Protein-Protein Interactions in the Yeast Pheromone Response Pathway: Ste5p Interacts With All Members of the MAP Kinase Cascade

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Manuscript received May 23, 1994
Accepted for publication July 21, 1994

ABSTRACT

We have used the two-hybrid system of Fields and Song to identify protein-protein interactions that occur in the pheromone response pathway of the yeast *Saccharomyces cerevisiae*. Pathway components Ste4p, Ste5p, Ste7p, Ste11p, Ste12p, Ste20p, Fus3p and Kss1p were tested in all pairwise combinations. All of the interactions we detected involved at least one member of the MAP kinase cascade that is a central element of the response pathway. Ste5p, a protein of unknown biochemical function, interacted with protein kinases that operate at each step of the MAP kinase cascade, specifically with Ste11p (an MEKK), Ste7p (an MEK), and Fus3p (a MAP kinase). This finding suggests that one role of Ste5p is to serve as a scaffold to facilitate interactions among members of the kinase cascade. In this role as facilitator, Ste5p may make both signal propagation and signal attenuation more efficient. Ste5p may also help minimize cross-talk with other MAP kinase cascades and thus ensure the integrity of the pheromone response pathway. We also found that both Ste11p and Ste7p interact with Fus3p and Kss1p. Finally, we detected an interaction between one of the MAP kinases, Kss1p, and a presumptive target, the transcription factor Ste12p. We failed to detect interactions of Ste4p or Ste20p with any other component of the response pathway.

As a prelude to conjugation, yeast **a** and α cells communicate via secreted peptide pheromones and receptors for those pheromones. Binding of pheromone to receptor activates a signaling pathway that is common to **a** and α cells and serves to transmit information received at the cell surface to intracellular targets. These targets effect substantial physiological changes in the responding cell, including arrest of the mitotic cell cycle in the G_1 phase and transcription induction of a set of genes whose products catalyze the mating event (reviewed by SPRAGUE and THORNER 1992; KURJAN 1992; ERREDE and LEVIN 1993; FIELDS 1990).

Components that operate in the pheromone signaling pathway have been identified primarily by the isolation of mutants that show altered pheromone response. Epistasis experiments involving loss-of-function and gain (or increase)-of-function mutants have allowed the relative position of the components to be deduced (Figure 1) (BLINDER *et al.* 1989; WHITEWAY *et al.* 1990; STEVENSON *et al.* 1992; CAIRNS *et al.* 1992; LEBERER *et al.* 1992; GARTNER *et al.* 1992; HASSON *et al.* 1993; DOLAN and FIELDS 1990; ZHOU et al. 1993). Moreover, in many cases the cloning and sequencing of the genes encoding these components has provided clues as to their biochemical functions (TEAGUE *et al.* 1986; CHALEFF and TATCHELL 1985). Together these studies imply that the response pathway is largely linear in character and uses biochemical modules homologous to those found in signaling pathways from animal species. The receptors are members of the seven transmembrane receptor family (JENNESS *et al.* 1983; BURKHOLDER and HARTWELL 1985; NAKAYAMA *et al.* 1985; HAGEN *et al.* 1986) and couple to a heterotrimeric G protein (DIETZEL and KURJAN 1987; MIYAJIMA *et al.* 1987; NAKAFUKU *et al.* 1987; JAHNG *et al.* 1988; WHITEWAY *et al.* 1989; BLUMER and THORNER 1990). The signal is transmitted from the activated G protein to a MAP kinase cascade by the action of Ste20p, a serine/threonine protein kinase (LEBERER *et al.* 1992; RAMER and DAVIS 1993), and Ste5p, a protein of unknown function (PERLMAN *et al.* 1993; LEBERER *et al.* 1993). The mechanism by which Ste20p and Ste5p link the G protein to the MAP kinase cascade is not known and may well involve as yet unidentified proteins. The MAP kinase cascade is composed of Ste11p (CHALEFF and TATCHELL 1985; RHODES *et al.* 1990), an MEKK homolog (LANGE-CARTER *et al.* 1993), Ste7p (CHALEFF and TATCHELL 1985; TEAGUE *et al.* 1986), an MEK homolog (BOULTON *et al.* 1990), and Fus3p and Kss1p, two related MAP kinase homologs (ELION *et al.* 1990; COURCHESNE *et al.* 1989; CREWS *et al.* 1992). Elements of this MAP kinase cascade have been reconstituted *in vitro*. In particular, Ste11p phosphorylates Ste7p (NEIMAN and HERSKOWITZ 1994), and Ste7p phosphorylates Fus3p (ERREDE *et al.* 1993). The pathway bifurcates after the MAP kinases, one branch leading to transcription induction and the other branch leading to cell cycle arrest. The MAP kinases have partially redundant functions. Both Fus3p and Kss1p appear to phosphorylate

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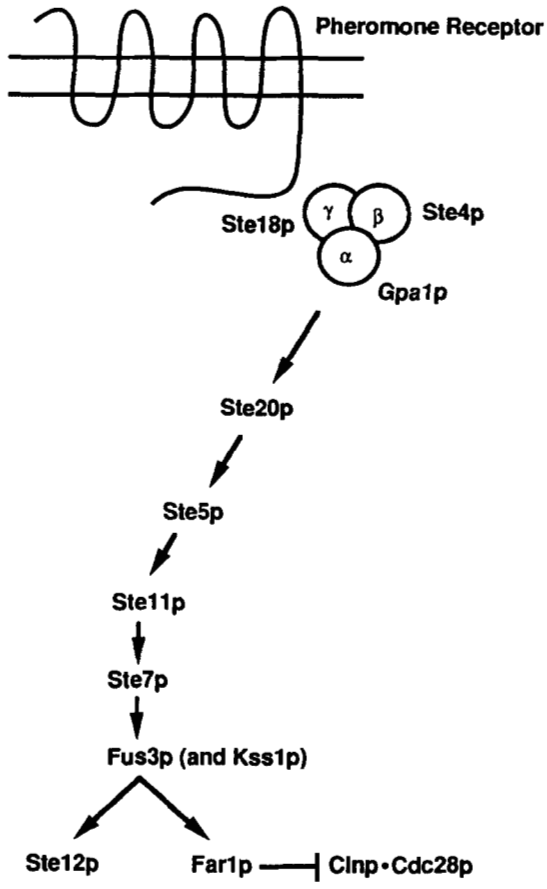


FIGURE 1.—A schematic of the pheromone response pathway. The binding of pheromone to the receptor results in the exchange of GTP for GDP on the α subunit of the G protein, and its dissociation from the β and γ subunits. The immediate target of $G_{\beta\gamma}$ is not known but ultimately the signal is transmitted to two MAP kinase homologs, Fus3p and Kss1p. Both of these kinases affect the activity of the transcription factor Ste12p, leading to increased transcription of genes required for mating and cell cycle arrest. Fus3p, but apparently not Kss1p, phosphorylates Far1p, which then inhibits Clnp·Cdc28p complexes, leading to cell cycle arrest. Lines with arrowheads indicate stimulation of the activity of the next protein; lines with terminal bars indicate inhibition of the activity of the next protein.

Ste12p (ELION *et al.* 1993; J. THORNER, personal communication), a transcription factor for pheromone responsive genes (ERREDE and AMMERER 1989; DOLAN *et al.* 1989; SONG *et al.* 1991), and thereby promote transcription induction. Fus3p, but not Kss1p, can also phosphorylate Far1p (PETER *et al.* 1993; TYERS and FUTCHER 1993). Phosphorylated Far1p can bind to and inhibit the activity of the Clnp·Cdc28p complex, providing one means by which pheromone treatment leads to arrest of the cell division cycle.

Despite the considerable understanding of the pheromone response pathway summarized above, major questions remain unanswered. Foremost among these is how the G protein is linked to the MAP kinase cascade. In addition, very little is known about how the activity of the pathway is regulated. During signal transmission, a biochemical signal must be passed from one pathway component to the next, but the nature of these biochemical events is not known except in the case of the MAP kinases, which must be phosphorylated on two key residues by Ste7p (GARTNER *et al.* 1992). Conversely, following an initial burst of signal transmission, the activity of the pathway components is attenuated (reviewed by SPRAGUE and THORNER 1992), allowing the cells to recover from pheromone treatment and resume growth should mating not occur. Again, very little is known about the mechanisms that attenuate the pathway signal. However, it has been proposed that Fus3p participates in this attenuation process in two ways. First, once activated by Ste7p, Fus3p in turn phosphorylates and thereby inactivates Ste7p (ZHOU *et al.* 1993). Second, *MSG5*, a gene whose transcription is induced in response to pheromone, is thought to encode a protein phosphatase that dephosphorylates and inactivates Fus3p (DOI *et al.* 1994).

To gain insight into the issues raised above, we have asked whether known pathway components interact *in vivo* by using the two hybrid system developed by FIELDS and SONG (1989). We find that Ste5p interacts with Ste11p, Ste7p and Fus3p, leading to the suggestion that Ste5p serves as a scaffold to facilitate interactions between the members of the MAP kinase cascade. In this way, Ste5p may make signal transmission, signal attenuation, or both, more efficient. We also show that Ste11p interacts with itself through an amino terminal domain that serves a negative regulatory function. A dominant, activated form of Ste11p containing an amino acid substitution in the amino terminal domain displayed increased interaction with Ste5p and decreased interaction with Fus3p and Kss1p. These altered interactions suggest ways in which the activity of Ste11p may be regulated.

MATERIALS AND METHODS

Strains, media and microbiological techniques: The yeast strains used in this work are isogenic with GGY::171 (Table 1) (GILL and PTASHNE 1987). Several deletion derivatives of this strain were constructed by one step gene replacement at the relevant loci (ROTHSTEIN 1991), using *far1Δ::ADE2*, *ste4Δ::ADE2*, *ste5Δ::ADE2*, *ste7Δ::ADE2*, *ste11Δ::ADE2* and *fus3Δ::ADE2* DNA constructions (see below). For most two-hybrid assays, the GGY::171 *far1Δ::ADE2* strain was used, because a number of the Gal4p-Ste fusion proteins caused constitutive activation of the pheromone response pathway. Deletion of *far1* suppresses G1 arrest normally associated with activation of the pheromone response pathway (CHANG and HERSKOWITZ 1990).

Yeast and bacterial strains were propagated using standard methods. YEPD and SD media have been described (ROSE *et al.* 1990). Yeast transformations were performed by the lithium acetate method as described by SCHIESTL and GIETZ (1989).

Plasmids: Plasmids (Table 2) were constructed by standard methods (SAMBROOK *et al.* 1989). Unless otherwise indicated, all two-hybrid fusions were cloned into the *Bam*HI site of pGAD2F (CHEIN *et al.* 1991) or pMA424 (MA and PTASHNE 1987).

AD (activation domain)-Ste7p (pSL2168) and BD (binding domain)-Ste7p (pSL1962) were created by polymerase chain

TABLE 1

Yeast strains

Strain	Genotype	Source
GGY1::171	*MATα GAL1-lacZ@URA3 gal4Δ gal80Δ his3 leu2 ade2*	GILL and PTASHNE (1987)
SY2509	GGY1::171 *far1::ADE2*	This study
SY2510	GGY1::171 *ste4::ADE2*	This study
SY2756	GGY1::171 *ste5::ADE2*	This study
SY2597	GGY1::171 *ste7::ADE2*	This study
SY2598	GGY1::171 *ste11::ADE2*	This study
SY2596	GGY1::171 *fus3::ADE2*	This study
SY2366	**a**/α GGY1::171	Lab Strain

TABLE 2

Plasmids

Plasmid	Relevant markers	Source
pMA424	*HIS3* 2μ *pADH1 GAL4 (1–147)*	MA and PTASHNE (1987)
pKB84.7	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE20*	K. CLARK and M. WHITEWAY
pSL1962	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE7*	This study
pSL2121	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE11*	This study
pSL2019	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE5*	This study
pSL2174	*HIS3* 2μ *pADH1 GAL4 (1–147)-FUS3*	This study
pSL2120	*HIS3* 2μ *pADH1 GAL4 (1–147)-KSS1*	This study
pSL2167	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE7 (1–172)*	This study
pSL2173	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE11 (1–435)*	This study
pSL2478	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE11K444R*	This study
pSL2479	*HIS3* 2μ *pADH1 GAL4 (1–147)-STE11P279SK444R*	This study
pGAD2F, 3F	*LEU2* 2μ *pADH1 GAL4 (768–881)*	CHEIN *et al.* (1991)
pKB40–1	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE4*	CLARK *et al.* (1993)
pRL222	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE20*	K. CLARK and M. WHITEWAY
pSL2091	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE11*	This study
pSL2168	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE7*	This study
pSL2289	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE5*	This study
pSL2175	*LEU2* 2μ *pADH1 GAL4 (768–881)-FUS3*	This study
pSL2122	*LEU2* 2μ *pADH1 GAL4 (768–881)-KSS1*	This study
pSL2205	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE12*	This study
pSL2170	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE7 (172–515)*	This study
pSL2172	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE11 (1–435)*	This study
pSL2477	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE11K444R*	This study
pSL2480	*LEU2* 2μ *pADH1 GAL4 (768–881)-STE11P279SK444R*	This study
pCAD1, 2, 3	*LEU2* 2μ *pADH1 GAL4 (768–881)*	This study
pSL2280	*LEU2* 2μ *pADH1 STE12-GAL4 (768–881)*	This study
pSL2281	*LEU2* 2μ *pADH1 STE11(1–435)-GAL4 (768–881)*	This study
pXT1	*LEU2* 2μ *KSS1*	COURCHESNE *et al.* (1989)
pSL2200	*far1::ADE2*	This study
pSL2199	*ste4::ADE2*	This study
pSL2327	*ste5::ADE2*	This study
pSL2222	*ste11::ADE2*	This study
pSL2270	*ste7::ADE2*	This study
pSL2223	*fus3::ADE2*	This study

reaction (PCR) amplification of the *STE7* coding sequence from pSL1951 (STEVENSON *et al.* 1992) using the primers, 5′-CCGGATCCTGGTCATGTTTCAACGAAAG-3′ and 3′-CTTTCTAGTTGGGTAACTTACGGATCCCG-5′.

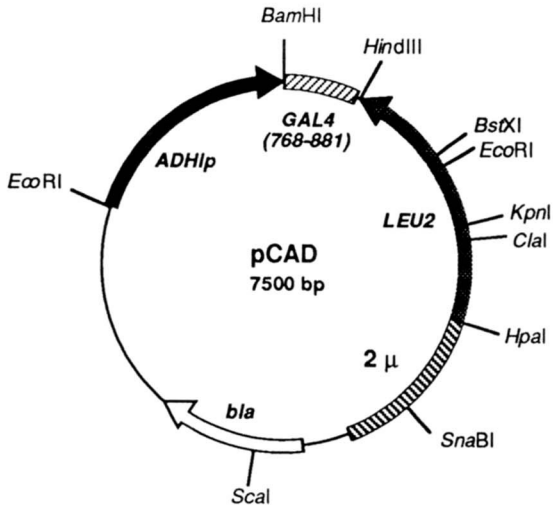
AD-Fus3p (pSL2175) and BD-Fus3p (pSL2174) were created by PCR amplification of the *FUS3* open reading frame from pYEE81 (ELION *et al.* 1990) using the primers 5′-G-CGGATCCAGATGCCAAAGAGAATTG-3′ and 3′-CCTTGCTTTATAAATCAATCCTAGGCG-5′.

AD-Kss1p (pSL2122) and BD-Kss1p (pSL2120) were created by PCR amplification of the *KSS1* sequence from pXT1 (COURCHESNE *et al.* 1989) using the primers 5′-CCGGATCCAGATGGCTAGAACCATAAC-3′ and 3′-TACTTCTGGTACCTTATCCTAGGCC-5′.

AD-Ste5p (pSL2289) was constructed by cutting pSL1979 [*Kpn*I-*Bam*HI *STE5* fragment in pTZ18 (MEAD *et al.* 1986)] with *Kpn*I, which removes the first 24 amino acids of Ste5p, and inserting a *Bam*HI linker to place the *STE5* coding sequence in-frame with the Gal4p sequence of pGAD3. BD-Ste5p (pSL2019) was created by cloning an *Eco*RI-*Bam*HI *STE5* fragment from pSL1979 into the *Eco*RI-*Bam*HI sites of pMA424.

AD-Ste11p (pSL2091) and BD-Ste11p (pSL2121) were generated by cutting pSL1877 (STEVENSON *et al.* 1992) with *Sal*I-*Xba*I, filling the overhangs with Klenow fragment and tailing with *Bam*HI linkers. The *Acc*I site immediately upstream of the *STE11* ATG codon was replaced previously with a unique *Sal*I by site directed mutagenesis.

AD-Ste12p (pSL2205) was constructed by adding *Bam*HI linkers to a 1.4-kb *Sty*I fragment of *STE12* (SONG *et al.* 1991), followed by cloning the fragment into pGAD2F. Ste12p-AD (pSL2280), with the Gal4p transcription activation domain at



	GAL4 codon 768	
G GAT CCG	GCC AAT	pCAD1
GG ATC CCG	GCC ATT	pCAD2
GGA TCC	GCG GCC ATT	pCAD3

FIGURE 2.—Restriction map of the carboxyl-terminal activation domain plasmid. Fusion proteins with the Gal4p transcriptional activation domain at the carboxyl terminus of a protein of interest are created by cloning into the unique *Bam*HI site. The three vectors differ in the reading frame of the *Bam*HI cloning site, as indicated.

the carboxyl terminus of Ste12p, was constructed by cloning the same 1.4-kb *Bam*HI fragment into pCAD2 (see below).

AD-Ste4p (pKB40.1) has been described previously (CLARK *et al.* 1993). BD-Ste20p (pKB84.7) and AD-Ste20p (pRL22) were generous gifts from KAREN CLARK and MALCOLM WHITEWAY.

To place the Gal4p transcription activation domain at the carboxyl terminus of a desired protein, a unique series of vectors, pCAD1, 2 and 3 (pSL2277, 2278, and 2279, respectively) were created (Figure 2). Like the pGAD vectors, these are 2μ-*LEU2* vectors that express the fusion proteins from the *ADH1* promoter. The pCAD vectors were made by introducing an *Eco*RV site at codon 768 of *GAL4*, by site directed mutagenesis, and inserting *Bam*HI 8-, 10- and 12-mer linkers. The resulting Gal4p transcription activation domains were then subcloned as a *Bam*HI-*Hin*dIII fragment behind the *ADH1* promoter contained in YEp351 (HILL *et al.* 1986). Hybrid genes specifying fusion proteins are made by cloning into the unique *Bam*HI site.

Gal4p fusions with the amino terminus of Ste11p were generated by inserting a *Bam*HI linker into the unique *Bsm*I site of pSL1966 [*Bam*HI *STE11* fragment in Bluescript II KS$^-$ vector (Stratagene)], allowing nucleotides encoding amino acids 1–435 to be liberated as a 1.3-kb *Bam*HI fragment. This fragment was then cloned into the *Bam*HI site of pMA424, pGAD2F and pCAD2.

The amino terminus of Ste7p was cloned into pMA424 and pGAD2F as a *Bam*HI-*Bgl*II fragment from pSL1963. The Ste7p kinase domain was cloned into pGAD3F as a *Bgl*II-*Bam*HI fragment from pSL1963.

Fusion proteins of Ste11p containing the K444R mutation were constructed by subcloning a 1.8-kb *Eco*RI-*Nco*I fragment from pNC192 (RHODES *et al.* 1990) into pSL1966, which contains the *Bam*HI *STE11* fragment used to construct pSL2205 and pSL2121, creating pSL2479 (BD fusion) and pSL2477 (AD fusion). The P279S substitution encoded by *STE11-1* was introduced into pSL2479 and pSL2477 as a 0.6-kb *Eco*RI-*Hin*dIII fragment from pSL1654 (STEVENSON *et al.* 1992).

A collection of plasmids were used to make gene disruptions in strain GGY::171. pSL2199 is a modification of p121 (WHITEWAY *et al.* 1989) in which a 2.2-kb *Bgl*II *ADE2* fragment from pAZS11 (STOLZ and LINDER 1990) replaces the *LEU2* sequences, producing a *ste4Δ::ADE2* disruption allele. The *far1Δ::ADE2* (pSL2200) construction was generated by replacing the *Bam*HI-*Hin*dIII fragment of *FAR1* with the 2.2-kb *ADE2* fragment from pAZS11. The *ste11::ADE2* allele (pSL2222) was constructed by replacing the *Pst*I-*Hin*dIII fragment of *STE11* with a 2.2-kb *Bgl*II *ADE2* fragment. A *fus3Δ::ADE2* disruption plasmid (pSL2223) was created by inserting the same *ADE2* fragment into the *Bgl*II site of *FUS3*. pSL2270 contains a *ste7Δ::ADE2* disruption in which the 2.5 kb *Bgl*II *ADE2* fragment from pAZS10 (STOLZ and LINDER 1990) replaces the *Bgl*II-*Cla*I fragment of *STE7*.

Anti-Gal4p antibodies: *GAL4 Eco*RI-*Sal*I DNA fragments encoding amino acids 1–147 (DNA binding domain) or 768–881 (transcriptional activation domain) were made by PCR using pMA424 and pGAD2F as templates. These fragments were then cloned in pMAL-c2 (New England Biolabs, Beverly, Massachusetts) creating pSL2236 [pMAL-c2-Gal4(1–147)p) and pSL2237 (pMAL-c2-Gal4(768–881)p]. The fusion proteins were expressed and purified from *E. coli* as described in the pMAL protein purification system literature. Purified protein was used as an antigen to generate monoclonal antibodies (mAbs) from mice by standard techniques (MARUSICH 1988). mAb 6E10-A7 (IgG1,κ) recognizes an epitope present in Gal4(1–147) and mAb 7E10-G10 (IgG2b, κ) recognizes an epitope present in Gal4(768–881).

Immunoblots: To prepare protein extracts for immunoblotting, 5–10 OD units of cells grown at 30° in selective medium were pelleted, washed with sterile water, immediately frozen on dry ice, and then stored at −70°. Cell pellets were thawed by suspension in 100 μl of cracking buffer (8 M urea, 5% sodium dodecyl sulfate (SDS), 40 mM Tris-HCl, pH 6.8, 0.1 mM EDTA, 1% β-mercaptoethanol, 0.4 mg/ml bromphenol blue), prewarmed to 60°, and transferred to a 1.5-ml microcentrifuge tube containing an 80-μl volume of glass beads. Samples were heated at 70° for 10 min, followed by vortexing for 1 min. Then 10–30 μl of the supernatant fraction from a 5-min microcentrifuge spin was fractionated on an 8% SDS-polyacrylamide gel electrophoresis (PAGE) gel (LAEMMLI 1970). Protein was transferred to nitrocellulose and probed with monoclonal antibodies against the Gal4p DNA binding domain and the Gal4p transcription activation domain. Primary antibodies were detected with an horseradish peroxidase (HRP)-conjugated donkey anti-mouse immunoglobulin (IgG) secondary antibody (Jackson ImmunoReasearch, West Grove, Pennsylvania), followed by the ECL chemiluminescent system (Amersham Corp., Arlington Heights, Illinois).

β-Galactosidase assays: Preliminary evaluations for interacting proteins were done by assay of β-galactosidase activity on plates. Transformation plates were replica plated onto M63GV-Leu-His plates (1.36% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 2% glucose, 2% agar, 0.5 mg/l $FeSO_4$ $7H_2O$, plus amino acids and vitamins. Adjusted to pH 7.0 with 1 M KOH) to which had been applied 300 μl of a 10 mg/ml solution of X-gal in dimethylformamide. The use of M63GV plates allowed more robust growth of the GGY::171 strains than SSX plates (CHEIN *et al.*

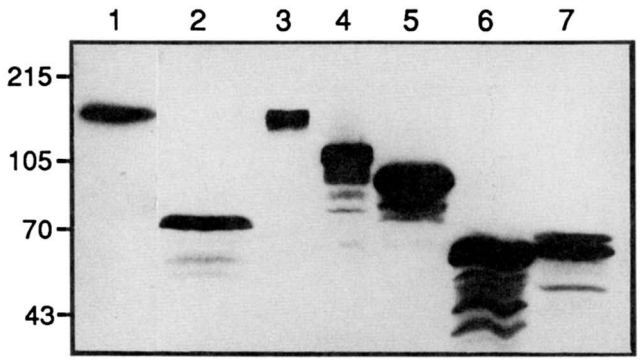


FIGURE 3.—Immunoblot analysis of two-hybrid proteins. Equal amounts of cell extract (5×10^6 cell equivalents) from transformants of SY2366 were immunoblotted with either mAb 6E10-A7 (lane 1) or mAb 7E10-G10 (lanes 2, 3, 4, 5, 6, 7, 8) as described in MATERIALS AND METHODS. Lane (1) BD-Ste5p; (2) AD-Ste4p; (3) AD-Ste20p; (4) AD-Ste11p; (5) AD-Ste7p; (6) AD-Fus3p; (7) AD-Kss1p. The positions of protein molecular mass standards, in kilodaltons, are shown.

1991), without compromising the ability to observe a positive signal for β-galactosidase induction.

For β-galactosidase assays in liquid, strains carrying the two-hybrid plasmids were grown to mid-log phase at 30° in selective medium (*e.g.*, SD-His-Leu). Cultures were then diluted into YEPD medium and incubated for 4–5 hr at 30° before preparation and assayed for β-galactosidase activity as described previously (JARVIS *et al.* 1988). Typically, four individual transformants of each plasmid combination were selected for liquid assay.

RESULTS

Protein-protein interactions revealed by the two-hybrid system: To identify the protein-protein interactions that occur in the pheromone response pathway, the two-hybrid system of FIELDS and SONG (1989) was used. Hybrid proteins contained either the DNA binding domain (BD) of Gal4p (amino acids 1–147) or the transcription activation domain (AD) of Gal4p (amino acids 768–881) fused to the proteins of interest. Initially we made hybrid genes that fused essentially the entire open reading frame of Ste4p, Ste20p, Ste5p, Ste11p, Ste7p, Ste12p, Fus3p and Kss1p to the BD, the AD, or both, of Gal4p. Upon transformation into yeast, the hybrid genes directed the synthesis of the appropriate fusion proteins (Figure 3, and data not shown). Moreover, each of the fusion proteins was functional as revealed by the ability of the hybrid genes to complement a deletion of the corresponding pathway gene (data not shown). The hybrids were then expressed in all pair-wise combinations in the *Saccharomyces cerevisiae* strain GGY::171 (GILL and PTASHNE 1987), or a derivative, which contains an integrated *GAL1-lacZ* reporter construct. Formation of a complex between the two fusion proteins should reconstitute the activity of Gal4p, causing transcription of the *GAL1-lacZ* reporter construct.

The results are compiled in a matrix shown in Table 3 and can be organized by five summary statements. First, several interactions that were expected based on biochemical or genetic studies were detected by this *in vivo* assay. In particular, BD-Ste7p interacted with both Fus3p and Kss1p (Table 3, row 4), and Kss1p appeared to interact with Ste12p, although in these initial tests the level of β-galactosidase activity promoted by the latter interaction was barely above background (see below). Second, we failed to detect interaction between two pairs of proteins that have been shown to interact *in vitro*, namely Ste11p with Ste7p (NEIMAN and HERSKOWITZ 1994) and Fus3p with Ste12p (ELION *et al.* 1993). Naturally, however, the failure to detect an interaction cannot be taken as evidence that it does not occur.

Third, and more strikingly, we observed interaction of BD-Ste5p with Ste11p, Ste7p, and Fus3p (Table 3, row 1). Although genetic epistasis experiments have suggested that Ste5p and Ste11p might act sequentially in the pathway (HASSON *et al.* 1993; STEVENSON *et al.* 1992), there was no evidence Ste5p might interact with the more distal members of the MAP kinase cascade. These findings suggest a role for Ste5p in the response pathway (see DISCUSSION).

Fourth, and also unexpectedly, AD-Ste11p interacted with both MAP kinases, Fus3 and Kss1p (Table 3, column 4). Finally, the two hybrid analysis failed to reveal interactions between either Ste4p (the G_β subunit) or Ste20p and any other component of the pathway tested.

As noted above, the initial evidence for interaction between Kss1p and Ste12p was equivocal (Table 3, column 8). We reasoned that in some cases the orientation of the Gal4p activation domain with respect to the remainder of the fusion protein might preclude transcription activation of the reporter even though the binding domain and activation domain hybrids were interacting. Accordingly, we constructed a new set of vectors (see MATERIALS AND METHODS) that would allow the activation domain to be placed at the carboxyl terminus of the hybrid rather than at the amino terminus, as results from use of the pGAD vectors. Indeed, when a Ste12p-AD hybrid was expressed from this new vector in combination with the BD-Kss1p hybrid, much more robust activation of the reporter was observed (Table 3, column 9). However, this Ste12p-AD hybrid still failed to reveal interactions with other pathway components, including Fus3p.

Protein-protein interactions occur in the absence of pathway components: The observation that the *GAL1-lacZ* reporter was activated by co-expression of a Ste5p hybrid with hybrids not only to Ste11p, but also to the more downstream proteins in the MAP kinase cascade, Ste7p and Fus3p, was unexpected. This observation could reflect direct interaction between Ste5p and Ste7p or Fus3p, or it could reflect indirect interaction mediated by a bridging protein. For example, perhaps wild-type Ste11p binds to both the Ste5p hybrid and the Ste7p hybrid and thereby promotes reconstitution of a active

TABLE 3

Two-hybrid matrix of protein-protein interactions

Gal4p (1–147) Fusions	Units of β-galactosidase activity[a,b] with Gal4p (768–881) hybrid fusions								
	Ste4p	Ste20p	Ste5p[c]	Ste11p	Ste7p	Fus3p	Kss1p	AD-Ste12p	Ste12p-AD
Ste5p[d]	–[e]	–	–	1112	333	120	–	–	–
Ste20p	–	–	–	–	–	–	–	–	–
Ste11p	–	–	28	65	–	–	30	–	–
Ste7p	–	–	93	–	–	89	249	–	–
Fus3p	–	–	–	44	–	–	–	–	–
Kss1p	–	–	–	1050	163	–	–	2	147

[a] β-Galactosidase activity was determined as described in MATERIALS AND METHODS. The values reported are the average of assays of five independent transformants. One standard deviation was typically 30–50% of the mean value.

[b] Unless otherwise indicated, β-galactosidase assays were performed in strain SY2509.

[c] Gal4p (768–881)-Ste5p fusions were expressed from the less active promoter of pGAD3.

[d] Two-hybrid interactions with BD-Ste5p were assayed in SY2066, an **a**/α diploid version of GGY::171, due to the toxicity of Ste5p overexpression in the haploid strains. We note that the BD-Ste5p fusion activates transcription from the *GAL1-lacZ* independent of the presence of a fusion protein bearing the Gal4p-AD. Therefore β-galactosidase levels with BD-Ste5p have been adjusted for this basal induction.

[e] "–" indicates <1 unit of β-galactosidase activity.

transcription factor at the *GAL1-lacZ* reporter. Similarly, the interactions we detect between Ste11p and Fus3p or Kss1p could be indirect. To test the possibility that these (or other) interactions might be indirect, we re-examined the ability of some pairs of hybrid proteins to activate the reporter in strains carrying deletions at *STE4, STE5, STE7, STE11* or *FUS3*. As shown in Figure 4, these deletions had little or no effect on interaction between the hybrids. In no case was the interaction abolished.

Several details are worth noting. First, we could not co-express the Ste5p and Ste11p hybrids in many of the strains. Presumably, simultaneous expression of high levels of these proteins activates the pheromone response pathway and precludes isolation of transformants capable of expressing both hybrids. Mutation of the catalytically crucial residue K444 of Ste11p (HANKS *et al.* 1988) to arginine allowed co-expression and therefore allowed interaction to be assessed (Figure 4A). Second, we could not express the BD-Ste5p hybrid in *ste4*Δ and *ste5*Δ strains, again presumably because this hybrid activates the pathway. This activation cannot occur if the pathway is blocked downstream of Ste5p, *e.g.*, in *ste11*Δ and *ste7*Δ mutants (Figure 4B). Finally, we could not co-express the AD-Ste5p and BD-Ste7p hybrids in a *fus3*Δ strain. One possible explanation for this failure is that Fus3p has a role not only in signal transmission, but also in signal attenuation, acting at least in part to reduce the activity of Ste7p. In this view, the highly expressed Ste7p hybrid is hyperactive in *fus3*Δ mutant strains compared to *FUS3* wild-type strains, and this hyperactivity is toxic to the cell. Mutation of the catalytically crucial K220 residue of Ste7p did not solve this problem because Ste7p hybrids bearing this mutation are expressed only at very low levels (data not shown).

The overall conclusion from these experiments is that the interactions we detect do not require bridging events involving known pathway components.

Protein-protein interactions of Ste7p and Ste11p can be mapped to distinct domains: The DNA sequences of *STE7* and *STE11* predict that each encode a protein comprised of two domains: a carboxyl-terminal catalytic domain with protein kinase activity, and an amino-terminal, presumably regulatory, domain. A regulatory role has been documented most clearly for the Ste11p amino-terminal domain (Figure 5). Truncations and amino acid substitutions in this region of Ste11p cause constitutive activation of the pheromone response pathway (STEVENSON *et al.* 1992; CAIRNS *et al.* 1992). To determine whether the interactions observed above for Ste7p or Ste11p could be ascribed to particular domains of the proteins, hybrids containing either the amino-terminal or carboxyl-terminal domains were tested against the full-length hybrids of Table 3.

As shown in Table 4, the amino terminal domain of Ste7p (residues 1–172) interacted with Fus3p and Kss1p, whereas the carboxyl terminal catalytic domain (residues 172–515) interacted with Ste5p. Conversely, the amino-terminal regulatory domain of Ste11p (residues 1–435) interacted with Ste5p, with itself, and perhaps with Kss1p. Initially, we did not detect an interaction between the regulatory domain of Ste11p and any of the hybrid proteins shown to interact with the full length Ste11p hybrid (Table 3). However, fusion of the Gal4p-AD to the carboxyl terminus of Ste11p(1–435) allowed detection of an interaction between two Ste11p regulatory domains. We have not tested the interaction spectrum of the Ste11p carboxyl-terminal catalytic domain. For reasons that are unclear, we are unable to obtain transformants expressing Gal4p hybrids of either wild-type Ste11p kinase domain or the K444R substitution.

***STE11-1* point mutation alters interactions of Ste11 protein:** An amino acid substitution, P279S, in the amino-terminal domain of Ste11p activates the pheromone response pathway constitutively (STEVENSON *et al.* 1992). To determine if the activated phenotype of this mutation correlates with an alteration in the ability of Ste11p to form protein complexes, a hybrid protein in which the Gal4p-AD was fused to Ste11pP279SK444R

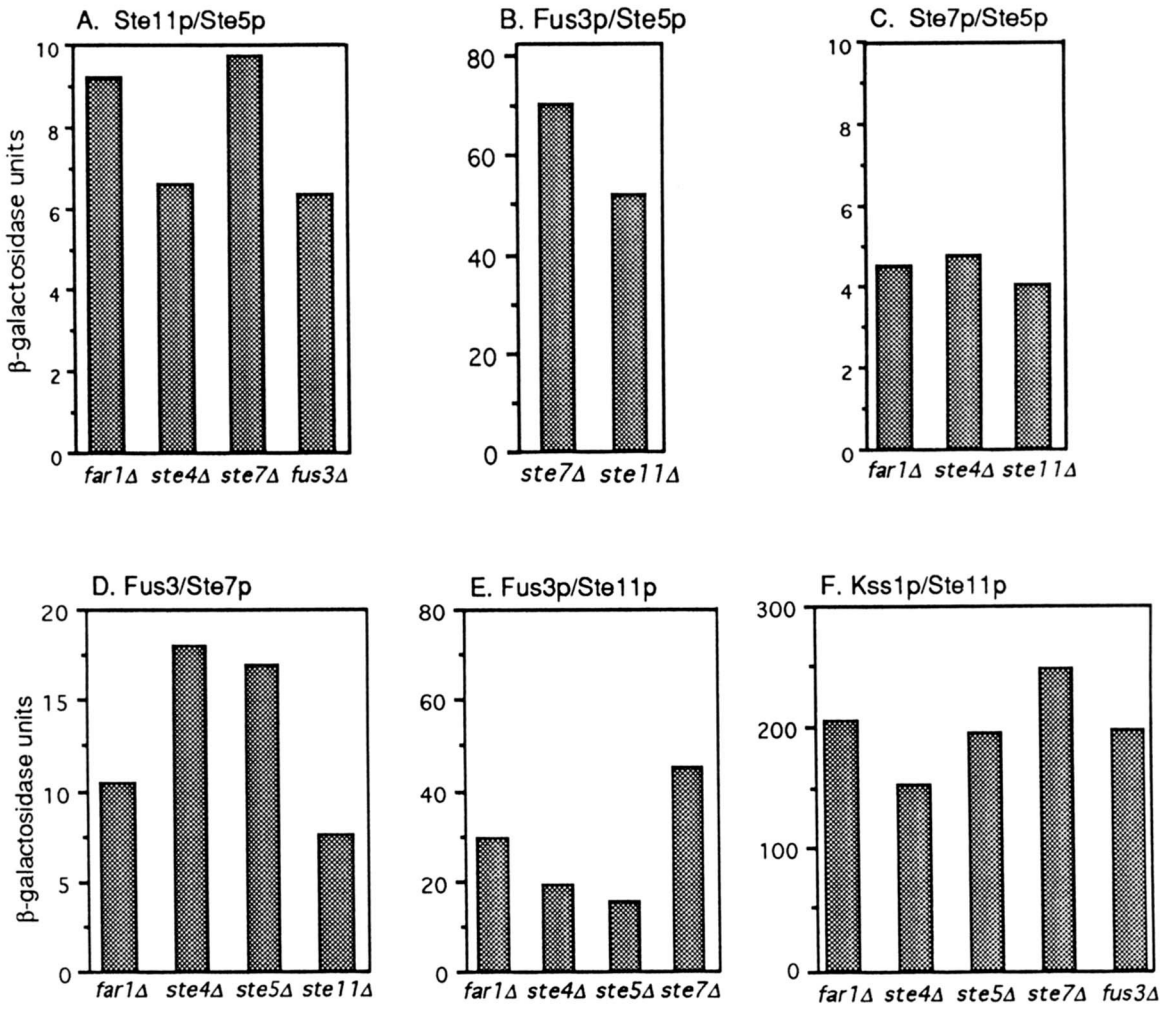


FIGURE 4.—Protein-protein interactions in strains deleted for signal transduction components. The indicated strains were co-transformed with plasmids: (A) pSL2121 (BD-Ste11p) and pSL2289 (AD-Ste5p); (B) pSL2175 (AD-Fus3p) and pSL2019 (BD-Ste5p); (C) pSL2162 (BD-Ste7p) and pSL2289 (AD-Ste5p); (D) pSL2168 (AD-Ste7p) and pSL2175 (AD-Fus3p); (E) pSL2174 (BD-Fus3p) and pSL2091 (AD-Ste11p); (F) pSL2120 (BD-Kss1p) and pSL2091 (AD-Ste11p). Five independent transformants were selected at random and assayed for β-galactosidase activity as described in MATERIALS AND METHODS. One standard deviation was typically 10–30% of the mean value. Basal activation of *GAL1-lacZ* by the BD-Ste5p fusion has been subtracted from the values reported above. The strains used were: SY2509 (*far1*Δ), SY2510 (*ste4*Δ), SY2756 (*ste5*Δ), SY2597 (*ste7*Δ), SY2598 (*ste11*Δ) and SY2596 (*fus3*Δ).

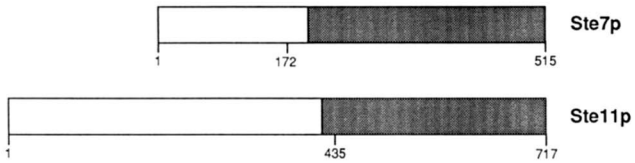


FIGURE 5.—Domain structure of Ste7p and Ste11p. The amino acids contained in the Gal4p fusions of Table 4 are indicated. Designation of the catalytic domains are from HANKS *et al.* (1988).

was tested in the two-hybrid system. The K444R substitution was used so that the effect of the P279S substitution could be evaluated without possible complications that could arise from constitutive activation of the pathway. Quantitative β-galactosidase assays were performed on SY2509 (GGY::171 *far1*) containing either Ste11p, Ste11pK444R or Ste11pP279SK444R hybrid proteins, plus the BD- or AD-fusion to be tested. Only hybrid proteins shown to form complexes with wild-type Ste11p hybrid protein were examined in this experiment. The P279S substitution had a profound effect on Ste11 protein interactions. β-Galactosidase activity was elevated 16-fold, with respect to both wild-type Ste11p and Ste11K444Rp hybrid proteins, when the Ste11pP279SK444R hybrid was co-expressed with the Ste5p hybrid (Figure 6). Conversely, interactions with Fus3p and Kss1p were decreased to 1 and 16%, respectively, of the β-galactosidase levels seen with Ste11pK444R. Interactions of the mutant Ste11 fusion proteins with another Ste11 fusion protein, mutant or wild-type, were unchanged.

To determine whether the interaction properties of the P279S substitution could be due to effects on the level of expression of the hybrid protein, immunoblot analysis was performed. Extracts were prepared from

TABLE 4

Interactions of Ste11p and Ste7p domains

	Ste5p[a]	Ste11p	Fus3p	Kss1p	AD-Ste11p (1–435)	Ste11p (1–435)-AD
Ste7p (1–172)[b]	—[c,d]	—	62	212	—	—
Ste7p (172–515)	687	—	—	—	—	—
Ste11p (1–435)	136	43	—	2	—	94

[a] β-galactosidase assays with Ste5 fusion proteins were performed in strain SY2366. Background *GAL1-lacZ* activation by the BD-Ste5p hybrid was subtracted from the reported values.

[b] Plasmids used were: pSL2167 (BD-Ste7p(1–172)), pSL2170 (AD-Ste7p(172–515)), pSL2173 (BD-Ste11p(1–435)), pSL2172 (AD-Ste11p(1–435)), plus the indicated hybrid protein fused to either Gal4p DNA binding domain (BD) or transcription activation domain (AD) (Table 2).

[c] Values represent the average units of β-galactosidase activity of five independent transformants of strain SY2509. One standard deviation was typically 30% of the mean value.

[d] "—" indicates <1 unit of β-galactosidase activity.

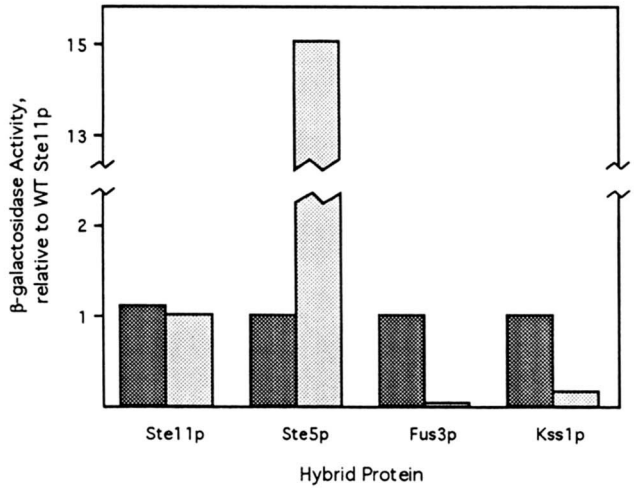


FIGURE 6.—Effect of P279S substitution on Ste11 protein-protein interactions. Strain SY2509 (*far1*Δ) was co-transformed with plasmids bearing hybrid proteins of either, Ste11pK444R (darkly shaded bars), or Ste11pP279SK444R (lightly shaded bars), plus the indicated hybrid protein. Five independent transformants were selected at random and assayed for β-galactosidase activity as described in MATERIALS AND METHODS. The reported values have been normalized to the β-galactosidase activity found with the wild-type Ste11p hybrid. The plasmids used were: Ste11p interactions–pSL2121 (BD-Ste11p), pSL2477 (AD-Ste11pK444R), and pSL2480 (AD-Ste11pP279SK444R); Ste5p interactions–pSL2289 (AD-Ste5p), pSL2121 (BD-Ste11p), pSL2479 (BD-Ste11pK444R), and pSL247X (BD-Ste11pP279SK444R); Fus3p interactions–pSL2174 (BD-Fus3p), pSL2091 (AD-Ste11p), pSL2477 (AD-Ste11pK444R), and pSL2480 (AD-Ste11pP279SK444R); Kss1p interactions–pSL2120 (BD-Kss1p), pSL2091 (AD-Ste11p), pSL2477 (AD-Ste11pK444R), and pSL2480 (AD-Ste11pP279SK444R).

SY2509 transformants expressing either Ste11pK444R or Ste11pP279SK444R, and the Ste5p, Kss1p or Fus3p hybrid proteins. Western blots were probed with monoclonal antibodies to the Gal4p DNA binding and transcription activation domains. Steady-state expression of the Ste11pP279SK444R fusion was not significantly different than the Ste11pK444R fusion in any of the two-hybrid combinations tested (Figure 7, compare lanes 1, 3 and 5 with lanes 2, 4 and 6). Thus, the P279S mutation does not stabilize the Ste11pP279SK444R fusion in combination with Ste5p hybrid, nor does it decrease the expression in combination with either Fus3p or Kss1p hybrid proteins. Steady-state expression of Kss1p, Fus3p or Ste5p hybrid proteins was identical in combination with both Ste11pK444R and Ste11pP279SK444R (data not shown). In addition, identical results were obtained in β-galactosidase assays with strains deleted for *STE7* and *STE11*, indicating that perturbation of the pheromone response pathway was not causing the apparent changes in affinity (data not shown). Thus, the change in expression of the *GAL1-lacZ* reporter in cells bearing the Ste11pP279SK444R hybrid compared to cells bearing the Ste11pK444R hybrid appears to reflect a change in the affinity of Ste11pP279SK444R for Ste5p, Fus3p and Kss1p. We cannot exclude the possibility that the P279S substitution affects activation of the reporter by influencing the ability of the hybrid protein to enter the nucleus or interact with the transcription machinery. However, the finding that the substitution has opposite effects on apparent interaction of Ste11p with Ste5p and with Fus3p or Kss1p is most easily accommodated by supposing that there is a change in the affinity of the protein interactions.

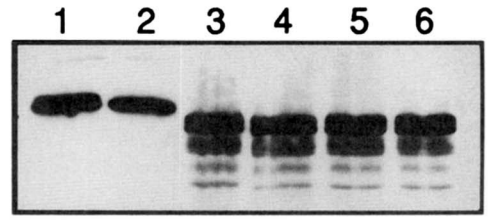


FIGURE 7.—Steady state expression of Ste11pK444R and Ste11pP279SK444R hybrid proteins. Equal amounts of cell extracts (5×10^6 cell equivalents) from transformants used in Figure 5 were immunoblotted with monoclonal antibodies to either to Gal4p(1–147) (lanes 1 and 2), or to Gal4p(768–881) (lanes 3, 4, 5 and 6). The plasmids used are the same as those described in the legend to Figure 5. Lanes 1, 3 and 4 contain Ste11pK444R. Lanes 2, 5 and 6 contain Ste11pP279SK444R.

DISCUSSION

We have utilized the two hybrid technique of FIELDS and SONG (1989) to examine the protein-protein inter-

actions among components of the yeast pheromone response pathway. Our results indicate that multiple protein-protein interactions occur involving Ste5p, Ste11p, Ste7p, Fus3p and Kss1p, all of which are required for signal transmission and mating. Some of these interactions, for example between Ste5p and Ste11p or between Ste7p and Fus3p, were expected because these pairs of proteins were argued on genetic grounds to control subsequent steps in the pheromone response pathway. Our results, therefore, support the idea that these proteins actually interact *in vivo* and imply that there are not as yet unidentified proteins that function between these particular pairs. The fact that these interactions occurred in strains deleted for possible bridging proteins further supports this conclusion, but the involvement of unknown proteins in mediating the observed protein interactions cannot be formally excluded. Other pairs of interactions, for example between Ste5p and Ste7p or between Ste11p and Fus3p, were not expected given the current linear view of the pheromone response pathway deduced from epistasis experiments (Errede *et al.* 1993; Stevenson *et al.* 1992). These interactions have interesting implications for the organization of the response pathway. Three general conclusions emerge from our work. First, we found that Ste5p, a protein of unknown function, interacts with protein kinases that operate at each step of the MAP kinase cascade, specifically Ste11p, Ste7p and Fus3p. Ste5p-Ste11p interaction occurs via the amino-terminal regulatory domain of Ste11p, whereas the Ste5p-Ste7p interaction occurs via the carboxyl-terminal region of Ste7p. Second, both Ste7p and Ste11p make contact with Fus3p and Kss1p. The Ste7p-Fus3p and Ste7p-Kss1p interactions occur via the amino-terminal regulatory domain of Ste7p. Finally, we detect protein-protein interactions between two Ste11p molecules via their amino-terminal regions. Below we discuss the implications of these findings for signal transmission and its regulation.

Interaction between Ste5p and members of the MAP kinase cascade: The finding that Ste5p interacts with Ste11p, Ste7p and Fus3p suggests that one role of Ste5p in signal transmission is to serve as a scaffolding protein and facilitate interactions between the members of the MAP kinase cascade. This finding is in accord with studies that show that Ste5p and Fus3p co-immunoprecipitate *in vitro* (Kranz *et al.* 1994). In addition, recent work from Elaine Elion's laboratory provides complementary genetic and biochemical support for the conclusion that Ste5p interacts with each of these protein kinases (E. A. Elion, personal communication). The interaction of Ste5p with each of these protein kinases may result from transient interactions events, or it may indicate that Ste5p forms a stable multimeric complex containing each of these protein kinases. These possibilities cannot be distinguished by the two hybrid system. The existence of a multimeric signaling complex involving elements of a MAP kinase cascade has recently been demonstrated in a mammalian signaling pathway, where Raf-1 kinase was shown to form a complex with its effector, Ras-GTP, and with its target, an MEK (Vojtek *et al.* 1993; Zhang *et al.* 1993; Moodie *et al.* 1993).

In its proposed role as a scaffold, Ste5p may make both signal propagation and signal attenuation more efficient. In addition, and perhaps more interestingly, in this role Ste5p may help to minimize cross-talk with other MAP kinase cascades and thus ensure the integrity of the pheromone response pathway. In support of this idea, partially constititutive forms of Ste7p can suppress deletion of *BCK1*, which encodes the Ste11p equivalent in the yeast protein kinase C (*PKC1*)-activated MAP kinase pathway (Lee and Levin 1992), if the *STE5* gene is also deleted (B. Yashar and B. Errede, personal communication).

The interaction of Ste5p with Ste7p and Ste11p involves specific domains of these latter proteins, a finding that suggests additional possible physiological roles for these protein-protein contacts. Ste5p makes contact with the amino-terminal region of Ste11p, which has been implicated in modulating Ste11p kinase activity (Stevenson *et al.* 1992; Cairns *et al.* 1992). In this light, it is interesting to note that a partially constitutive version of Ste11p, Ste11pP279S, appears to interact better with Ste5p than does the non-mutant protein. In contrast to the Ste5p-Ste11p interaction, the Ste5p-Ste7p interaction involves the catalytic domain of the Ste7p, suggesting that Ste5p is a substrate of the Ste7p kinase. Because Ste7p functions after Ste5p in the response pathway, the putative phosphorylation of Ste5p by Ste7p may serve to attenuate the pathway signal.

Together, our findings expand the possible roles of Ste5p in the pheromone response pathway. First, Ste5p may simply serve as a passive scaffold to organize the MAP kinase cascade. In this view, the activity of Ste5p does not change in response to pheromone; rather, an upstream component relays the signal directly to Ste11p. Second, the ability of Ste5p to serve as a scaffold may be influenced by the activity of the pathway. Interaction with the MAP kinase cascade may be enhanced when the pathway is initially activated and reduced when the pathway signal is attenuated. The interactions that we detect by the two hybrid system do not change when the cells are treated with pheromone. We note, however, that the fusion proteins are expressed at substantially higher levels than are the native proteins. If activation of the pathway altered the interaction of only a small fraction of the fusion proteins, this would not be detected in our analysis. Finally, Ste5p may have roles in pheromone response in addition to its role as a scaffolding protein. For example, perhaps an upstream component modifies or alters the conformation of Ste5p such that Ste5p becomes competent to relieve the inhibitory effect of the Ste11p amino-terminal domain and thereby activates the MAP kinase cascade. The

analysis of dominant constitutive alleles of *STE5*, such as the one recently reported by HASSON *et al.* (1994), may help distinguish among these possible roles for Ste5p.

Interaction among members of the MAP kinase cascade: Both Ste11p and Ste7p were found to interact with the two MAP kinases, Fus3p and Kss1p. The interaction of Ste11p with these MAP kinases was unexpected because they are not adjacent to Ste11p in the pathway as it is currently understood. Moreover, purified Ste11p cannot phosphorylate Kss1p or Fus3p (ERREDE *et al.* 1993). Perhaps the interactions reflect part of the mechanism whereby the pathway signal is attenuated. Support for this possibility comes again from the properties of the partially constitutive Ste11pP279S mutant. Hybrids containing this form of Ste11p interact very poorly with Fus3p or Kss1p hybrids. The inability of Ste11pP279S to interact with either Kss1p or Fus3p could in part account for the partial constitutivity of this version of Ste11p, if Fus3p and Kss1p negatively influence Ste11p activity. Indeed, it is conceivable that the absence of this negative influence on the mutant Ste11p could account for its greater ability to associate with Ste5p. Even in a cell that has not been stimulated with pheromone, there is some active Kss1p and Fus3p, which could therefore reduce the level of interaction between Ste5p and wild-type Ste11p below that seen for mutant forms of Ste11p that are immune to this negative influence.

Ste7p was found to interact with both Fus3p and Kss1p, in accord with reported interactions detected by *in vitro* assays that show that Fus3p and Kss1p are substrates for the Ste7p kinase (NEIMAN and HERSKOWITZ 1994; ERREDE *et al.* 1993). Surprisingly, however, the interaction that we detected *in vivo* involved the amino-terminal, presumptive regulatory region of Ste7p. Perhaps this region of Ste7p is required for substrate recognition. Another possibility is that the interaction reflects phosphorylation of Ste7p by Fus3p (and presumably by Kss1p as well), which occurs after pheromone stimulation and may be another part of the attenuation mechanism (ZHOU *et al.* 1993; STEVENSON *et al.* 1992). Naturally, these two possibilities are not mutually exclusive.

In conclusion, our experiments to detect interactions *in vivo* among the components of the pheromone response pathway have suggested a specific role for the Ste5p protein, a heretofore enigmatic member of the response pathway. In addition, the experiments revealed a number of unexpected interactions that may be important for regulating the propagation of the signal through the pathway.

We thank STAN FIELDS for providing the plasmids necessary for creating the two hybrid fusions; MIKE MARUSICH and BETH PRESCOTT of the University of Oregon Monoclonal Antibody Facility for preparing the mAbs described in this report; KIRK EKENA and members of the TOM STEVENS laboratory for initial characterization of the mAbs; ELAINE ELION and JEREMY THORNER for communicating results prior to publication; KAREN CLARK and MALCOLM WHITEWAY for providing plasmids, and members of the laboratory for stimulating discussions. This work was supported by research (GM 30027) and training (GM 07759) grants from the U.S. Public Health Service.

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Communicating editor: M. JOHNSTON