). The Kss1_{\text{AEF}}, Kss1_{\text{Dloop}}, and Kss1_{\text{Y231C}} fusions exhibited near wild-type two-hybrid methodology had a $K_D$ of $5 \text{ nm}$ (Bardwell et al. 1996).

To delineate the Kss1-binding domain of the 688-residue Ste12 protein, we used Kss1 as bait to screen a random library and recovered seven different Ste12 isolates, including amino- and/or carboxy-terminal truncations (Cook et al. 1996). These isolates shared a common segment (residues 298-473). This portion of Ste12 lacks both the amino-terminal DNA-binding domain and the carboxy-terminal element that mediates cooperativity with the Mcm1 transcription factor, yet contains part of the transactivating region and a segment implicated in binding to Ste12. Two such mutants were identified. The

**Figure 2.** Direct binding of Kss1 to Ste12 in vitro. Radiolabeled Kss1, Kss1_{\text{Y231C}}, and myc epitope-tagged Ste12 (meSte12) were prepared by in vitro translation, partially purified by ammonium sulfate precipitation, and portions for Kss1 and Kss1_{\text{Y231C}} (5% of the amount added in the immunoprecipitation (pptn) reactions, and for meSte12, 20%; Input) were resolved on a 10% SDS-polyacrylamide gel. Samples (2 pmol) of Kss1 and Kss1_{\text{Y231C}}, each accompanied by $\sim 200 \mu g$ of total protein from the rabbit reticulocyte lysate, were immunoprecipitated with the anti-c-Myc mAb 9E10 either in the absence (lanes 4,6) or presence (lanes 5,7,8) of 2 pmol of Ste12 protein, and the resulting immunoprecipitates were analyzed on the same gel. The percentage of the input Kss1 derivatives bound in the reactions corresponding to lanes 4–7 was, respectively, 0.6, 1.7, 0.5, and 0.7; and of meSte12 in lanes 5,7,8: 37.2, 30.7, and 35.1.

**Figure 3.** Structure and properties of the Kss1_{\text{Dloop}} and Kss1_{\text{Y231C}} derivatives. Amino acid sequence of Kss1 and homology to yeast Fus3 and rat Erk2 in the affected regions. Dashes (−) indicate single-residue gaps; identities are boxed.
Unphosphorylated MAPK represses transcription

Figure 4. Kss1loop and Kss1Y231C are specifically defective in binding to Ste12. (A) Two-hybrid interaction of alleles of KSS1 with DIG1, DIG2, and STE12. Strain MA/V303a was cotransformed with pKSS1–GDB (W.T.), pkss1(AEF)–GDB (encoding the T183A, Y185F derivative), pkss1(Y231C)–GDB, pkss1loop–GDB, or the plasmid encoding the GDB domain only (Δ); and with pGAD–DIG1(7–452), pGAD–DIG2(56–323), or pGAD–STE12(191–478); and β-galactosidase-specific activity was measured as described in the legend to Fig. 1B. Values are normalized to that observed for the interaction of wild-type KSS1 with DIG1, DIG2, or STE12 (41, 49 and 52 nmoles/min per milligram of protein, respectively). None of the KSS1–GDB alleles displayed an appreciable two-hybrid interaction with an empty GAD plasmid (data not shown). (B) In vitro binding of Kss1, Kss1 mutants, and Fus3 to Ste12, Ste7, and Dig1. Radiolabeled DIG1213–452, and bound with greater efficiency than Kss1 loop did not bind to GST–Ste7 or GST–Dig1 at a level well above nonspecific binding to GST (Fig. 4B). Fus3 did not bind detectably to purified GST–Ste12298–473 in this assay, although it bound with about the same efficiency as Kss1 to GST–Dig1213–452, and bound with greater efficiency than Kss1 to GST–Ste71–172 (Fig. 4B). Compared with Kss1, Fus3 also exhibited a weaker association with Ste12 in yeast cell extracts (data not shown).

Finally, as judged by coimmunoprecipitation, radiolabeled Kss1loop (Fig. 2) and Kss1loop (data not shown) were unable to interact detectably with full-length, epitope-tagged and radiolabeled Ste12.

Binding of Kss1 to Ste12 is required for Kss1 to inhibit Ste12

Having demonstrated that Kss1loop and Kss1Y231C are specifically defective in binding to Ste12 (but fully able to interact with Ste7, Dig1, and Dig2) allowed us to assess whether direct association between Kss1 and Ste12 is involved in Kss1-mediated inhibition of invasive growth. For this purpose, alleles of KSS1 (Fig. 5A, a) carried on low copy (CEN) plasmids were introduced into a haploid strain JCY137 (ste7 Δ fus3Δ). In this strain, even normal Kss1 remains unphosphorylated as a result of the absence of Ste7. Hence, wild-type, catalytically inactive and unactivatable Kss1 derivatives all behaved as potent inhibitors of invasive growth (Fig. 5A, c) and of FRE-mediated transcription (Fig. 5B). In marked contrast, both Kss1loop and Kss1Y231C permitted a substantial decrease in invasive growth (Fig. 5A, c) and a measurably elevated level of FRE-mediated transcription (Fig. 5B). All of these Kss1 derivatives were produced at about the same level as endogenous Kss1 (Fig. 5C).

When introduced into a ste7Δ/fus3Δ kss1Δ/kss1Δ diploid strain, wild-type KSS1, as well as the catalytically inactive (Y24F) and unactivatable (AEF) alleles potently inhibited formation of filaments (pseudohyphae), whereas the Y231C and A100 alleles permitted at least some filamentation (data not shown). These findings suggest that Kss1 binding to Ste12 mediates Kss1-imposed inhibition of invasive growth in diploids, as in haploids. However, in diploids expressing Kss1Δ24F, reintroduction of Ste7 only weakly stimulated filamentation (data not shown).
establish that Ste7 relieves this inhibition via phosphorylation of Kss1. These observations predict that phosphorylation of Kss1 decreases its affinity for Ste12. To test this prediction, the ability of phosphorylated and unphosphorylated Kss1 to bind in vitro to Ste12 was examined.

To obtain extracts containing roughly equivalent amounts of total Kss1, but different amounts of phosphorylated Kss1, lysates were prepared from three different \( \Sigma 1278b \)-derived strains: wild-type cells overproducing Kss1 that contain a readily detectable level of phosphorylated Kss1 (Fig. 6, lane 1); the same cells treated with \( \alpha \)-factor mating pheromone that contain an increased level of phosphorylated Kss1 (Fig. 6, lane 2); and, an otherwise isogenic ste7\( \Delta \) strain overexpressing Kss1 from the same plasmid that contains no detectable phosphorylated Kss1 (Fig. 6, lane 3). Because Kss1 is overproduced, the bulk of the Kss1 pool remains unphosphorylated, even in pheromone-treated cells (L. Bardwell and J.G. Cook, unpubl.). Total and phosphorylated Kss1 were detected as in Figure 1, quantitated by immunoblotting of serial dilutions of the samples, and normalized to the input level of Kss1 (which was used as the standard).

As predicted, phosphorylated Kss1 clearly bound less well to GST–Ste12 than the bulk unphosphorylated Kss1 (Fig. 6, lanes 7–9). The amount of unphosphorylated Kss1 bound was ~25% of input, whereas only 1%–2% of the input phosphorylated Kss1 bound. The >10-fold difference in relative retention between phosphorylated and unphosphorylated Kss1 indicates that the phosphorylated form of Kss1 must have a correspondingly weaker affinity for Ste12. This effect was specific to the Kss1-

Phosphorylation of Kss1 reduces its affinity for Ste12

The preceding results provide strong support for the conclusion that direct physical interaction between Kss1 and Ste12 is required for Kss1-mediated inhibition of invasive growth. Moreover, the findings presented above...
Ste12 interaction because phosphorylated and unphosphorylated Kss1 associated with GST–Ste7 at the same efficiency (∼25% of input) (Fig. 6, lanes 10–12), consistent with our previous finding that Kss1–Ste7 interaction is unaffected by pheromone treatment (Bardwell et al. 1996). As expected, neither phosphorylated nor unphosphorylated Kss1 bound to GST alone (Fig. 6, lanes 4–6).

Discussion

Modulation of transcription factor function by protein kinase-mediated phosphorylation is a ubiquitous regulatory strategy. Phosphorylation-independent modes of protein kinase-mediated regulation are feasible because protein kinases often form relatively stable complexes with their substrates.

This investigation addressed two main mechanistic questions. First, how does unactivated Kss1 MAPK inhibit invasive growth? Second, how do upstream signals relieve this inhibition? We found that Kss1 binds directly to the Ste12 transcription factor and thereby represses its function (Fig. 7A). Phosphorylation of the activation loop of Kss1 by the Ste7 MEK weakens Kss1–Ste12 interaction, and is sufficient to relieve Kss1-mediated repression (Fig. 7B). Thus, we have demonstrated that an unactivated kinase isoform potently inhibits a transcription factor, and that activation of the kinase lifts this inhibition.

Two nuclear proteins—Dig1 and Dig2—bind to Kss1 (and Fus3) and to Ste12 (Cook et al. 1996; Tedford et al. 1997). In addition, Dig1 and Dig2 show an interaction with Tec1 in the two-hybrid assay (M. Lorenz and J. Heitman, pers. comm.). Although it has been suggested that the Dig proteins bridge the interaction between the MAPKs and Ste12, we have shown here that Kss1 can bind directly to Ste12 in the absence of any other yeast proteins. Moreover, our recent results (L. Bardwell, J.G. Cook, J.X. Zhu-Shimoni and J. Thorner, in prep.) suggest that the Kss1–Ste12 interaction promotes the recruitment of Dig1 and Dig2, which act as negative regulators of Ste12 function (Cook et al. 1996; Tedford et al. 1997), because, for example, Kss1-mediated repression is defective in the absence of Dig1 and Dig2. Hence, the Dig proteins may constitute (or attract) factors that directly block Ste12 (and/or Tec1) action, whereas Kss1 serves primarily to stabilize the Dig–Ste12–Tec1 complex (Fig. 7A).

It seems likely, therefore, that phosphorylation of Kss1 relieves repression by a dual mechanism. First, as we have demonstrated, phosphorylation of Kss1 by Ste7 weakens Kss1–Ste12 interaction and presumably, therefore, weakens Dig binding to (or Dig action on) Ste12 and/or Tec1 (Fig. 7B). Second, derepression of Ste12 may be further reinforced by phosphorylation of Ste12, Tec1, and/or Dig1/Dig2 by activated Kss1 (Fig. 7C). Phosphorylation of these proteins may further weaken these complexes and promote interaction of Ste12/Tec1 with the transcription machinery. It has been shown that both Ste12 and Dig1 can be phosphorylated in a Kss1-dependent manner in immune complexes (Cook et al. 1996; Madhani et al. 1997) and that both GST–Ste12,298–473 (containing the Kss1-binding site) and GST–Dig1,213–452 (containing the Kss1- and Ste12-binding sites) serve as efficient in vitro substrates for purified Kss1 (L. Bardwell, unpubl.). In fact, Kss1 catalytic activity is required for optimal invasive growth in haploids, in which repression by Fus3 must also be prevented, and for efficient filamentous growth in diploids, in which the ratio of unphosphorylated Kss1 (and the Dig1 and Dig2 proteins) to Ste12 is perhaps higher than in haploids because of the reduced expression of STE12 in diploids (Fields and Herskowitz 1987). In toto, these events provide a switch-like mechanism to achieve commitment to a differentiated state.

Ste12 is the target of Kss1-mediated inhibition of invasive growth

The conclusion that Ste12 is the direct target of Kss1-mediated inhibition of invasive growth is supported by multiple observations. First, both here and elsewhere (Cook et al. 1997), we found a remarkable correlation between the extent of inhibition caused by alleles of Kss1 (and by Fus3) and the level of expression of a Ste12-dependent reporter gene (FRETY1–lacZ). Second, as we and others have found, Kss1 associates with Ste12 in cell extracts (Madhani et al. 1997) and in the two-hybrid as-

Figure 7. Model for Kss1-mediated regulation of Ste12 in invasive growth. (A) Unphosphorylated Kss1 binds directly to Ste12, and to Dig1 and Dig2, thereby stabilizing Dig1/2–Ste12 complexes and potentiating Dig-mediated repression of Ste12. (B) Phosphorylation of Kss1 by Ste7, in response to upstream signals, causes a conformational change in Kss1 that weakens its association with Ste12, and consequently reduces Dig1/2 interaction with Ste12–Tec1. (C) Phosphorylated and activated Kss1 further reinforces the transition, presumably by phosphorylating Dig1/2, Ste12, and/or Tec1, permitting full derepression.
say (Printen and Sprague 1994; Cook et al. 1996). This interaction is direct because, as we showed here, Kss1 and Ste12 translated in vitro can associate. Third, Kss1 mutants specifically deficient in binding to Ste12 that we generated exhibited greatly reduced inhibition of invasive growth and FRE-mediated transcription in vivo. In a complementary approach, Madhani et al. (1997) isolated Kss1 mutants that displayed hyperfilamentation and found that some were defective in formation of Ste12-containing complexes. [Because, under some in vitro conditions, association of Kss1 with Ste12 is dependent on the presence of the Dig proteins (Telford et al. 1997; Mike Tyers, pers. comm.), the alleles identified by Madhani et al. (1997) could be defective in Kss1–Dig binding, rather than in Kss1–Ste12 binding per se, or both.] Fourth, we found that Fus3, which is a weaker inhibitor of invasive growth than Kss1 (Cook et al. 1997), displays a correspondingly weaker binding to Ste12. Fifth, Ste7-mediated phosphorylation of Kss1 on T183 and Y185 was both necessary and sufficient to prevent Kss1-mediated inhibition and, correspondingly, reduced the affinity of Kss1 for Ste12.

Phosphorylated Kss1 exhibits substantially reduced, yet still detectable, affinity for Ste12. The residual binding of activated Kss1 to Ste12 may expedite Kss1-mediated phosphorylation of Ste12, Tec1, and/or Dig1 and Dig2. Such a docking function has been suggested for the Ste7–Kss1, Jnk-Jun, and Erk–Elk interactions (Bardwell and Thorner 1996; Bardwell et al. 1996; Kalunki et al. 1996; Yang et al. 1998).

The activation loop of Kss1 controls Ste12 binding and inhibition

The activation loop of MAPKs is flexible, and its conformation dictates catalytic activity and contributes to substrate recognition (Cobb and Goldsmith 1995). Our results indicate that this segment of Kss1 controls its ability to repress Ste12. Binding of unphosphorylated Kss1 to Ste12 requires integrity of the activation loop. The Kss1-loop mutant, in which this segment was removed and replaced with an unrelated sequence, exhibited substantially reduced binding to Ste12 (but undiminished binding to Dig1, Dig2, and Ste7) and correspondingly, reduced ability to repress Ste12. A substitution (Y231C) in a residue that is conserved in most MAPKs caused the same reduction in specific binding and repression. This residue contacts the activation loop in unphosphorylated Erk2; but in phosphorylated Erk2, this contact has been broken (Zhang et al. 1995; Canagarajah et al. 1997).

The activation loop of rat Erk2 refolds following MEK-mediated phosphorylation (Canagarajah et al. 1997). Thus, Ste7-mediated phosphorylation of the activation loop of Kss1 provides a simple mechanism to relieve Kss1-imposed repression. We demonstrated here that phosphorylation of T183 and Y185 in Kss1 is necessary for Ste7 to relieve Kss1-mediated inhibition of invasive growth in vivo, and weakens Kss1–Ste12 binding in vitro. This dual phosphorylation is sufficient to relieve Kss1-mediated inhibition of haploid invasive growth, even when Kss1 is not catalytically active. Thus, residues in the activation loop of Kss1 may be in direct contact with Ste12. However, in Erk2, the state of the activation loop affects the conformation of the active site, the P1 specificity pocket, the extended carboxyl terminus, and the so-called MAPK insertion (Zhang et al. 1995; Canagarajah et al. 1997). Hence, other regions of Kss1 could participate in Ste12 recognition. In this regard, two of the mutations (D249G and E260G) isolated by Madhani et al. (1997) are located in the MAPK insertion region.

Phosphorylation-induced refolding of an activation segment situated between conserved subdomains VII and VIII causes conformational changes required for catalysis and for access of substrates to the active site in many protein kinases (Johnson et al. 1996; Canagarajah et al. 1997). Our results argue that such structural rearrangements can also diminish the binding of a kinase to a target, with important physiological consequences.

Generality of MAPK-mediated inhibition

Our work demonstrates that regulation of a transcription factor by a MAPK involves direct protein kinase-transcription factor binding (and recruitment by the bound kinase of other regulatory factors). How general is this mechanism? Relatively stable interactions between mammalian MAPKs and transcription factors have been observed (Gupta et al. 1996; Kalunki et al. 1996; Yang et al. 1998). It was proposed that binding of SAPK/Jnk may inhibit c-Jun function directly (Gupta et al. 1996), although this suggestion is at odds with some recent results (May et al. 1998). Nonetheless, the inhibitory role of Kss1 was revealed initially by use of genetic approaches that are more difficult in organisms less tractable than S. cerevisiae. It seems likely, therefore, that the mechanism characterized here will be found in other protein kinase signaling pathways that act as developmental switches, but may not have been readily discernible heretofore.

Materials and methods

Yeast strains and media

Yeast strains used in this work are shown in Table 1. Standard yeast media were prepared as described (Bardwell et al. 1996), except that, in synthetic complete medium, twice the recommended level of nutritional supplements was used.

Plasmid constructions and recombinant DNA methods

Plasmids YEpU (Cook et al. 1997) and YEp PT–KSS1 (Bardwell et al. 1996) are described in the citations given. YEpU–FTyZ, containing the FRE+ lacZ reporter gene (and the URA3 gene) on a 2μ DNA plasmid, has been described elsewhere (Cook et al. 1997). The URA3 gene was excised from YEpU–FTyZ by digesting with Sse8387 I and Xmal and replaced with the LEU2 gene on a Ps1–Xmal fragment excised from pBJ252 (Jones and Prakash 1990), to yield YEpL–FTyZ.

To construct YCpU–KSS1, an EcoRI–SphI fragment contain-
ing KSS1 and its promoter was excised from plasmid YEp-KSS1 (Ma et al. 1995) and inserted into the corresponding sites of YCpU. YCpU–kss1(Y231C) was generated by use of the BsiEI fragment from YEp-KSS1 (Bardwell et al. 1996) to replace the corresponding fragment of pGEM4Z–KSS1. Only two plasmids (designated p141 and p242) met all three criteria. Nucleotide sequence analysis revealed that both of these plasmids (most likely siblings from the mutagenized DNA library) contained a single A-to-G transition at nucleotide 692 of the KSS1 ORF, resulting in a tyrosine-to-cysteine substitution at residue 231.

Table 1. S. cerevisiae strains used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| JCY100 | MATαhis3Δ::hisG leu2Δ::hisG trp1Δ::hisG ura3-52 | Cook et al. (1997) |
| JCY107 | JCY100 ste7Δ::ura3 | J.C. Hicks (Cold Spring Harbor Laboratory) |
| JCY130 | JCY100 kss1Δ::hisG fus3Δ::TRP1 | Cook et al. (1997) |
| JCY137 | JCY100 ste7Δ::ura3 kss1Δ::hisG fus3Δ::TRP1 | Cook et al. (1997) |
| Other strains | | |
| DC17 | MATαhis1 | J.C. Hicks (Cold Spring Harbor Laboratory) |
| BJ2168 | MATαleu2 trp1 ura3-52 prb1-1112 pep3-3 prc1-407 gal2 | J.C. Hicks (Cold Spring Harbor Laboratory) |
| MaV103a | MATαleu2-3,112 trp1-901 hisΔ200 ade2-101 gal4Δ gal80Δ SPA1Δ::URA3 GAL1:: lacZ GAL1:: HIS3@lys2 can1Δ cyh2Δ | Cook et al. (1997) |

Reverse two-hybrid screen

The kss1(Y231C) allele was isolated in a reverse two-hybrid screen by essentially the method described by Vidal and colleagues (Vidal et al. 1996). Briefly, a library of mutagenized kss1-GDB plasmids was created (Greener et al. 1996) by growing this plasmid for ~25 generations in E. coli mutator strain XL-1 Red (Stratagene), using carbenicillin to select for the plasmid. Plasmid DNA was prepared and used to transform yeast strain MaV103a carrying plasmid pGAD–STE12(191-478). MaV103a contains three GAL promoter-driven reporter genes (URA3, HIS3, and E. coli lacZ). Transformants (19,000) were screened for their ability to grow on agar medium containing 0.1% 5-FOA, thus selecting for loss of URA3 expression, and thereby for drastic reduction of the KSS1–STE12 two-hybrid interaction. The 218 colonies so obtained were then tested for those that could grow on agar medium containing 20 μg 3-amino-1-H-1,2,4-triazole (3-AT), but not on 50 μg 3-AT, thus screening for clones that have diminished, but not completely abolished, HIS3 expression (and thereby a greatly reduced, but not completely null, KSS1–STE12 two-hybrid interaction). From the resulting five clones, the pKSS1–GDB plasmids were rescued, retransformed into MaV103a along with pGAD–DIG17-452, and scored for lacZ expression to identify Kss1 mutants that fully retained the ability to interact with Dig1. Only two plasmids (designated p141 and p242) met all three criteria. Nucleotide sequence analysis revealed that both of these plasmids (most likely siblings from the mutagenized DNA library) contained a single A-to-G transition at nucleotide 692 of the KSS1 ORF, resulting in a tyrosine-to-cysteine substitution at residue 231.

Transcription and translation in vitro

Transcription and translation reactions in vitro, and the partial purification of translation products by ammonium sulfate precipitation, were performed as described previously (Bardwell et al. 1992, 1996). Preparation of in vitro-translated Kss1, Kss1loop, Fus3, and Fus3p was described elsewhere (Bardwell et al. 1996). The Kss1(Y231C) mRNA was transcribed from plasmid pGEM4Z–kss1(Y231C) linearized with Ndel. The mKss1 mRNA was transcribed from plasmid pGEM4Z–mKss1 linearized with SalI.
GST-fusion protein production and binding assays
GST–Ste7L1–172 and GST–Dig113–462 were prepared from E. coli as described elsewhere (Bardwell et al. 1996; Cook et al. 1996). GST–Ste12298–473 was expressed from plasmid pEG-105 in yeast strain B2168 and purified as follows: Cultures (250 ml) were grown overnight in selective medium containing 2% galactose and 0.2% sucrose, and harvested. Cell pellets were resuspended in buffer B (Bardwell et al. 1996) containing 5 mM diithiothreitol (DTT), and extracts were prepared as described previously (Bardwell et al. 1996). The extracts were adjusted to a final concentration of 1.5 % (wt/vol) N-lauroyl-sarcosine (sarcosyl) and rocked at 4°C for 15 min. Triton X-100 was then added to a final concentration of 3.0% (vol/vol), followed by a further 15 min of rocking. The lysate was centrifuged for 5 min at 13,000g at 4°C to remove insoluble material. To the resulting clarified extract, 0.1 ml of a 50% slurry of glutathione-Sepharose (Pharmacia) was added, and the mixture was rotated for 1 hr at 4°C. The glutathione-Sepharose beads were collected, and washed thoroughly with buffer B containing 5 mM DTT and 0.75 M potassium acetate, followed by buffer B containing 5 mM DTT. Bound proteins were eluted with three 10-min incubations at room temperature in 50 μl of freshly prepared 10 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0). The eluate fractions were pooled (150 μl total) and frozen at −70°C. Alternatively, fusion proteins bound to glutathione-Sepharose beads were stored on ice in buffer B containing 5 mM DTT and 1 mg/ml BSA until needed. GST–Ste12298–473 protein purified from yeast by this method was judged to be >90% pure by Coomassie blue staining and to be free of contaminating Dig1, Dig2, Kss1, or Fus3 by immunoblotting. GST was purified from both yeast and E. coli. No difference in the binding properties of GST purified from these two sources was found.

Binding assays using GST fusions and in vitro-translated proteins were as described elsewhere (Cook et al. 1996). Binding assays using GST fusions and yeast cell extracts were performed by our published procedure for immunoprecipitation from yeast cell extracts (Bardwell et al. 1996), except that glutathione-Sepharose-bound fusion proteins were used in place of Protein A/G-antibody beads, and 1 mg/ml BSA and 5 mM DTT were present in all buffers.

Other methods and reagents
Expression of the FRET13–lacZ reporter gene was determined as described elsewhere (Cook et al. 1997). The bioassay for haploid invasive growth has been described (Roberts and Fink 1994). Growth and harvesting of yeast cultures for biochemical analysis, preparation of cell extracts, and coimmunoprecipitation assays were performed exactly as described previously (Bardwell et al. 1996). Anti-GST antisera was purchased from Santa Cruz Biotechnologies. The rabbit polyclonal antisera used for analysis of Kss1 (Ma et al. 1995) has been described. Anti-phospho-MAPK antisera (Khokhlatchev et al. 1997), initially a gift of Erik Cobb and thereafter obtained from a commercial source (anti-ACTIVE-MAPK antisera; Promega), was used at a dilution of 1:1300 with overnight incubation at 4°C.

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