Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast *Saccharomyces cerevisiae*

Jeanette Gowen Cook,¹ Lee Bardwell,¹ Stephen J. Kron,² and Jeremy Thorner^{1,3}

¹Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3202 USA; ²Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637 USA

Haploid cells of budding yeast *Saccharomyces cerevisiae* respond to mating pheromones by inducing genes required for conjugation, arresting cell cycle progression, and undergoing morphological changes. The same cells respond to nutrient deprivation by altering budding pattern and inducing genes required for invasive growth. Both developmental alternatives to vegetative proliferation require the MAP kinase Kss1 and the transcriptional transactivator Ste12. Using a two-hybrid screen for gene products that interact with Kss1, two homologous and previously uncharacterized loci (*DIG1* and *DIG2*, for down-regulator of invasive growth) were identified. *DIG2* is pheromone-inducible, whereas *DIG1* is constitutively expressed. Dig1 colocalizes with Kss1 in the nucleus, coimmunoprecipitates with Kss1 from cell extracts in a pheromone-independent manner, and is phosphorylated by Kss1 in immune complexes in a pheromone-stimulated manner. Kss1 binds specifically to a GST–Dig1 fusion in the absence of any other yeast protein. Using the two-hybrid method, both Dig1 and Dig2 also interact with the other MAP kinase of the pheromone response pathway, Fus3. However, neither *dig1* or *dig2* single mutants, nor a *dig1 dig2* double mutant, have a discernible effect on mating. In contrast, *dig1 dig2* cells constitutively invade agar medium, whereas a *dig1 dig2 ste12* triple mutant does not, indicating that Dig1 and Dig2 share a role in negatively regulating the invasive growth pathway. High-level expression of Dig1 suppresses invasive growth and also causes cells to appear more resistant to pheromone-imposed cell cycle arrest. Ste12 also binds specifically to GST–Dig1 in the absence of any other yeast protein. Collectively, these findings indicate that Dig1, and most likely Dig2, are physiological substrates of Kss1 and suggest that they regulate Ste12 function by direct protein–protein interaction.

[Key Words: Protein kinase; two-hybrid screen; protein–protein interaction; signal transduction; gene regulation; developmental control]

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Eukaryotic cells respond to diverse extracellular signals, such as growth factors, nutrients, and environmental stresses, by altering their growth and morphology accordingly. These cues are transmitted from the cell surface through multitiered and interconnected signaling pathways to various intracellular target molecules. Haploid cells of the budding yeast *Saccharomyces cerevisiae* have a number of developmental options in addition to their mitotic growth and division. One such alternative developmental program is the mating process, which occurs in response to secreted peptides (mating pheromones). The multicomponent signaling cascade required

for pheromone response is perhaps the best understood signal transduction pathway known in any eukaryotic organism (Bardwell et al. 1994b), and components of this pathway are evolutionarily conserved in metazoans (Dhanasekaran et al. 1995). The mating pheromones bind to cell type-specific, G protein-coupled receptors. Receptor occupancy stimulates release of G $\beta\gamma$ (Whiteaway et al. 1989), which is necessary to activate downstream events. One of the proposed effectors of $\beta\gamma$ is Cdc24, a guanine–nucleotide exchange factor (Zhao et al. 1995), which acts on Cdc42, a Rho-like small GTPase (Simon et al. 1995). Cdc42 is able to bind to (and presumably stimulate) Ste20 (Leberer et al. 1992), a homolog of the mammalian protein–serine kinase p65^{PAK} (Manser et al. 1994; Martin et al. 1995). Ste20 activates an archetypal MAP kinase (MAPK) cascade (Cairnes et

³Corresponding author.

al. 1992; Errede et al. 1993; Neiman and Herskowitz 1994), consisting of Ste11 (a MEK kinase), Ste7 (a MEK), and two MAPKs, Kss1 and Fus3, that are partially redundant in function (Elion et al. 1991; Ma et al. 1995). Hence, Ste20 functions as a MEK kinase kinase (Wu et al. 1995). Each of the four different classes of protein kinase in this MAPK cascade has been shown to associate with the Ste5 protein, which appears to serve, at least in part, as a platform or scaffold for these other signaling components (Choi et al. 1994; Marcus et al. 1994; Printen and Sprague 1994; Leeuw et al. 1995). Both Kss1 and Fus3 efficiently phosphorylate Ste7 (Bardwell et al. 1996; Errede and Ge 1996), presumably as part of a feedback mechanism for modulating the activity of this enzyme. Likewise, Fus3 has been shown to phosphorylate Ste5 (Kranz et al. 1994). However, only two downstream substrates of Kss1 and Fus3 have been identified previously. These targets are the Cdk inhibitor Far1 (Chang and Herskowitz 1990, 1992), which is phosphorylated most efficiently by Fus3 (Elion et al. 1993; Peter et al. 1993; Tyers and Futcher 1993) and the Ste12 transcription factor (Elion et al. 1993; L. Bardwell, J.G. Cook, and J. Thorner, unpubl.). As a result of these modifications (and presumably others) cell cycle progression is arrested in the G₁ phase, the expression of multiple genes is induced, and dramatic changes occur in cell polarity and morphology in preparation for cytoplasmic and nuclear fusion (for review, see Sprague and Thorner 1992; Kurjan 1993).

As a developmental response to changes in nutrient conditions, *S. cerevisiae* cells can undergo a dimorphic transition (for review, see Kron and Gow 1995). Under conditions of nutrient deprivation, especially nitrogen limitation, MATa/MAT α cells convert from their unicellular, ovoid yeast form to a more elongated, filament-like pseudohyphal form (Gimeno et al. 1992; Blacketer et al. 1993). This reversible differentiation process is characterized by the formation of elongated cells that bud synchronously in a unipolar fashion and are capable of invading solid agar medium (Kron et al. 1994). In contrast, haploid cells form only short filaments and display invasive growth only on rich agar medium (Roberts and Fink 1994). Furthermore, only a subset of laboratory strains of *S. cerevisiae*, specifically those derived from the Belgian Σ 1278 lineage (Wiame et al. 1985), are capable of displaying such changes in response to nutrient deprivation. In this strain background, it has been shown that various components of the same MAPK pathway that is required for mating are also necessary to manifest filamentous and invasive growth. Ste20, Ste11, Ste7, and Ste12 are all required for both the diploid and haploid versions of pseudohyphal and invasive growth (Liu et al. 1993; Roberts and Fink 1994). Kss1 appears to have a supportive rather than essential role in pseudohyphal growth in diploids, and Fus3 is totally dispensible for this process (Liu et al. 1993). In marked contrast, in haploid cells Kss1 is required for invasive growth, whereas Fus3 negatively regulates this process (Roberts and Fink 1994). Thus, rather than serving essentially redundant roles as they do in the pheromone response pathway,

Kss1 and Fus3 have opposite functions in the signaling pathway that leads to invasive growth by haploid cells.

Given that Kss1 and Fus3 are responsible for transmitting two different kinds of signals, each of which elicits a multiplicity of cellular responses, it seemed reasonable to conclude that these MAPKs should have multiple target substrates, as well as multiple regulatory and accessory factors. As one approach for identifying novel proteins that interact with Kss1, we used the two-hybrid screen (Fields and Song 1989; Bartel and Fields 1995). Here, we describe the isolation and characterization of two novel genes, *DIG1* and *DIG2*, which encode proteins that occupy the same cellular compartment as Kss1, associate with Kss1, and are substrates of Kss1 in vitro. We further demonstrate using genetic analysis that Dig1 and Dig2 are negative regulators of invasive growth by showing that loss of Dig1 and Dig2 permits invasive growth by haploid strains that are normally incapable of undergoing this developmental switch. Moreover, we found that Dig1 can associate with Ste12 in the absence of any other yeast protein, suggesting that Dig1 (and presumably Dig2) negatively regulates the Ste12-dependent expression of genes required for invasive growth by direct association with Ste12.

Results

Identification of gene products that interact with Kss1

To perform a two-hybrid screen to identify novel proteins that interact with Kss1, we initially fused two different DNA-binding domains to the amino terminus of Kss1. Both Gal4 DNA-binding domain (GDB)–Kss1 and LexA DNA-binding domain–Kss1 chimeras were constructed. Although both fusion proteins were expressed at a readily detectable level, neither was able to complement the mating defect of a MATa *kss1* Δ *fus3* Δ double mutant (data not shown). In addition, neither fusion protein, when expressed at a high level, was able to cause the formation of a turbid halo in the standard agar diffusion bioassay for pheromone-induced G₁ arrest (Julius et al. 1983), despite the fact that even many catalytically inactive mutants of Kss1 are able to do so (Ma et al. 1995). Moreover, neither fusion, when coexpressed with a construct (generously provided by S. Fields, University of Washington, Seattle, WA) in which full-length Ste12 (a known Kss1 target) was fused to the carboxyl terminus of the sequence encoding the Gal4 transcriptional activation domain (GAD), was able to generate a reproducibly positive signal indicative of an authentic interaction (data not shown). These results suggested that the structure and/or function of Kss1 is highly sensitive to alterations at its amino terminus.

To avoid this problem, a third fusion was prepared in which the GDB was fused to the carboxy-terminal end of full-length Kss1. Immunoblot analysis using a rabbit polyclonal anti-Kss1 antiserum (Ma et al. 1995) detected a protein species that migrated at the molecular weight expected for this fusion protein (data not shown). This

Table 1. *S. cerevisiae* strains

Strain	Genotype	Source
YPH499	<i>MATa ade2-101^{oc} his3-Δ200 leu2-Δ1 lys2-801^{am} trp1-Δ63 ura3-52</i>	Sikorski and Hieter (1989)
YPH500	<i>MATα</i> (otherwise isogenic to YPH499)	Sikorski and Hieter (1989)
YPH501	<i>MATa/MATα</i> (YPH499 × YPH500)	Sikorski and Hieter (1989)
Y190	<i>MATa ade2-101^{oc} cyh2 gal4 gal80 his3 leu2-3, 112 trp1-901 ura3-52</i> <i>URA3::GAL1-lacZ LYS2::GAL1-HIS3</i>	S. Elledge (Baylor University, Houston, TX)
DK499 ^a	<i>YPH499 sst1-Δ5::hisG</i>	D. Kaim
YDM210 ^b	<i>YPH499 fus3-6::LEU2 sst2Δ::ura3</i>	D. Ma
YDM230	<i>YPH499 kss1Δ::hisG fus3-6::LEU2</i>	Ma et al. (1995)
YDM400 ^c	<i>YPH499 sst2Δ::ura3</i>	Ma et al. (1995)
YDM420 ^d	<i>YPH499 kss1Δ::hisG sst2::ura3</i>	D. Ma
JCY3	<i>YPH499 dig1-Δ1::HIS3</i>	This work
JCY4	<i>YPH499 dig2-Δ1::TRP1</i>	This work
JCY5	<i>YPH499 dig1-Δ1::HIS3 dig2-Δ1::TRP1</i>	This work
JCY6 ^e	<i>MATα</i> otherwise isogenic to JCY5	This work
YD4 ^f	<i>MATa/MATα dig1/dig1 dig2/dig2</i> (JCY5 × JCY6)	This work
JCY512 ^g	<i>YPH499 dig1-Δ1::HIS3 dig2-Δ1::TRP1 ste12::LEU2</i>	This work
JDY3 ^g	<i>YPH499 ste12::LEU2</i>	J.L. Davis
10560-4A	<i>MATa his3::hisG leu2::hisG trp1::hisG ura3-52</i>	G.R. Fink (MIT, Cambridge, MA)
Σ1278b	<i>MATa/MATα ura3-52/ura3-52 leu2::hisG/leu2::hisG</i>	Liu et al. (1993)
BJ2168	<i>MATa gal2 leu2 pep4-3 prb1-1112 prc1-407 trp1 ura3-52</i>	Jones (1991)
DC14	<i>MATa his1</i>	J.B. Hicks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)
DC17	<i>MATα his1</i>	J.B. Hicks

^aStrain YPH499 was transformed by D. Kaim (this laboratory) with a DNA fragment containing the *sst1-Δ5::hisG::URA3::hisG* allele from plasmid pJGsst1, which has been described in detail elsewhere (Reneke et al. 1988), selecting for Ura⁺ transformants and testing for acquisition of super-sensitivity to pheromone-induced growth arrest (Chan et al. 1983), and subsequently selecting for a *ura3*⁻ derivative using medium containing 5-fluoro-orotic acid (5-FOA) (Boeke et al. 1984).

^bStrain YDM210 was constructed in two steps by D. Ma (this laboratory). YPH499 was transformed with an *NheI*–*HindIII* fragment containing the *fus3-6::LEU2* allele from plasmid pYEE98 (Elion et al. 1990), and disruption of the chromosomal *FUS3* gene was confirmed by Southern hybridization, yielding YDM200 [YPH499 *fus3-6::LEU2*]. To generate YDM210, the *sst2Δ::ura3* allele present in plasmid pBC14 was introduced into YDM200 by the two-step gene transplacement method of Scherer and Davis (1979), as described previously (Ma et al. 1995), and confirmed by Southern hybridization and by acquisition of pheromone hypersensitivity.

^cYDM400 was incorrectly indicated as *URA3*⁺ in Table 1 of Ma et al. (1995).

^dStrain YDM420 was constructed in two steps by D. Ma (this laboratory). YPH499 was transformed with the *kss1Δ::hisG::URA3::hisG* allele (Ma et al. 1995), and disruption of the chromosomal *KSS1* locus was confirmed by Southern hybridization. A Ura⁻ derivative was selected on 5-FOA medium, yielding YDM600 [YPH499 *kss1::hisG*]. To generate YDM420, the *sst2Δ::ura3* allele was introduced into YDM600, as described in footnote b.

^eJCY5 was transformed with a multicopy plasmid expressing the *HO* gene (Herskowitz and Jensen 1991) from the *GAL1* promoter, propagated briefly on galactose, plated on medium containing glucose and 5-FOA, and screened for colonies that mated reproducibly as *MATα*, one of which was designated JCY6.

^fTo generate a homozygous *dig1/dig1 dig2/dig2 MATa/MATα* diploid, individual cells of JCY5 and JCY6 were juxtaposed by micro-manipulation and the resulting diploid clones were confirmed by their lack of mating phenotype.

^gStrain JCY5 was transformed with a *Bam*HI fragment from plasmid pLB1367 (gift of G. Ammerer, Research Institute of Molecular Pathology, Vienna, Austria), in which an internal 1.1-kb *Xba*I fragment of *STE12* is replaced with the *LEU2* gene, and Leu⁺ transformants were scored for sterility that could be complemented by a plasmid [YEp352GAL-*STE12*] expressing *STE12* at its uninduced level on raffinose-containing medium (gift of S. Fields, University of Washington, Seattle, WA). Strain JDY3 was constructed in the same manner by J.L. Davis (this laboratory) by transformation of YPH499.

Kss1–GBD chimera was able to complement weakly the mating defect of *MATa kss1Δ fus3Δ* tester strain and was able, when overproduced, to cause a turbid halo in the plate assay for pheromone response (data not shown). Most significantly, the Kss1–GDB fusion also consistently scored positive for interaction with the tester GAD–Ste12 chimera (data not shown) and with various other GAD–Ste12 fusions in the two-hybrid test (see Fig. 1). Because the Kss1–GDB carboxy-terminal fusion behaved more like native Kss1 than either of the amino-

terminal fusions, it was used to conduct a two-hybrid screen for interacting gene products.

For this purpose, strain Y190 (Table 1), which contains two *GAL* promoter-driven reporter genes (yeast *HIS3* and *Escherichia coli lacZ*), was cotransformed with a plasmid encoding the Kss1–GDB fusion and either a library of yeast cDNAs fused to the GAD coding sequence or a library of random yeast genomic DNA fragments fused to the same sequence. Positive clones were initially selected via their robust level of *HIS3* expression, as judged

Figure 1. A two-hybrid screen for proteins that interact with Kss1 identifies two novel genes, *DIG1* and *DIG2*. Serial 10-fold dilutions (left to right) of exponentially grown cultures ($\sim 10^7$ /ml) of strain Y190 cotransformed with a plasmid [pJGC2] expressing a Kss1–GDB chimera and a plasmid expressing either GAD alone (top row), a GAD–Ste12 fusion (second row), a GAD–Dig1 fusion (third row), or a GAD–Dig2 fusion (bottom row), obtained from library screening, were spotted onto plates containing SCGlc-Trp-Leu-His medium and the various concentrations of 3-AT indicated. Because 3-AT is a competitive inhibitor of the His3 enzyme, the level of 3-AT resistance is an indicator of the magnitude of chromosomal *GAL_{UAS}::HIS3* reporter expression and, thus, of the strength of the interaction between Kss1–GDB and the GAD-containing fusion protein.

by their ability to grow on agar medium containing 50 mM 3-amino-1*H*-1,2,4-triazole (3-AT), a competitive inhibitor of the His3 enzyme (Klotkowski and Wiater 1965), and then subjected to a secondary screen for vigorous production of β -galactosidase, as judged by the generation of blue color in permeabilized colony replicas using the standard 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) indicator dye (Miller 1972). Library plasmids from the reproducibly positive isolates were recovered and characterized using conventional recombinant DNA methods and direct nucleotide sequence analysis (Sambrook et al. 1989).

In the collection of plasmids obtained in this fashion from $\sim 6 \times 10^6$ transformants screened, a total of 11 encoded GAD–Ste12 fusions, representing seven distinct isolates. The recovery of Ste12-containing fusions from a random screen further validated the conclusion that the Kss1–GDB chimera was able to interact with an authentic Kss1 substrate. This result also suggested that the fusion would also be capable of identifying other proteins that associate with Kss1 in a bona fide manner. Indeed, in this same screen (Fig. 1), we obtained seven independent isolates that encoded different, but overlapping, segments of the same novel gene, which we designated *DIG1*, as well as four independent isolates of a second, homologous gene, which we termed *DIG2*, based on the phenotypes of null alleles in these genes (see below). Our analysis of the complete DNA sequences of *DIG1* and *DIG2* confirmed the open reading frames for these coding sequences (GenBank accession nos. U44030 and U33050, respectively), which were obtained as the result of the recently completed international effort to

determine the nucleotide sequence of the entire *S. cerevisiae* genome. None of the GAD–Dig1 and GAD–Dig2 fusions was able to yield a positive two-hybrid interaction when coexpressed with several other DNA-binding chimeras, including GDB fusions to a mammalian protein kinase (Raf), a mammalian protein kinase substrate (Rb), and two different nuclearly localized yeast proteins (Fpr3 and Rad3) (data not shown). These results demonstrated that the interaction of GAD–Dig1 and GAD–Dig2 with Kss1–GDB was specific.

As judged by the level of resistance to 3-AT or the degree of blue color on X-Gal filter replicas, the strength of the association between the GAD–Dig1 and GAD–Dig2 fusions and the Kss1–GDB chimera was not altered detectably by treatment of the Y190 cells (which are MATa) with α -factor mating pheromone (data not shown). Likewise, the signal observed was not appreciably different when GAD–Dig1 or GAD–Dig2 were coexpressed with a catalytically inactive Kss1 (K42R)–GDB derivative. Thus, the interaction of GAD–Dig1 and GAD–Dig2 with Kss1–GDB appeared to be largely constitutive and was neither dependent on, nor attenuated by, the protein kinase activity of Kss1. As judged by the two-hybrid method, both the GAD–Dig1 and GAD–Dig2 fusions were able to interact with a GDB–Fus3 fusion (Printen and Sprague 1994) (data not shown).

Sequence and expression of the *DIG1* and *DIG2* genes

DIG1 resides on the left arm of chromosome 16, adjacent to *MNN9* and *DIG2* is located on the right arm of chromosome 4, adjacent to *PHO8*. An alignment of the deduced amino acid sequences of the 452-residue Dig1 (calculated molecular weight of 49,360) and the 323-residue Dig2 (calculated molecular weight of 36,883) polypeptides showed that they are homologous (Fig. 2). The two proteins share 27% amino acid sequence identity and 42% similarity, if standard conservative amino acid substitutions are considered. Both the proteins are highly charged, rich in proline, and contain multiple occurrences of serine–proline and threonine–proline, which are the minimal consensus sites for phosphorylation by the proline-directed MAPKs (Alvarez et al. 1991; Clark-Lewis et al. 1991; Davis 1993). The smallest segment of either protein that was recovered as an isolate in the two-hybrid screen was the carboxy-terminal half of Dig1 (residues 213–452). The two proteins share a reasonable degree of similarity over this region (aside from insertions in the significantly longer Dig1 protein), and the majority of the serine–proline and threonine–proline sites (four of six in Dig1 and three of five in Dig2) fall in this same segment of each protein. These considerations suggest that the carboxy-terminal portions of Dig1 and Dig2 are sufficient for their interaction with Kss1. Other than their homology to each other, Dig1 and Dig2 bear no extensive resemblance to any other proteins in currently available data bases.

Expression of *DIG1* and *DIG2* was examined by RNA hybridization analysis in two different laboratory strain backgrounds: an isogenic set of S288C-derived MATa

Figure 2. Dig1 and Dig2 are homologous proteins. The deduced amino acid sequences of the *DIG1* and *DIG2* gene products were aligned using the FAST-P algorithm (Lipman and Pearson 1985), and then optimized by eye. Identical residues are given as white-on-black letters; conventional conservative amino acid substitutions (D,E; K,R,H; F,Y,W; Q,N;; S,T; L,V,I,M; P,G,A; D,N; E,Q; A,S,C) are shaded. Dashes indicate single-residue gaps inserted to optimize the alignment. The smallest segment of Dig1 isolated as a GAD fusion in the two-hybrid screen is underlined. Potential MAP kinase phosphorylation sites (Ser-Pro or Thr-Pro) are indicated by the solid circles above (for Dig1) and below (for Dig2) each line.

(YPH499), *MAT α* (YPH500), and *MATa/MAT α* (YPH501) strains; and a *MATa* haploid and *MATa/MAT α* diploid of the Σ 1278 lineage. The $\sim$ 1.9-kb *DIG1* mRNA was readily detectable and, like *KSS1* (Courchesne et al. 1989), was expressed at approximately the same level in all three cell types and unaffected by α -factor treatment of *MATa* cells (Fig. 3A). In contrast, basal expression of the $\sim$ 1.4-kb *DIG2* transcript was barely detectable in all three cell types, but was markedly elevated (at least threefold) in *MATa* cells treated with α -factor (Fig. 3B). Not unexpectedly, the sequence of the 5'-flanking region of the *DIG2* coding sequence contains one perfect and

one seven out-of-eight match to the consensus pheromone response element (Ste12-binding site) (Van Arsdell and Thorner 1987; Dolan et al. 1989). The apparent sizes of both mRNAs are more than sufficient to encode the *DIG1* and *DIG2* open reading frames.

Dig1 is a nuclear protein

We have shown previously that the bulk of the cellular pool of Kss1 is found in the nucleus (Ma et al. 1995). If Dig1 and Dig2 interact with Kss1 in vivo, then these proteins should occupy the same subcellular compartment as Kss1. Both Dig1 and Dig2 contain extended

Figure 3. Expression of *DIG1* and *DIG2*. Total RNA (50 μ g) from the strains indicated (see also Table 1), grown and treated with pheromone as described in Materials and Methods, was extracted, resolved by agarose gel electrophoresis, blotted to a nylon membrane, and probed with 32 P-labeled internal fragments of either *DIG1* [A], *DIG2* [B], or *CMD1* (insert).

blocks of basic amino acids (residues 83–102 and 88–105, respectively) in a context very similar to that present in the nuclear localization signals found in other yeast nuclear proteins (Osborne and Silver 1993). To address the localization of Dig1, a rabbit polyclonal anti-Dig1 antiserum was raised against a bacterially expressed GST–Dig1 fusion protein, as described in Materials and Methods. This antiserum recognized a doublet of bands with a mobility on SDS–PAGE corresponding to an apparent molecular mass of ~62 kD (Fig. 4). This doublet represents the *DIG1* gene product because these bands were absent in two independently derived *dig1Δ* strains and were greatly elevated (along with a series of degradation products) when Dig1 was overexpressed from the *GAL1* promoter (Fig. 4). The apparent size of Dig1 based on gel mobility is significantly larger than its calculated molecular weight, which is a frequently observed anomaly for highly charged and proline-rich proteins (for example, see Benton et al. 1994). Neither the steady-state level nor the electrophoretic mobility of Dig1 were detectably altered by prior pheromone treatment of the cells (data not shown).

To determine the subcellular distribution of Dig1, we used indirect immunofluorescence (Fig. 5). When *DIG1*⁺ cells were fixed and stained, bright fluorescence was seen that was congruent with that observed for the DNA dye, 4',6-diamidino-2-phenylindole (DAPI), indicating that Dig1 is located predominantly in the nucleus. Even when Dig1 was markedly overexpressed from the *GAL1* promoter, the signal (now much brighter) was still confined to the nucleus. Faint fluorescence observed outside the nucleus was strictly a result of nonspecific staining

Figure 4. Specificity of the anti-Dig1 antiserum. Samples of extracts (50 μ g total protein), prepared from YPH499 (wt), two independently generated isolates of JCY3 (*dig1Δ*), and YPH499 transformed with *pGAL1-DIG1* (right lane), grown in SC medium containing 2% galactose and 0.2% sucrose (and, where necessary, lacking uracil to maintain selection for the plasmid), were resolved on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblotting with rabbit polyclonal anti-Dig1 antibodies as described in Materials and Methods.

Figure 5. Dig1 is localized to the nucleus by indirect immunofluorescence. Exponentially growing cultures of YPH499 (A), YPH499 transformed with *pGAL1-DIG1* (B), or the *dig1Δ* strain, JCY3 (C), were grown in SC medium containing 2% galactose and 0.2% sucrose with appropriate nutritional supplements, fixed, permeabilized, and stained with the DNA dye, DAPI (left column) or with rabbit polyclonal anti-Dig1 antibodies followed by FITC-labeled goat anti-rabbit immunoglobulin (right column), as described in Materials and Methods.

by the antiserum because only the same diffuse signal was seen when *dig1Δ* cells were examined. As observed previously for Kss1 itself (Ma et al. 1995), localization of Dig1 was unaffected by α -factor treatment of *MATa* cells (data not shown). Dig1 was still localized to the nucleus in a *kss1Δ fus3Δ* double mutant; hence, nuclear localization of Dig1 does not depend on phosphorylation by, or trafficking in a complex with, either of these MAPKs.

Kss1–Dig1 complexes are detectable in cell extracts

The interaction between Kss1–GDB and GAD–Dig1 observed genetically, and the colocalization of native Dig1 with Kss1 in the nucleus, suggested that Dig1 and Kss1 have an opportunity to interact physically in the cell. To determine whether Kss1 and Dig1 associate in vivo, we analyzed whether the two proteins could be coimmunoprecipitated from cell extracts. Toward this end, we expressed in cells either normal Kss1 or a version of Kss1 containing at its carboxyl terminus a 10-residue c-Myc epitope tag (Kss1–myc) that is recognized by the highly specific anti-c-Myc monoclonal antibody (mAb) 9E10 (Evan et al. 1985). To enhance the sensitivity of detec-

tion, all constructs were expressed at an elevated level from the *GAL1* promoter. When extracts of cells expressing Kss1-myc were subjected to immunoprecipitation with mAb 9E10, a readily detectable amount of Kss1 was recovered, as determined by immunoblotting with rabbit polyclonal anti-Kss1 serum (Fig. 6A, left). In contrast, in control cells expressing the untagged Kss1, no Kss1 was immunoprecipitated by mAb 9E10, as expected. When cells expressing Kss1-myc were also cotransformed with a plasmid expressing *DIG1*, a readily detectable amount of Dig1 was found in the Kss1-myc precipitates (Fig. 6A, right). No Dig1 was immunoprecipitated by mAb 9E10 when *DIG1* was coexpressed with the untagged Kss1, confirming that the coimmunoprecipitation observed was a result of an authentic association between Dig1 and Kss1-myc. Exposure of the cells to pheromone prior to preparation of the lysates only slightly enhanced (if at all) the amount of Dig1 present in a complex with Kss1-myc, in agreement with our finding that pheromone treatment did not affect the interaction between Kss1-GDB and GAD-Dig1, as judged by the two-hybrid method.

Dig1 is a phosphoacceptor substrate for Kss1

We have demonstrated elsewhere (Bardwell et al. 1996) that the phosphotransferase activity of Kss1 toward coimmunoprecipitated and exogenously added substrates can be measured in immune complex kinase assays. For this purpose, the immune complexes obtained as described above, which were bound on beads (see Materials and Methods), were incubated in buffer containing Mg^{2+} and $[\gamma\text{-}^{32}P]\text{ATP}$, and the resulting radiolabeled species were resolved by SDS-PAGE and examined by autoradiography. To determine whether Dig1 is a substrate for Kss1, Dig1 was co-overexpressed with c-Myc epitope-tagged versions of wild-type Kss1 and a catalytically unactivatable variant, Kss1 (T183A Y185F) (Gartner et al. 1992; Ma et al. 1995). A doublet of phosphorylated bands of the appropriate apparent molecular mass to be Dig1 was observed when Dig1 was coexpressed with normal Kss1-myc, but only when this MAPK was activated by prior treatment of the cells with pheromone (Fig. 6B, cf. lanes 7 and 8); these species were absent when Dig1 was coexpressed with the nonactivatable Kss1-myc, whether or not the cells were pretreated with pheromone (Fig. 6B, lanes 9 and 10). Even when *DIG1* was expressed at its endogenous level, the phosphorylated Dig1 species were detectable, albeit faintly, in the immune complexes prepared from cells expressing normal Kss1-myc that had been pheromone-treated (Fig. 6B, cf. lanes 3 and 4), but not in cells expressing the unactivatable Kss1-myc mutant before or after pheromone treatment (Fig. 6B, lanes 5 and 6). Thus, the Dig1 that coimmunoprecipitates with active Kss1-myc is a target substrate for this MAPK.

Kss1 and Dig1 physically associate

To determine whether the association of Dig1 with Kss1 detected by coimmunoprecipitation from cell extracts

represents a direct interaction between these two proteins and does not require any other yeast protein, we

Figure 6. Coimmunoprecipitation of Dig1 with Kss1 and phosphorylation of Dig1 by Kss1. (A) Protease-deficient strain BJ2168 was transformed with either YE p GAL-*KSS1* (no tag) (Ma et al. 1995) or YE p TG-*KSS1*H6myc (*Kss1-myc*), along with pGAL1-*DIG1* (*Dig1*), where indicated, grown in SC containing 2% galactose and 0.2% sucrose with appropriate supplements to maintain selection for the plasmids, treated with 6 μ M α -factor (+) or not (–) for 15 min, as indicated, harvested, and lysed, and samples of the resulting extracts were immunoprecipitated with anti-c-Myc mAb 9E10, by procedures described in detail previously (Bardwell et al. 1996). The resulting immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with rabbit polyclonal antisera directed against either Kss1 (left) or Dig1 (right). (B) Protease-deficient strain BJ2168 was transformed either with YE p GAL-*KSS1* (no myc tag) (Ma et al. 1995), with YE p TG-*KSS1*H6myc (wt), or with the nonactivatable mutant YE p TG-*KSS1*(AEF)H6myc (*TY-AF*), along with either a vector alone (YE p 352GAL) (Benton et al. 1990) or pGAL1-*DIG1*, as indicated, grown in SC containing 2% galactose and 0.2% sucrose with appropriate supplements to maintain selection for the plasmids, treated with 6 μ M α -factor (+) or not (–) for 15 min, as indicated, harvested, and lysed, and samples of the resulting extracts were immunoprecipitated with anti-c-Myc mAb 9E10, by procedures described in detail previously (Bardwell et al. 1996). The resulting immune complexes were resuspended in protein kinase assay buffer, incubated with Mg^{2+} and $[\gamma\text{-}^{32}P]\text{ATP}$ for 10 min, and then analyzed by SDS-PAGE and autoradiography, also as described in Bardwell et al. (1996). Doublet representing radiolabeled Dig1 species indicated by brackets (lanes 4 and 8).

synthesized Kss1 by in vitro translation and tested its ability to bind to beads carrying either glutathione *S*-transferase (GST) alone, a GST-Dig1(213–452) fusion, or, as a positive control, a GST-Ste7(1–172) fusion (Fig. 7A). We have demonstrated previously that the amino-terminal noncatalytic domain of Ste7 (residues 1–172) binds tightly and specifically to both Kss1 and Fus3 in solution (Bardwell et al. 1996). Kss1 was able to bind strongly to both the GST-Dig1(213–452) and the GST-Ste7(1–172) fusions, but not to GST alone (Fig. 7A). Thus, the ability of Dig1 to associate physically with Kss1 was demonstrated by four independent criteria: two-hybrid interaction, coimmunoprecipitation, ability to serve as a phosphoacceptor substrate, and direct binding in vitro.

DIG1 and DIG2 encode negative regulators of invasive growth

Given that the *DIG1* gene product is found in the nucleus, associates with Kss1, and can be phosphorylated by Kss1, it was of obvious interest to determine whether Dig1 plays any role in either of the two signaling pathways in which Kss1 functions (mating and invasive growth) by examining the phenotype of Dig1-deficient cells. Towards this end, we constructed the *dig1-Δ1::HIS3* allele in which most of the *DIG1* coding sequence is replaced with the *HIS3* gene (see Materials and Methods). Given that *DIG2* is homologous to *DIG1*, and also was isolated by virtue of its interaction with Kss1 as

detected in the two-hybrid screen, we suspected that it might share some partially redundant function with *DIG1*. Hence, we also constructed the *dig2-Δ1::TRP1* allele by replacing most of the *DIG2* coding sequence with the *TRP1* gene (see Materials and Methods). These constructs were used for DNA-mediated transformation of both diploid and haploid recipients of appropriate genotype. We found, first, that neither *DIG1* nor *DIG2* are essential genes, and that even a *dig1 dig2* double mutant shows no overt deficiency in growth rate under a wide variety of conditions. Second, we found that both *dig1* and *dig2* single mutants, as well as *dig1 dig2* double mutants, mated with an efficiency indistinguishable from otherwise isogenic *DIG1⁺ DIG2⁺* cells, and showed a pheromone sensitivity identical to the same controls (data not shown).

Because we could not detect any discernible mating-associated phenotype, we examined whether *dig1* and *dig2* mutations have any effect on haploid invasive growth. Unlike otherwise isogenic control haploids (and *dig1* and *dig2* single mutants), the *dig1 dig2* double mutant displayed the ability to undergo invasive growth because it was able to penetrate beneath the surface of an agar plate containing rich medium (Fig. 8A). This result was striking because the S288C-derived parental strains (Table 1) in which the *dig1 dig2* double mutant was generated have not been reported previously to manifest haploid invasive growth under any condition tested. The *dig1Δ dig2Δ* cells remaining in the agar after the wash procedure used to reveal invasive growth were examined

Figure 7. In vitro binding of Kss1 and Ste12 to GST-Dig1 in vitro. (A) ^{35}S -Kss1 and ^{35}S -Ste12 were prepared by in vitro translation, partially purified by ammonium sulfate precipitation, and portions (10% of the amount added in the binding reactions, "input") were subjected to SDS-PAGE in a 10% polyacrylamide gel (lanes 1 and 2). Samples (1 pmole) of the same proteins, each accompanied by $\sim 60 \mu\text{g}$ of total protein from the rabbit reticulocyte lysate, were incubated with approximately equal amounts of bacterially expressed GST (lanes 3 and 6), GST-Dig1_{213–452} (lanes 4 and 7), or GST-Ste7_{1–172} (lanes 5 and 8) bound to glutathione-Sepharose beads. Bead-bound protein complexes were isolated and analyzed on the same gel. Migration positions of molecular mass markers are shown in kilodaltons on the left. The percentage of the input ^{35}S -labeled protein bound in the reactions corresponding to lanes 3–8, respectively, was 0.3, 4, 12, 0.6, 12, and 0.5. (B) ^{35}S -Ste12 and c-Fos were prepared as described in A and portions (2% of the amount added in the binding reactions, "input") were analyzed by SDS-PAGE as in (A) (lanes 2 and 3). Samples (1 pmole) of the same proteins were incubated with $\sim 25 \text{ pmole}$ of purified GST (lanes 4 and 6) or GST-Dig1_{213–452} (lanes 5 and 7) which had been rebound to glutathione-Sepharose beads, and the resulting bead-bound protein complexes were analyzed in the same gel. Marker proteins of the indicated molecular masses (in kilodaltons) were also resolved on the same gel (lane 1). The percentage of the input ^{35}S -labeled protein bound in the reactions corresponding to lanes 4–7, respectively, was 0.4, 4, 0.2, and 0.3.

Figure 8. *DIG1* and *DIG2* are negative regulators of invasive growth. (A) YPH499 (WT) and the otherwise isogenic *dig1Δ* single mutant (JCY3), *dig2Δ* single mutant (JCY4), and *dig1Δ dig2Δ* double mutant (JCY5) derivatives, as indicated, were streaked on a rich medium (YPD) plate, incubated for three days at 30°C and photographed (left column), and then washed under a water stream to reveal invasive growth and photographed again (right column). (B) A *ste12* mutation blocks the invasive growth manifested by a *dig1Δ dig2Δ* double mutant. YPH499 (WT) and the otherwise isogenic *ste12* single mutant (JCY3), *dig1Δ dig2Δ* double mutant (JCY5), and *dig1Δ dig2Δ ste12Δ* triple mutant (JCY512) derivatives, as indicated, were grown and tested for invasive growth as in A. (C) Expression of either *DIG1* or *DIG2* rescues the invasive growth manifested by a *dig1Δ dig2Δ* double mutant. Strain JCY5 (*dig1Δ dig2Δ*) was transformed with a vector (YE352), plasmid pGAL1-*DIG1*, or YE352 carrying the *DIG2* gene under the control of its endogenous promoter, grown on plates containing SC-Ura medium with 2% galactose and 0.2% sucrose, and examined for invasive growth as in A.

under a microscope. In this population, a small proportion (~1%) were clearly elongated and in short filament-like chains (data not shown), as observed during invasive growth by other haploid strains (Roberts and Fink 1994).

It has been shown that the expression of genes required for invasive growth by yeast cells of the Σ 1278 lineage requires the action of the Ste12 transcription factor (Liu et al. 1993; Roberts and Fink 1994). To determine whether the invasive growth manifested by the *dig1 dig2* double mutant was mediated by the same pathway, we examined the behavior of a *dig1 dig2 ste12* triple mutant. The presence of the *ste12* mutation completely abolished invasive growth by the *dig1 dig2* cells (Fig. 8B), indicating that the loss of Dig1 and Dig2 activates the authentic haploid invasive growth pathway and does not promote invasive growth via some novel

mechanism. Likewise, the invasive growth phenotype displayed by the *dig1Δ dig2Δ* cells was completely reversed by multicopy plasmids expressing either *DIG1* (from the *GAL1* promoter) or *DIG2* (from its endogenous promoter) (Fig. 8C). Thus, Dig1 and Dig2 share a common role in inhibiting invasive growth, at least in the genetic background of certain yeast strains.

Effects of *DIG1* overexpression

The *DIG1* gene was also isolated independently in a screen for yeast cDNAs that, when overexpressed, caused haploid cells to appear more resistant to pheromone-imposed G_1 arrest (S.J. Kron, unpubl.). Indeed, wild-type *MATa* cells, transformed with a plasmid expressing the *DIG1* coding sequence from the inducible *GAL1* promoter, formed markedly turbid halos in the standard agar diffusion halo assay for pheromone-induced cell cycle arrest when grown on galactose-containing medium, whereas cells transformed with the empty vector formed larger and completely clear halos (Fig. 9A). Turbid halos are indicative of either increased resistance to pheromone action, or acceleration of the adaptation and recovery process, or both (Reneke et al. 1988). High-level expression of *DIG1* did not appear to cause turbid halos by inducing expression of *SST2* (Dietzel and Kurjan 1987), a gene involved pivotally in the adaptation and recovery process (Dohlman et al. 1996), because *DIG1* overexpression caused turbid halos even in an *sst2Δ* mutant (Fig. 9A). In strains lacking either *Kss1* (YDM420) or *Fus3* (YDM210), overproduction of Dig1 still caused turbid halos (data not shown). Conversely, co-overexpression of either *KSS1* or *FUS3* from appropriate plasmids (Ma et al. 1995; Bardwell et al. 1996) did not ameliorate the effect of *DIG1* overexpression (data not shown), suggesting that it does not block pheromone response by titrating out the MAPKs. Overexpression of *DIG2*, at least at the level achieved from a multicopy plasmid and driven by its own promoter, which is pheromone-inducible (Fig. 3B), had no detectable effect on halo size or turbidity (data not shown).

Because we demonstrated that both Dig1 and Dig2 act to negatively regulate haploid invasive growth in cells of the Σ 288C background, and because *Kss1* is also required for this process in haploids of the Σ 1278 lineage (Roberts and Fink 1994), which invade the agar of a rich medium plate quite vigorously, we tested whether overproduction of Dig1 or Dig2 could inhibit invasive growth by Σ 1278 haploid (Fig. 9B). Overexpression of *DIG1* from the *GAL1* promoter clearly suppressed invasive growth on a plate containing synthetic complete medium with galactose as the carbon source, whereas cells carrying the empty vector, or cells overexpressing *DIG2* (at the level achieved from a multicopy plasmid and driven by its own promoter in the absence of pheromone), did not. Thus, it appears that at least Dig1 can act as a negative regulator of haploid invasive growth in more than one yeast strain background.

To determine whether Dig1 and Dig2 act as negative regulators of the pseudohyphal growth manifested by

Figure 9. Effect of *DIG1* overexpression on pheromone response and invasive growth and effect of *DIG1* and *DIG2* loss of function on diploid growth. (A) Equivalent amounts ($\sim 2 \times 10^5$ cells) of exponentially growing cultures of YDM400 transformed with either a vector (YEp352GAL) or pGAL1-*DIG1*, as indicated, were inoculated in top agar on plates containing SC-Ura medium with 2% galactose and 0.2% sucrose, overlaid with sterile cellulose discs containing either 2 or 0.5 μ g of synthetic α -factor (Peninsula Labs or Star Biochemicals), as indicated, and incubated at 30°C for 2 days. (B) A haploid strain of the Σ 1278 lineage, 10560-4A (see Table 1), was transformed with either a vector control (YEp352GAL), or pGAL1-*DIG1*, or YEp352-*DIG2*, as indicated, streaked on SC-Ura medium with 2% galactose and 2% raffinose, incubated at 30°C for two days and then at room temperature for 2 days, photographed (left), rinsed under a gentle stream of distilled water and photographed again (right). (C) Diploid strains YPH501, YD4 and Σ 1278b (see Table 1) were grown on synthetic low-ammonia dextrose plates (Gimeno et al. 1992) supplemented with uracil, histidine, adenine, and lysine. Representative colonies were photographed.

diploid cells, we constructed a homozygous *dig1/dig1 dig2/dig2* *MATa/MATa* diploid in the YPH strain background. We examined the effect of the loss of Dig1 and Dig2 in this background because YPH strains do not show pseudohyphal growth under any condition tested previously. Unlike the parental strain (YPH501), the colony morphology of the *dig1/dig1 dig2/dig2* double mutant diploid displayed elements of filamentous growth that resembled those of the Σ 1278 diploid strain grown under the same conditions (Fig. 9C), suggesting that Dig1 and Dig2 may also contribute to the negative regulation of pseudohyphal growth in diploids.

Dig1 can physically associate with Ste12

Our findings raised the possibility that Dig1 (and Dig2) could negatively regulate haploid invasive growth by act-

ing directly on Ste12 to block its action. First, Dig1 was found in the nucleus, like Ste12. Second, overexpression of Dig1 squelched two different Ste12-dependent pathways, invasive growth and mating pheromone response. Third, a *ste12* mutation was epistatic to the constitutive invasive growth displayed by a *dig1 dig2* double mutant. To determine whether Ste12 is capable of binding to Dig1 in vitro, radiolabeled Ste12 was prepared by in vitro translation and tested for its ability to bind to GST alone, to GST-Dig1(213–452), and to GST-Ste7(1–172) (Fig. 7A and B). As additional controls, we tested the ability of another transcriptional transactivator, radiolabeled c-Fos, to associate with these same proteins. Radiolabeled Ste12 was very efficiently bound by the beads carrying GST-Dig1(213–452), but was not retained to any significant extent by the beads carrying either GST alone or GST-Ste7(1–172) (Fig. 7A). As expected, c-Fos was not bound by either GST alone or GST-Dig1(213–452) (Fig. 7B). Thus, Ste12 can associate specifically with Dig1 in the absence of any other yeast protein.

Discussion

We used the two-hybrid screen to isolate two previously unidentified gene products, Dig1 and Dig2, that interact with the MAPK Kss1. We have examined the product of the *DIG1* gene in detail because *DIG1* is constitutively expressed (unlike the *DIG2* gene, which is expressed at a reasonable level only upon pheromone induction). Dig1 appears to be an authentic substrate of Kss1 as judged by several criteria: (1) Both Dig1 and Kss1 are found in the nucleus; (2) Dig1 associates with Kss1 sufficiently tightly to be coimmunoprecipitated from cell extracts along with this MAPK; (3) Dig1 is phosphorylated in immune complexes in a Kss1-dependent manner; and (4) Kss1 can bind tightly to a GST-Dig1 fusion in vitro. Because Dig2 also was isolated by virtue of its ability to interact with Kss1 in vivo, is homologous to Dig1, and shares a common regulatory function with Dig1 (as judged by genetic analysis), it seems likely that Dig2 is also a target substrate of Kss1, although this supposition has not been tested directly yet. Because Kss1 is required for haploid invasive growth (Roberts and Fink 1994), and the phenotype of *dig1 dig2* double mutants revealed that both proteins are involved in negatively regulating this pathway, it is possible that Kss1-dependent phosphorylation of these proteins is involved in counteracting their inhibitory function, thereby promoting invasive growth. This role for Kss1 is consistent with the fact that *KSS1* is expressed in all three cell types and is not a pheromone-inducible gene (Courchesne et al. 1989).

Because both GAD-Dig1 and GAD-Dig2 fusions also interacted with a GDB-Fus3 construct in the context of the two-hybrid scheme, it is possible that both proteins are also substrates of Fus3. It has been shown that Fus3 action exerts a negative effect on haploid invasive growth (Roberts and Fink 1994) and that *FUS3*, like *DIG2*, is a pheromone-inducible gene (Elion et al. 1990). Hence, phosphorylation by Fus3 may have some role in enhancing the inhibitory action of Dig2 (and perhaps

Dig1), and/or in antagonizing the effects of Kss1-mediated phosphorylation of these proteins, to ensure that the invasive growth pathway is fully suppressed when cells are undergoing the mating process. Alternatively, Fus3 association with Dig1 and Dig2 may simply sterically prevent phosphorylation of these proteins by Kss1.

We favor the model, assumed in the preceding discussion, that: (1) Dig1 and Dig2 are downstream targets of Kss1; (2) they act as negative regulators because they bind to Ste12 and modify its function to prevent the expression of Ste12-dependent genes required for invasive growth (but not those required for mating); and (3) phosphorylation of Dig1 and Dig2 by Kss1 (which presumably becomes activated during nutrient limitation) relieves the inhibitory action of these regulators (Fig. 10). We favor this view for several reasons. First, Dig1 is a nuclear protein. Second, *ste12* mutants do not display invasive growth (Roberst and Fink 1994), and we showed that a *ste12* mutation was epistatic to the constitutive invasiveness displayed by *dig1 dig2* double mutant.

Figure 10. Models for Dig1 and Dig2 action. (A) In the absence of an appropriate signal, Dig1 (and Dig2) interact with Ste12 bound in a hypothetical complex with Tec1 (Gavrias et al. 1996) and prevent activation of promoter elements (filamentous growth response elements, or FREs; H. Madhani and G.R. Fink, in prep.) required for the expression of genes necessary for invasive growth, but not genes required for mating. Upon an appropriate signal, Kss1 is activated, phosphorylates Dig1 (and Dig2), thereby preventing inhibition of Tec1–Ste12 (and perhaps permitting full phosphorylation of Ste12), allowing expression from FREs. (B) Alternatively, Dig1 (and Dig2) may bind to Ste12 in such a way as to block its ability to associate with Tec1 under normal growth conditions. Under conditions of nutrient deprivation, Kss1-dependent phosphorylation dissociates Dig1 (and Dig2), thereby allowing Tec1 to interact with Ste12, thus promoting gene expression from FREs. See Discussion for further details.

Third, both Dig1 and Ste12 are phosphorylated by activated Kss1 in vitro (L. Bardwell, unpubl.). Fourth, MAPK-dependent phosphorylation has been shown to relieve the inhibitory effect of at least one other negative transcriptional regulator, the *Drosophila* Yan protein (Rebay and Rubin 1995). Finally, we have found that Ste12 prepared by in vitro translation can be retained by GST–Dig1 immobilized on beads, but not by GST alone or by another GST fusion protein. Thus, our results provide a critical link between MAPK activation and regulation of the transcriptional events required for induction of the invasive growth pathway.

Because Ste12 acts at the promoters of genes required for both mating pheromone response and for invasive growth, yet Dig1 and Dig2 binding to Ste12 affects the action of this transcriptional regulator only at the latter class of promoters, it is likely that this differential effect of Dig1 and Dig2 reflects some intrinsic difference between how Ste12 acts at these two different classes of promoters (Laloux et al. 1994; Mösch et al. 1996). Indeed, another transcriptional regulator, Tec1 (Gavrias et al. 1996), that is required for invasive growth and that acts in cooperation with Ste12 (H. Madhani and G.R. Fink, in prep.), has been described recently. Hence, we favor the view that Dig1 (and Dig2) act either by associating specifically with a presumptive Tec1–Ste12 complex to block its function (Fig. 10A) or by associating with Ste12 in such a way as to prevent formation of a Tec1–Ste12 complex (Fig. 10B). Thorough characterization of the interactions of all of these proteins will be required to distinguish between these different proposed mechanisms.

Yeast stocks derived from strain S288C, such as the YPH series (Sikorski and Hieter 1989), do not normally manifest either pseudohyphal growth in the diploid state or invasive growth in the haploid state under any laboratory condition. In contrast, strains of the Σ 1278 pedigree readily form, as diploids, pseudohyphae on medium containing low nitrogen (Gimeno et al. 1992) and, as haploids, invade the agar on plates containing standard rich medium (Roberts and Fink 1994). We have noted that the Σ 1278-derived strains invade agar more vigorously than the *dig1 dig2* S288C-derived cells on rich medium; however, on synthetic medium, the converse is true (J.G. Cook, unpubl.). The genetic differences between these two strain backgrounds are not fully understood. Mutations in a number of loci, including genes that are also required for the pheromone response pathway, can abolish the ability of the Σ 1278-derived strains to undergo any dimorphic transition (Liu et al. 1993; Roberts and Fink 1994). Because S288C-derived haploids are mating-component, loss of these functions cannot be responsible for their inability to display invasive growth. Conversely, mutations in a wide variety of genes, including those encoding a presumptive protein kinase, *ELM1* (Blacketer et al. 1993), a candidate phosphoprotein phosphatase subunit, *CDC55* (Healy et al. 1991; Blacketer et al. 1995), and a so-called septin (Chant 1996) or neck filament protein, *CDC12* (Blacketer et al. 1995), allow S288C-derived strains to display pseudohyphal growth as diploids and invasive growth as haploids. As we have

shown here, the list of negative regulators of these developmental processes now includes one demonstrated (Dig1) and one suspected (Dig2) nuclear protein. All cells presumably must express at least the *CDC55* and *CDC12* genes, because they are essential for viability; so, the loss of these proteins cannot be the reason why a *dig1 dig2* double mutant haploid acquires the ability to invade agar.

DIG1 and *DIG2* mRNA levels in Σ 1278 strains appeared similar to those in the YPH strains. It is possible, however, that these transcripts encode inactive forms of the Dig1 and Dig2 proteins, in analogy to the defective *HO* gene product produced by heterothallic strains of *S. cerevisiae* (Herskowitz et al. 1992). Alternatively, in S288C strains, Dig1 and Dig2 may be altered such that they are insensitive to the signal that elicits invasive growth, and, thus, S288C-related cells remain incompetent to undergo this transition even under appropriate conditions. However, both of these possibilities seem unlikely because *DIG1* overexpression partially suppressed (and *DIG2* expression did not prevent) invasive growth in a Σ 1278 haploid. A more likely scenario, perhaps, is that the mechanism that triggers inactivation of Dig1 and Dig2 in response to nutrient limitation is defective in S288C strains. For example, in S288C, the Ste11–Ste7–Kss1 MAPK cascade may be less fully activated in response to nutrient limitation than in Σ 1278 strains and, thus, unable to overcome the Dig1/Dig2-imposed block. In any event, based on the sources of the libraries screened, both the cDNA and genomic sequences described here are from the S288C lineage, which is the DNA sequence determined in the recently completed *S. cerevisiae* genome project (M. Cherry, pers. comm.).

In the case of filamentous growth by diploids, nitrogen starvation appears to be the signal that induces cells to undergo the transition from the yeast form to the pseudohyphal form (Gimeno et al. 1992). The signal that triggers invasive growth by haploids has not been identified. It seems clear that the decrease in some nutrient and/or the accumulation of some metabolite is responsible for inducing haploids to undergo invasive growth because, initially, a haploid colony grows almost exclusively on the surface of the agar and begins to invade only after several days of incubation, supporting the view that these morphological transitions provide a mechanism whereby an otherwise nonmotile cell can “forage” for more nutrients (Gimeno et al. 1992) (or escape the toxic effect of “pollutants”).

Just as signals propagated by the same MAPK cascade allow haploids to mate when stimulated by pheromones or permit cells to undergo invasive growth when induced by the right nutrient conditions, there are other cell types in which activation of the same MAPK pathway elicits quite different cellular responses. For example, the MAPK Erk2 is activated when PC12 cells are treated with epidermal growth factor (EGF), leading to mitogenesis and cellular proliferation, but also when the same cells are treated with nerve growth factor (NGF), leading to a cessation of growth and neuronal differentiation

(Gotoh et al. 1990; Traverse et al. 1992). How different extracellular signals activate the same MAPK cascade, yet are deciphered differently, is not fully understood in any organism, although the duration and extent of MAPK activation may dictate, in part, the nature of the cellular response (Traverse et al. 1994). The available evidence indicates that Dig1 and Dig2 affect developmental decisions at the level of gene regulation (rather than playing a role in preventing the execution of any of the morphological events required for cellular differentiation processes). The unique genetic tractability of *S. cerevisiae* provides a valuable opportunity to investigate how the Dig1 and Dig2 proteins block expression from specific promoters, how that blockade is removed by Kss1 action, and how these events contribute to the ability of a single MAPK pathway to evoke different developmental outcomes.

Materials and methods

Strains and growth conditions

Standard yeast media were prepared as described (Sherman et al. 1986), except that, in synthetic minimal medium, twice the recommended level of nutritional supplements was used. Yeast strains used in this work are shown in Table 1. The *dig1-Δ1::HIS3* allele was constructed in plasmid pGEX-18 (see below) by replacing an internal 0.7-kb *XbaI*–*SacI* fragment of *DIG1* (encoding residues 166–419) with the *HIS3* gene extracted from plasmid pJJ215 (Jones and Prakash 1990). This construct was excised as a 2.6-kb *EcoRI* fragment and used for DNA-mediated transformation of a *MATa*/*MATα* strain (YPH501). His⁺ colonies were selected, and disruption of the chromosomal *DIG1* locus on one homolog was confirmed by restriction enzyme digestion and hybridization analysis (Southern 1975). The resulting heterozygous diploid was sporulated, and the tetrads so generated were dissected, to yield strain JCY3. The *dig2-1Δ::TRP1* allele was generated by a method utilizing the polymerase chain reaction (PCR) (Lorenz et al. 1995). For this purpose, the *TRP1* gene on plasmid pRS304 (Sikorski and Hieter 1989) was amplified by PCR using primers 103-KO1 (5'-GG-TAAGCCTCCTACCATTACGACTTCTCCAGCAGAGAA-AACCGTACCCGCAGATTGTACTGAGATGC-3') and 103-KO2 (5'-CCTTCTTGTCTGTTGTATCATCGGCATCGTCTCG-TTAAGTGAAGCGCCCTCCTTACGCATCTGTGCGG-3'), each of which possesses homology both to *DIG2* (at their 5' ends) and to the pRS vector series (at their 3' ends). The resulting 1.2-kb PCR product (which replaces codons 91–267 of the *DIG2* coding sequence with *TRP1*) was used to transform YPH501 to Trp⁺, and disruption of the chromosomal *DIG2* locus on one homolog was confirmed by PCR amplification using primers corresponding to sequences flanking the *DIG2* gene and genomic DNA isolated from the transformants or from the parental strain as the template. The resulting heterozygous diploid was sporulated, and the tetrads so generated were dissected, to yield strain JCY4. A *dig1Δdig2Δ* double mutant (JCY5) was constructed by transforming strain JCY3 with the *dig2-Δ1::TRP1* PCR product and confirming the gene replacement by PCR amplification.

Plasmid constructions and recombinant DNA methods

E. coli strain DH5α (Hanahan 1983) was used for the propagation of plasmids. A plasmid to express the Kss1–GDB chimera from the constitutive *PGK1* promoter (Hitzeman et al. 1980)

was constructed in several steps. First, codons 1–147 of *GAL4* were amplified using primers Gal4-147STOP (5'-CCTTAAGCTTTTACGATACAGTCAACTGTCT-3') and Kss1-Gal4 joint (5'-(CTAATGAAGACCATGGAAGGGAGCTCTATGAAGCTACTGTCTTCT-3') and a *GAL4*-containing plasmid pG12 (gift of A. Buchman, Stanford University School of Medicine, Stanford, CA), as the template. The resulting product, which encodes the Gal4 DNA-binding domain followed by a translation termination codon and an *SphI* site at its 3' end, and preceded at its 5' end by nucleotides corresponding to the six most carboxy-terminal codons of *KSS1* and a three-amino acid linker (Gly-Ser-Ser) was gel-purified, denatured, and used as the 3' primer in a second round of PCR along with primer Kss1-3 (5'-GAAATGGTCTCCGGAAGCCT-3') and, as the template, a DNA fragment containing the 3' portion of *KSS1* that includes an internal *XbaI* site. The resulting product was inserted into the *EcoRV* site in Bluescript KS+ (Stratagene) by blunt-end ligation, generating pBS-KCG. pBS-KCG was digested with *XbaI* and *SphI*, and the resulting fragment was used to replace the corresponding *XbaI*-*SphI* fragment in *YEpGAL-KSS1* (Ma et al. 1995), yielding plasmid pJGC1. pJGC1 was digested with *BamHI* and *SphI* and the resulting fragment containing the entire *KSS1*-*GDB* fusion was converted to blunt ends by incubation with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates, and then ligated into a *CEN*-containing vector, pRS314-*PGK* (gift of K. Blumer, Washington University, School of Medicine, St. Louis, MO) that had been cleaved with *EcoRI* and converted to flush ends by the same procedure. A resulting plasmid containing *KSS1*-*GDB* in the correct orientation was identified by restriction endonuclease cleavage site mapping and designated as pJGC2. An otherwise identical construction incorporating a catalytically inactive mutant form of Kss1 was generated by replacing the 1.0-kb *BsiWI*-*BspEI* fragment in pJGC2 with the corresponding fragment from *YEpKSS1(K42R)* (Ma et al. 1995).

To express *DIG2* from multicopy (2 μ m DNA-containing) vectors, first, a 2.2-kb *XhoI*-*Sall* fragment containing the entire *DIG2* coding sequence and its promoter was excised from a plasmid, pAL138 (gift of Y. Oshima, Osaka University, Osaka, Japan), that carries a large segment of the region of the *S. cerevisiae* genome that surrounds the *PHO8* locus, and inserted into the *Sall* site of *YEp352-HIS3*, a derivative of *YEp352* (Hill et al. 1986) in which *HIS3* replaces *URA3* (constructed by K. Kuchler, this laboratory), yielding *YEp352H-DIG2*. Second, an *EcoRI*-*SphI* fragment carrying the entire *DIG2* coding sequence and its promoter was excised from *YEpH-DIG2* and ligated into the corresponding sites in *YEp352*.

Plasmid p*GAL-DIG1*, originally designated p*GAL-Afr18*, was isolated from a library of yeast cDNAs under control of the *GAL1* promoter (Liu et al. 1992) by means of a selection for *MATa* transformants that were able to grow on plates, despite the presence of α -factor in the medium (S.J. Kron, unpubl.). To determine the complete nucleotide sequence of the *DIG1* coding region, a *BamHI*-*NotI* fragment was excised from p*GAL-DIG1* and inserted into Bluescript KS+. Various internal fragments were subcloned, internal deletions were generated, and the corresponding sequences were determined using primers for T3 and T7 DNA polymerases, as appropriate. Where necessary, custom, synthetic oligonucleotide primers were prepared to obtain overlapping sequences and to resolve any ambiguities. The nucleotide sequence of *DIG2* was confirmed in a similar manner.

Multicopy plasmids to express epitope-tagged versions of wild-type Kss1 and a nonactivable variant were constructed in several steps. First, a novel expression vector (*YEpGal112GAL*) was constructed by excising an *EcoRI*-*BamHI* fragment con-

taining the *GAL1* promoter from plasmid pG12 (Nicolet and Friedberg 1987) and inserting it into the corresponding sites of *YEpGal112* (a *TRP1*- and 2- μ m DNA-containing plasmid) (Gietz and Sugino 1988). A *BamHI*-*SphI* fragment carrying the entire *KSS1* coding sequence was excised from *YEpGAL-KSS1* (Ma et al. 1995) and inserted into the corresponding sites of *YEpGal112GAL*, yielding *YEpTG-KSS1*. A derivative of *YEpTG-KSS1* expressing a version of Kss1 carrying a (His)₆ tract and a c-Myc-epitope tag (Evan et al. 1985) at its carboxyl terminus was constructed as follows. Oligonucleotide LB52 (5'-CCCACCACCACCACCACCGGTGAACAAAAGTTGATCTCCGAAGAAGACTTGGCTTAAG-3') was annealed to LB53 (5'-TCGACTTAAGCCAAGTCTTCTTCGGAGATCAACTTTTGTTCACCGTGCTGGTGGTGGTGGTGGGAGCT-3'; partial *Sall* and *SacI* sites underlined) to generate a double-stranded oligonucleotide (LB52/53). A *BamHI*-*SacI* fragment containing the entire *KSS1* coding sequence followed by the Gly-Ser-Ser linker was excised from pJGC1 and ligated, along with LB52/53, into pGEM4Z (Promega Biotech) that had been cut with *BamHI* and *Sall*, generating pGEM4Z-KSS1H6myc, which attaches a 21-residue extension (GSSHHHHHHGEQKLISEEDLA-COOH; c-Myc-epitope underlined) to the last residue (position 368) of the authentic Kss1 amino acid sequence. A *BamHI*-*SphI* fragment containing the *KSS1H6myc* allele was excised from pGEM4Z-KSS1H6myc and inserted into the corresponding sites of *YEpGal112GAL*, to yield *YEpTG-KSS1H6myc*. *YEpTG-KSS1H6myc* was tested for retention of biological function by two independent bioassays. As judged by a patch mating assay (Sprague 1991), the ability of *YEpTG-KSS1H6myc* to complement the sterility of a *kss1Δ fus3Δ* double mutant strain, YDM230 (Ma et al. 1995), was indistinguishable from that of *YEpTG-KSS1*. YDM230 carrying either plasmid formed dense patches of diploids under inducing conditions (on galactose medium), mottled patches of diploids under non-inducing conditions (on raffinose medium), and no diploids at all under repressing conditions (on dextrose medium). In the standard agar diffusion (halo) assay for pheromone response, high-level expression (on galactose medium) of both *YEpTG-KSS1* and *YEpTG-KSS1H6myc* resulted in substantial and indistinguishable inhibition of pheromone-imposed arrest (Courschesne et al. 1989; Ma et al. 1995). Plasmid *YEpTG-KSS1(AEF)H6myc* was constructed by replacing the 0.8-kb *BamHI*-*XbaI* fragment in *YEpTG-KSS1H6myc* with the corresponding fragment from *YEpGAL-KSS1(T183A, Y185F)* (Ma et al. 1995).

Two-hybrid screen

Strain Y190 harboring plasmid pJGC2 was transformed with either a library of yeast cDNAs fused to the carboxyl terminus of a construct expressing GAD (generously provided by S. Elledge, Baylor College of Medicine, Houston, TX) or a library of yeast genomic DNA fused to GAD (James et al. 1996) (generously provided by P. James and E.A. Craig, University of Wisconsin, Madison, WI). Fusions capable of interacting with Kss1 were selected on plates lacking histidine and containing 50 mM 3-AT (Sigma). The total number of transformants screened was estimated by plating a small fraction of the transformants on medium that selected only for the presence of the test plasmid (Trp⁺) and a library plasmid (Leu⁺). Approximately 1.5 million transformants of the cDNA library and about 4.5 million transformants of the genomic DNA library were screened. Positive clones were subjected to a secondary screen for *lacZ* expression using a nitrocellulose filter replica assay, essentially as described by Breeden and Nasmyth (1985). Extracts were prepared

from the reproducibly 3-AT^R LacZ⁺ cells and the size of the GAD chimera expressed was examined by SDS-PAGE and immunoblotting using an antiserum raised against the GAD portion (carboxyl terminus) of Gal4 (gift of S. Johnston, University of Texas Southwestern Medical Center, Dallas, TX). Plasmids encoding fusions detectably larger than the GAD domain itself were extracted from the yeast cells and recovered by transformation in *E. coli*. Specificity for interaction with Kss1 was confirmed by failure of the fusions expressed by the recovered library plasmids to give a positive signal when cotransformed with a vector-only control, or with GAD fusions to Raf (Van Aelst et al. 1993) (gift of M. Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); Rb (Durfee et al. 1993) (gift of T. Durfee, University of California, Berkeley, CA); Fpr3 (Benton et al. 1994) (gift of N. Dhillon, this laboratory); and Rad3 (Bardwell et al. 1994a). The nucleotide sequences of the inserts in these plasmids were partially determined using primer Gal4-850 (5'-GGAATCACTACAGGGATG-3') and compared with the *S. cerevisiae* genome data base using the BLAST-N search algorithm (Altschul et al. 1990).

Analysis of DIG1 and DIG2 mRNAs

To analyze mRNA levels, total RNA was extracted from cells harvested from exponentially growing cultures (200 ml), essentially as described by Köhler and Domdey (1991), with the minor modifications that, after precipitation and washing of the crude RNA with ethanol containing 0.01% diethylpyrocarbonate (DEPC), the resulting pellets were resuspended in 0.5 ml sterile water containing 1 μ l RNasin solution (Promega) and 1 mM dithiothreitol, and then adjusted to 0.3 M sodium acetate and 1% SDS, reprecipitated by addition of two volumes of ethanol containing 0.01% DEPC (yielding ~250 μ g RNA), and stored at -80°C. For pheromone induction, exponentially growing cultures (50 ml) of strain DK499 (Table 1) were treated with 0.21 μ M α -factor for 3 hr prior to harvest (after this treatment, virtually every cell was unbudded and had formed a mating projection). To analyze the RNA, samples (50 μ g total) were subjected to electrophoresis in a denaturing agarose gel containing 1% formamide, transferred to a nylon membrane (Boehringer Mannheim), affixed by baking at 80°C for 2 hr under vacuum, and annealed with an appropriate radiolabeled DNA probe. ³²P-labeled probes were prepared by use of the random primer method (Feinberg and Vogelstein 1984) using internal segments of *DIG1* (0.7-kb *Xba*I-*Sac*I fragment) or *DIG2* (0.7-kb *Eco*RV-*Nru*I fragment). Hybridization was conducted at 42°C overnight and washes were performed at 65°C using standard methods (Sambrook et al. 1989). Relative mRNA levels were quantitated using a PhosphorImager (Molecular Dynamics, Inc.) and normalized to the signal observed for the *CMD1* transcript (Davis et al. 1986) analyzed in the same fashion.

Preparation of anti-Dig1 antibodies

To generate a glutathione S-transferase (GST)-Dig1 fusion protein, a 0.8-kb fragment encoding the 240 most carboxy-terminal residues of Dig1 was excised from the polylinker of one of the *DIG1*-containing cDNA isolates by digestion with *Bgl*II and inserted into the *Bam*HI site in pGEX-5X (Pharmacia) downstream of and in-frame with the GST coding sequence, yielding pGEX-Dig1. Then, to generate a larger fusion, a 1.5-kb fragment containing all but the first six residues of Dig1 was excised from the polylinker of a different *DIG1*-containing cDNA isolate by digestion with *Bgl*II and inserted into the *Bam*HI site of pGEX-5X, yielding pGEX-18. To prepare antigen, expression of the GST-Dig1 fusion from plasmid pGEX-Dig1 was induced in a protease-deficient *E. coli* strain (BL21) (Studier 1991) by addition

of isopropyl- β -D-thiogalacto-pyranoside (IPTG) to a final concentration of 0.2 mM followed by incubation with aeration for 2 hr at 30°C. Cells were harvested, washed once with cold 1 $\times$ PBS (Harlow and Lane 1988), and resuspended in 1/20 volume 1 $\times$ PBS containing 10% glycerol and 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBF). Cells were disrupted by sonication followed by addition of Triton X-100 to a final concentration of 1%. Insoluble material was removed by centrifugation at 12,000 g, and the soluble GST-Dig1 fusion protein was purified by adsorption to and elution from glutathione-agarose beads (Pharmacia), essentially as directed by the manufacturer, except that elution was performed at 42°C in the presence of 1% Triton X-100 and 0.02% SDS. The purified protein was used as the immunogen to raise polyclonal antisera in adult female New Zealand White rabbits following standard immunization protocols (Harlow and Lane 1988).

Immunoblotting and immunofluorescence

For immunoblot analysis, samples (50–100 μ g total protein) were subjected to electrophoresis in a 10% SDS-polyacrylamide gel. Transfer of proteins to nitrocellulose was carried out by use of standard procedures. To detect Kss1, a rabbit polyclonal anti-Kss1 antiserum (Ma et al. 1995) was used at a dilution of 1:5000. To detect Dig1, the rabbit polyclonal antiserum described here was used at dilutions of either 1:2500 or 1:5000. Immobilized immune complexes were detected using a commercial chemiluminescence system (ECL, Amersham) and X-ray film.

Subcellular localization of Dig1 by indirect immunofluorescence and photomicroscopy was performed by methods described in detail elsewhere (Ma et al. 1995) using the rabbit polyclonal anti-Dig1 antiserum at dilutions of either 1:300 or 1:1000. The secondary antibodies [fluorescein isothiocyanate (FITC)-conjugated goat antirabbit immunoglobulin] were used at a dilution of 1:100.

Coimmunoprecipitation and protein kinase assay in immune complexes

A protease-deficient yeast strain (BJ2168) (Jones 1991) was transformed with either vector alone, YE_pGAL-KSS1, YE_pTG-KSS1H6myc, or YE_pTG-KSS1(AEF)H6myc, in the presence or absence of pGAL1-DIG1. Cells in the resulting cultures, grown at 30°C in appropriate selective medium to mid-exponential phase, were collected by centrifugation, resuspended in fresh prewarmed medium buffered at pH 3.5 with sodium succinate to retard proteolytic degradation of α -factor (Ciejeck and Thorner 1979), split into two equal portions, incubated with shaking at 30°C for 15 min, and then one portion was treated with a final concentration of 6 μ M α -factor and the other was left untreated, followed by incubation for an additional 15 min. Cells were harvested, and extracts were prepared as described in detail in Bardwell et al. (1996). Protein concentration in the resulting extracts was determined by a dye-binding method (Bradford 1976) using commercially available reagents (Bio-Rad) and bovine serum albumin (BSA) as the standard. A volume of extract containing 1 mg of total protein was precleared with a mixture of protein A and protein G conjugated to agarose beads, and then bead-bound immune complexes were prepared using the mAb 9E10 (Evan et al. 1985) and divided into two equal portions, which were used for immunoblotting and for in vitro protein kinase assays, using methods also described in detail in Bardwell et al. (1996). ¹⁴C-labeled molecular weight markers were obtained from Amersham.

Bioassays for pheromone response and invasive growth

An agar diffusion (halo) assay was used to measure pheromone-

induced growth arrest and recovery, as described in detail previously (Reneke et al. 1988). The procedure for revealing invasive growth by washing the surface of agar plates with a stream of water is described in detail elsewhere (Gimeno et al. 1992; Roberts and Fink 1994). To monitor invasive growth by haploid strains, rich medium (YPD) plates (Sherman et al. 1986) were typically used. To examine the effect of *DIG1* overexpression on invasive growth by a strain of the $\Sigma 1278$ lineage, SC-Ura medium (to maintain selection for the *DIG1*-containing plasmid) containing 2% galactose and 2% raffinose (to induce expression of *DIG1* from the *GAL1* promoter) was used, plates were incubated for three days, and were photographed immediately after washing.

In vitro protein interaction assays using GST fusions

For *in vitro* binding assays, GST fusion proteins were expressed in *E. coli* and bound to glutathione-agarose beads, as instructed by the manufacturer. Equal amounts of protein-coated beads were further incubated with 1 pmole of radiolabeled proteins, prepared by *in vitro* translation as described (Bardwell et al. 1996). Incubations were carried out at room temperature for 1 hr in 0.2 ml Buffer A (Bardwell et al. 1996) followed by two 1-ml washes with ice cold Buffer A. Bound proteins were eluted in SDS-PAGE sample buffer containing 5% SDS and 60 mM dithiothreitol (DTT). One half of each sample was loaded on a 10% polyacrylamide gel along with samples of the input *in vitro* translated proteins as markers. Alternatively, equal amounts of purified GST fusion proteins that had been eluted in 20 mM glutathione were diluted to 0.5 mM glutathione in Buffer A and rebound to glutathione-agarose beads at room temperature for 1 hr. The beads were collected and washed twice with ice-cold Buffer A prior to incubation with the radiolabeled proteins, as above.

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