The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5

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Activation of the *Saccharomyces cerevisiae* MAP kinase Fus3 is thought to occur via a linear pathway involving the sequential action of three proteins: Ste5, a protein of unknown function, Ste11, a MAPKK kinase homolog, and Ste7, a MAPK kinase homolog which phosphorylates and activates Fus3. In this report, we present evidence for a novel mechanism of Fus3 activation that involves a direct association with Ste5, a protein not predicted to interact with Fus3. First, overexpression of Ste5 suppresses *fus3* point mutations in an allele-specific manner and increases Fus3 kinase activity in vitro. Second, Ste5 associates with Fus3 in vivo as demonstrated by the two-hybrid system and by two methods of copurification. Third, Ste5 and Fus3 associate prior to pheromone stimulation even when Fus3 is inactive, and in strains lacking Ste7 and Ste11. Fourth, Ste5 is phosphorylated by Fus3 in purified complexes and copurifies with an additional protein kinase(s). These observations suggest the possibility that Ste5 promotes signal transduction by tethering Fus3 to its activating protein kinase(s).

[Key Words: *Saccharomyces cerevisiae*; MAP kinase; signal transduction; Fus3; Ste5]

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Haploid *Saccharomyces cerevisiae* cells exposed to mating pheromone undergo cell-cycle arrest at G₁/Start, transcriptional activation of mating-specific genes, and cell elongation prior to mating (for review, see Sprague and Thorner 1994). Genetic analysis has identified numerous signal transduction components which regulate these responses (Mackay and Manney 1974; Hartwell 1980; for review, see Kurjan 1992; Sprague and Thorner 1994). Based on genetic data, these signal transduction components have been ordered into a linear pathway upstream of the transcription factor, Ste12, that is activated and phosphorylated in response to pheromone (Dolan et al. 1989; Dolan and Fields 1990). In this linear pathway model, signal transduction initiates by pheromone binding to a cell surface receptor, activating a heterotrimeric G-protein, then continues successively through the putative protein kinase Ste20, the Ste5 protein, the protein kinases Ste11 and Ste7, and culminating in the activation of Fus3 and Kss1, related protein kinases that activate Ste12 (Blinder et al. 1989; Elion et al. 1990, 1991b; Whiteway et al. 1990; Cairns et al. 1992; Gartner et al.

1992; Leberer et al. 1992; Stevenson et al. 1992; Errede et al. 1993; Ramer and Davis 1993). Although this linear pathway is favored for its simplicity, interdependent or branched models have not been excluded (Elion et al. 1990; Stevenson et al. 1992; Elion et al. 1993).

The protein kinases Fus3, Kss1, Ste7, and Ste11 comprise a highly conserved regulatory module found in mammals as well as yeast (Errede and Levin 1993; Kosako et al. 1993; Lange-Carter et al. 1993; Neiman et al. 1993). Fus3 and Kss1 are homologues of MAP kinases (mitogen-activated protein kinases, MAPKs) or ERKs (extracellular signal-responsive kinases) that are activated by phosphorylation of tyrosine and threonine residues (Ray and Sturgill 1987; Boulton et al. 1991; for review, see Cobb et al. 1991; Pelech and Sanghera 1992a, 1992b; Posada and Cooper 1992). Fus3 is also phosphorylated on these residues in response to α -factor, and requires this modification for function (Gartner et al. 1992; Elion et al. 1993). Ste7 is related to the dual-specificity MAP kinase kinases (MAPKKs or MEKs, for MAP kinase/ERK kinase) (Crews and Erikson 1992; Seger et al. 1992; Kosako et al. 1993) that activate the MAP kinases (L'Allemain et al. 1992; Rossomando et al. 1992). Consistent with this, Ste7 immune complexes phosphorylate Fus3 in vitro (Errede et al. 1993; Zhou et al. 1993). Ste11 is

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related to the MAPK kinase regulator, MAPKKK or MEKK (Lange-Carter et al. 1993), although it has yet to be shown that Ste11 directly activates Ste7. Ste20 is a putative protein kinase which to date lacks a mammalian counterpart and physiological target (Leberer et al. 1992; Ramer and Davis 1993).

Ste5 is the only signal transduction component that lacks obvious homology to a protein of known function (Leberer et al. 1993; Mukai et al. 1993; Perlman et al. 1993), making its role difficult to assess. The *STE5* gene encodes a 917-residue open reading frame with limited homology to two types of proteins, fueling speculation about its function (Leberer et al. 1993; Mukai et al. 1993; Perlman et al. 1993). A 150-residue amino-terminal domain of Ste5 is related to Far1, suggesting that Ste5 and Far1 share a function in G_1 arrest (Leberer et al. 1993; Mukai et al. 1993; Perlman et al. 1993). This region overlaps a potential cysteine/histidine metal-binding motif, which together with a carboxy-terminal acidic domain, suggest that Ste5 is a transcription factor (Leberer et al. 1993; Mukai et al. 1993; Perlman et al. 1993; Sprague and Thorner 1994). Neither possibility is consistent with the current placement of Ste5 in the signalling cascade. Thus, while genetic experiments demonstrate that Ste5 is essential for signal transduction (MacKay and Manney 1974; Hartwell 1980), the function of Ste5 remains a mystery.

Ste5 is thought to act at a single step between the Ste20 and Ste11 protein kinases on the basis of genetic analysis with hyperactive forms of Ste5 and Ste11 (Cairns et al. 1992; Leberer et al. 1992; Stevenson et al. 1992; Hasson et al. 1994); however, several observations suggest a more complex picture. First, hyperactive forms of Ste11 do not fully suppress *ste5* mutations (Stevenson et al. 1992) and hyperactive forms of Ste5 only partially suppress G -protein mutants (Hasson et al. 1993). Second, overexpression of Ste5 suppresses *ste4* (i.e., G -protein; MacKay 1983), *fus3* (Elion et al. 1991a), and *ste20* (Leberer et al. 1993) mutations that block at different points in the signal transduction pathway. This ubiquitous pattern of suppression suggests that Ste5 function cannot be defined in simple linear terms. Therefore, more complicated models for the role of Ste5 in signal transduction, such as an interdependence with other signal transduction components (e.g., the G -protein, Ste11, or Fus3), cannot be excluded (Elion et al. 1991a; Stevenson et al. 1992; Hasson, Blinder, Thorner, Jenness, 1994).

We present here a connection between Ste5 and Fus3, proteins not previously thought to interact directly on the basis of classical genetic experiments. Overexpression of Ste5 suppresses multiple defects of *fus3* point mutants in an allele-specific fashion, and also increases Fus3 kinase activity in vitro, suggesting a direct interaction between the two proteins. Consistent with these results, Ste5 associates with Fus3 in vivo, independently of both pheromone-stimulation and Fus3 catalytic activity. Furthermore, Ste5 is phosphorylated by Fus3 and additional associated kinases in vitro. Taken together, these data suggest Ste5 associates in a complex with Fus3 and other protein kinase(s).

Results

Excess Ste5 suppresses G_1 arrest and transcription defects of fus3 mutants

We isolated the *STE5* gene in two screens for suppressors of *fus3* mutations. In the first screen, *STE5* was identified as a suppressor of the mating defect of the *fus3-3* mutant (Elion et al. 1991a). Subsequent analysis showed that *STE5* overexpression increases mating and *FUS1* transcription 1.5- to 2-fold, and partially suppresses the G_1 arrest defect of a *fus3* null mutant (Elion et al. 1991a). In contrast, excess *STE5* does not bypass a *fus3 kss1* double mutant, suggesting *STE5* either functions before Fus3 and Kss1, or requires either kinase for function (Elion et al. 1991a). *STE5* was also isolated as a suppressor of the α -factor resistance of the *fus3-2* mutant in a second screen that identified other efficient suppressors including *FAR1* and *STE12* (Elion et al. 1993; B. Satterberg and E.A. Elion, details to be published elsewhere). Subsequent analysis showed that overexpression of other upstream components (e.g., *STE2*, *STE4*, *STE7*, *STE11*, *STE20*) did not suppress the *fus3-2* mutant as efficiently as *STE5* (data not shown). Thus, *STE5* is a conspicuously strong suppressor of *fus3* mutations.

Ste5 activates Fus3 kinase activity in vitro

We determined the effect of Ste5 overexpression on Fus3 kinase activity using an immune complex kinase assay that employs a hemagglutinin (HA) epitope-tagged Fus3, Fus3-HA (Elion et al. 1993). A typical assay reveals a profile of five or more associated proteins that are phosphorylated when Fus3 is activated by pheromone (Fig. 1A, lane 2). Fus3 kinase activity increases approximately fivefold when Ste5 is overexpressed, as shown by a five- to sixfold increase in intensity of all phosphoproteins in the profile and the presence of at least three new associated substrates (apparent molecular mass of ~115 kD, ~95 kD, and ~55 kD; Fig. 1A, lanes 4,6). Ste5 overexpression also increases Fus3 kinase activity two- to threefold in the absence of pheromone induction (Fig. 1A, lanes 3,5). In contrast, a *ste5* deletion mutation blocks Fus3 kinase activity, as expected (data not shown).

Ste5 overproduction could cause an apparent increase in Fus3 kinase activity by several mechanisms. First, the kinase activity could be due to Ste5; however, no kinase activity is seen in immune, complexes prepared from strains with Fus3 lacking the HA epitope (Fig. 1A, lanes 7,8), nor in strains containing Fus3R42-HA, an inactive form of Fus3-HA (Elion et al. 1993; data not shown). Second, the effect could be an indirect consequence of an increase in the level of Fus3 due to enhanced activity of Ste12, which normally induces *FUS3* mRNA ~3-fold (Elion et al. 1991b). However, equivalent amounts of Fus3 are immunoprecipitated in the absence and presence of excess Ste5 (Figure 1B, cf. lanes 1 and 2 with 3–6). Third, Ste5 overproduction could simply increase the levels of the substrates that coprecipitate with Fus3. If this were the case, then Ste5 levels should not affect the

Figure 1. Effect of overexpression of *STE5* on Fus3 kinase activity. (A) Kinase assays on Fus3-HA immune complexes. Kinase reactions were performed as described in Materials and methods. Reactions were stopped by the addition of loading buffer and separated on an 8% (30:0.8 acrylamide/bis-acrylamide)-SDS gel. Shown is a 1 hr exposure of the autoradiograph. Numbers (left) mark positions of protein standards (in kilodaltons). Cells were induced for 1 hr with α -factor where indicated (+). Lanes 1 and 2 (JY147) = FUS3-HA (pYEE1102) + 2 μ vector (YEp24); lanes 3, 4 and 5, 6 (duplicate JY108 transformants) = FUS3-HA (pYEE1102) + *STE5* 2 μ (pJB223); lanes 7, 8 (JY106) = FUS3 (pYEE1100) + *STE5* 2 μ (pJB223). (B) Immunoblot of Fus3-HA immune complexes. Fus3-HA was detected with 12CA5 monoclonal antibody (Materials and methods). Samples are as in A. (C) Phosphorylation of casein by Fus3-HA immune complexes. Kinase reactions were performed in the presence of 1 μ g casein, as described (Elion et al. 1993). Samples were separated on a 10% (38:2 acrylamide: bis-acrylamide)-SDS gel. Radioactivity in A and C were quantitated using a PhosphorImager (Molecular Dynamics). Shown are relative values.

ability of Fus3 to phosphorylate an exogenous substrate. However, Fus3 also phosphorylates casein, a specific substrate of Fus3 (Elion et al. 1993), fivefold more efficiently in the presence of excess Ste5 (Fig. 1C). Thus, Ste5 is a limiting component for activation of Fus3.

Allele-specific suppression of *fus3* point mutations by *STE5*

The simplest explanation for both the suppression of *fus3* mutations and the increased Fus3 kinase activity is that Ste5 causes an increase in signal transduction which indirectly enhances activation of Fus3. To test this hypothesis, we compared the suppression of four *fus3* point mutations (Materials and methods; B. Satterberg and E.A. Elion, in prep.) by *STE5* and a dominant hyperactive allele of *STE11*, *STE11-4* (Stevenson et al. 1992). The *fus3* mutations were introduced into a *kss1* Δ strain to ensure that suppression be independent of Kss1, since it is redundant with Fus3 for signal transduction (Elion et al. 1991b). *STE11-4* efficiently restores α -factor sensitivity to *fus3-2*, *fus3-211*, and *fus3-212*, but has little effect on the most defective *fus3* mutant, *fus3-205* (Fig. 2). In contrast, *STE5* overproduction suppresses both *fus3-2* and *fus3-205*, but has little effect on *fus3-211* and *fus3-212*. The preferential suppression of the most severely defective mutant, *fus3-205*, by *STE5* is also reflected in the relative levels of restored *FUS1* expression in these mutants (Fig. 2, legend). Thus, the contrasting pattern of suppression of *fus3* mutants by *STE5* and *STE11-4* suggests the effect of excess Ste5 may not be due solely to a general increase in signal transduction. Indeed, while the suppression of *fus3* mutants by Ste5 is not allele-specific in the classical sense of one mutation compensating for a second (Jarvik and Botstein 1975), it is allele-specific with respect to the ability of overexpressed Ste5 to suppress different *fus3* point mutations, and may be a consequence of physical interactions between the two proteins.

Ste5 and *Fus3* interact in a two-hybrid system

The allele-specific pattern of *STE5* suppression suggested that Ste5 and Fus3 physically interact, perhaps as kinase/regulator or kinase/substrate. We therefore tested whether Fus3 and Ste5 interact in vivo, using a *lexA*-based (Zervos et al. 1993) two-hybrid system (Fields and Song 1989). Fus3 was fused to a *lexA* DNA-binding domain that binds a *lexA* operator within the promoter of a reporter β -galactosidase gene (pJK103, Kamens et al. 1990). Ste5 was fused to an acidic transcriptional activation domain that does not bind DNA ("B42," Ma and Ptashne 1987). Neither fusion protein alone induces >5–15 units of β -galactosidase expression (Table 1, Experiment I). In contrast, co-expression of both fusion proteins leads to a 100-fold increase in β -galactosidase activity (Table 1). Expression of β -galactosidase requires both Ste5 and Fus3, since other *lexA* and B42 fusion combinations do not induce β -galactosidase (e.g., *lexA*-

Figure 2. Effect of overexpressed *STE5* and hyperactive *STE11-4* on α -factor sensitivity of *fus3* mutants. Lawns of *MATa sst1Δ kss1Δ* strain EY966 containing mutations *fus3-2* (BY369), *fus3-205* (BY360), or *fus3-211* (BY366) and bearing either YEp24 (*URA3* 2 μ), pJB223 (*STE5 URA3* 2 μ), pSL1655 (*STE11-4*, integrated) were tested for α -factor sensitivity by a halo assay using 5 μ l of 0.1 mM α -factor in dimethylsulfoxide. Strains were grown in SC medium lacking uracil to select for all plasmids, including the integrated *STE11-4*. Plates were photographed after 24 hr at 30°C. We measured *FUS1* expression in a subset of these strains after a 1 hr exposure to α -factor with a *Fus1*- β -galactosidase reporter gene (pJB207). In the absence of excess *Ste5*, the *fus3-2*, *fus3-205*, and *fus3-211* strains produce equivalent low levels of β -galactosidase activity (1.5 ± 0.1 units). Excess *Ste5* has little effect on *FUS1* expression in the *fus3-211* strain (1.7 ± 0.1 units), a threefold effect in the *fus3-2* strain (4.8 ± 0.1 units) and a fourfold effect in the *fus3-205* strain (6.0 ± 0.2 units). Numbers are averages of duplicate transformants assayed twice.

bicoid, B42-CDC28 in Table 1). Thus, by the two-hybrid system, *Ste5* and *Fus3* proteins interact.

Ste5 and Fus3 interact in the absence of Ste7, Ste11, and Ste12

It is possible that the two-hybrid interaction we detect is not due to a simple association between *Fus3* and *Ste5*. For example, overexpression of *Ste5* could activate the endogenous signal transduction pathway and cause *lexA*-*Fus3* to associate with another protein, such as the transcription factor *Ste12*, a *Fus3* substrate (Elion et al. 1993). Alternatively, B42-*Ste5* could associate with *lexA*-*Fus3* via intermediary proteins such as *Ste11* or *Ste7*, which function between *Ste5* and *Fus3*. We therefore repeated the two-hybrid experiment in strains deleted for *STE7*, *STE11*, or *STE12*, using a *lexA*-dependent *LEU2* reporter gene, and assayed growth on plates lacking leucine. *Fus3* and *Ste5* still associate in the absence of *Ste7*, *Ste11*, or *Ste12* as shown by equivalent growth of *STE*, *ste7Δ*, *ste11Δ*, and *ste12Δ* strains on media lacking

leucine (data not shown). In addition, *Fus3* and *Ste5* interact equivalently in an isogenic *ste5* deletion strain (Table 1, Experiment II), indicating that endogenous *Ste5* is not required for their association. The fact that *Ste5* and *Fus3* still interact in the absence of these signal transduction proteins strongly suggests their association is direct.

Ste5 and Fus3 copurify

We investigated whether *Ste5* and *Fus3* copurify under conditions in which both proteins are functional, as predicted by the two-hybrid results. To detect *Ste5*, we constructed a fusion between glutathione-S-transferase and *Ste5* (GST-*Ste5*) that is expressed from the *GAL1* promoter and complements a *ste5Δ* strain for mating (see Materials and methods). *Fus3*-HA immune complexes were prepared from strains containing either GST or GST-*Ste5*, then immunoblotted with anti-GST antiserum. As shown in Figure 3A, GST-*Ste5*, but not GST alone, coprecipitates with *Fus3*-HA, from both *STE5* and *ste5Δ* strains (cf. lanes 3, 4, 6 and 7 to lane 5). The presence of GST-*Ste5* is not due to nonspecific binding to the antibody or protein-A-Sepharose as it is not recovered from strains lacking the epitope tag on *Fus3* (lane 1). Thus, the ability to copurify GST-*Ste5* with *Fus3*-HA is dependent on both *Ste5* and *Fus3*. Furthermore, *Ste5* and *Fus3* associate regardless of whether *Fus3* is active (α -factor-induced, lanes 4,7) or inactive (uninduced, lanes 3,6).

To confirm the specificity of the association between *Ste5* and *Fus3*, we did the complementary experiment of purifying GST and GST-*Ste5* with glutathione-agarose, and assaying for the presence of *Fus3*-HA. Identical results were seen: *Fus3*-HA copurifies with GST-*Ste5* but

Table 1. β -Galactosidase activity induced by interaction of *Fus3* and *Ste5* fusion proteins in a two-hybrid system

	Host strain	<i>lexA</i> fusion plasmid ^a	β -Galactosidase activity (units) ^b		
			B42 fusion plasmid ^a		
			Ø	CDC28	STE5
I	<i>STE5</i>	Ø	9	8	7
		bicoid	10	14	14
		<i>FUS3</i>	13	13	1200
II	<i>STE5</i>	<i>FUS3</i>	15	N.D.	310
	<i>ste5Δ</i>	<i>FUS3</i>	15	N.D.	360

[N.D.] Not determined.

^aSpecific plasmids are listed in Table 4. Ø refers to the vector alone. *LexA* vector is *LexA202PL*, *bicoid* fusion is *pHMØ*, *FUS3* is *LexA-FUS3*; B42 fusion vector is *pJG4-5*, *CDC28* fusion is *pYBS150*; *STE5* fusion is *pYBS146*.

^bValues are Miller units of β -galactosidase activity (Craven 1965), averaged from two experiments in which two independent plasmid-bearing transformants were assayed. All assays were done in yeast strain EGY048 (*STE5*) or a *ste5Δ* derivative carrying a *LacZ* reporter plasmid.

Figure 3. GST–Ste5 and Fus3–HA co-precipitate in vivo. Immunoblot analysis of GST–Ste5 in Fus3–HA immune complexes. (*Left*) Fus3–HA was immunoprecipitated with 12CA5 monoclonal antibody from *ste5Δ* (lanes 1–4) or *STE5*⁺ (lanes 5–7) strains carrying *FUS3*–HA on pYEE1102 (except lane 1, which has *FUS3* on pYEE1100) and either GST (lane 5) or GST–STE5 (lanes 1–4 and 6,7). Cells were pre-grown in either galactose (G) or dextrose (D), prior to being induced for 1 hr with α -factor, where indicated (+). Duplicate samples were run on separate gels for immunoblot analysis with either antiserum against GST (*top*) or 12CA5 (*bottom*). Note that the secondary antibody (goat anti-rabbit IgG–HRP) cross-reacts with the 12CA5 Ig in the immune complexes. Numbers (*left*) mark positions of protein standards (in kilodaltons). The expected position of GST protein is indicated. Strains are BY845 (lane 1), BY846 (lanes 2–4), BY 784 (lane 5), and BY786 (lanes 6,7) (*right*). Immunoblot

analysis of Fus3–HA associated with GST–Ste5. GST or GST–Ste5 was purified with glutathione–agarose (see Materials and methods), and duplicate samples were run on separate gels and immunoblotted with either 12CA5 (*top*) or affinity-purified rabbit antiserum against GST (*bottom*). Numbers mark positions of protein standards (in kilodaltons). Results are shown from two separate experiments. Cells were grown in either galactose (G) or dextrose (D), and were induced for 1 hr with α -factor before extract preparation, where indicated (+). Lanes 1–3 (BY846) = *ste5Δ* + GST–STE5 (pYBS186), lane 4 (BY844) = *ste5Δ* + GST (pEMBL–GST), lane 5 (BY784) = *STE5*⁺ + GST (pEMBL–GST), and lanes 6,7 (BY786) = *STE5*⁺ + GST–STE5 (pYBS186).

not with GST (Fig. 3A, cf. lanes 2 and 3 with 4; lanes 6 and 7 with 5). Again, the association between Ste5 and Fus3 could be detected in extracts of both α -factor-induced (lanes 3,7) and -uninduced (lanes 2,6) cells. Thus, Ste5 and Fus3 associate in vivo independently of pheromone induction.

Ste5 associates with a catalytically inactive form of Fus3

The association between Ste5 and Fus3 prior to pheromone stimulation (Fig. 3A) and in *ste* strains suggests that activation of Fus3 is not a prerequisite for association with Ste5. We tested this possibility by repeating both types of copurification with a catalytically impaired form of Fus3–HA, Fus3R42–HA. As shown in Figure 4, GST–Ste5 associates equivalently with both functional (Fus3–HA, lanes 3,4, left and right) and nonfunctional (Fus3R42–HA, lanes 6,7, left and right) forms of Fus3. Thus, Fus3 need not be catalytically active in order to associate with Ste5.

Fus3 phosphorylates Ste5 in purified complexes

We next determined whether Ste5 is phosphorylated by Fus3 as evidence of a direct association. Fus3 was first tested for its ability to phosphorylate Ste5 using the B42–Ste5 protein (Table 1) as an exogenous substrate. Equivalent amounts of B42–Ste5 and Fus3–HA were immunoprecipitated (Fig. 5, right) and tested in the kinase assay

(Elion et al. 1993). B42–Ste5 is only slightly phosphorylated when assayed alone (Fig. 5, left, lane 5) or in the presence of inactive Fus3R42–HA (lane 6). In contrast, the addition of active Fus3 causes a significant increase in the amount of B42–Ste5 phosphorylation (Fig. 5, left, lanes 2, 4). Equivalent results were seen when B42–Ste5 was prepared from either *fus3Δ* or *FUS3* strains (data not shown). Moreover, this phosphorylation is specific for Ste5, since B42 alone is not phosphorylated (lane 1 and data not shown).

The fact that Fus3 could phosphorylate Ste5 in vitro suggested that phosphorylation could be used to detect a direct association between the two proteins when they associate in vivo. We therefore performed kinase assays on Fus3–HA immunoprecipitated from strains containing GST–Ste5 (Fig. 6A, lanes 1–9). As seen previously for wild-type Ste5 (Fig. 1), overexpression of GST–Ste5 causes a large increase in Fus3 kinase activity both in the absence and presence of α -factor (Fig. 6A, cf. lanes 8 and 9 with 6 and 7), even under repressive conditions when GST–Ste5 is produced at very low levels (Fig. 6A, lanes 13,18). Significantly, overexpression of GST–Ste5 produces a prominent new phosphoprotein of ~120 kD, the size observed for GST–Ste5 (Fig. 6A, lanes 8,9), and consistent with the expression of GST–Ste5 (Fig. 6A, cf. lanes 8 and 9 with lanes 6 and 7). Furthermore, the ~120-kD phosphoprotein is present in extracts from both untreated (lane 8) and pheromone-treated (lane 9) cells, consistent with the association of Ste5 and Fus3.

To prove that the ~120-kD substrate is GST–Ste5, we

determined whether it could be purified with glutathione-agarose. Fus3—HA kinase reactions were solubilized and incubated with glutathione-agarose (see Materials and methods). Of the nine or more phosphoproteins,

only the 120-kD protein bound to glutathione-agarose (Fig. 6B, lane 2), demonstrating that it is GST-Ste5. Furthermore, treatment of the purified protein with thrombin, which recognizes a site engineered between GST

Figure 5. Phosphorylation of B42-Ste5 by Fus3-HA immune complexes. Kinase assays (*left*) were performed on B42-Ste5 and B42 immunoprecipitates alone (lane 5) or in the presence of Fus3-HA immune complexes. Strains containing the galactose-inducible fusion proteins B42 (BY872) or B42-Ste5 (BY873) were grown in galactose (G) or dextrose (D), as indicated. Fusion proteins were immunoprecipitated with 12CA5 monoclonal antibody from 200 M_g of yeast extract in RIPA buffer, then washed 5 times

before being added to Fus3-HA immune complexes. Fus3-HA immune complexes were prepared from Fus3-HA-containing strain EY960 (lanes 1-4) or fus3R42-HA (catalytically inactive)-containing strain EY1144 (lane 6). Fus3-HA strains were treated for 1 hr with a-factor where indicated (+). Samples were separated on an 8% (30:0.8 acrylamide/bis-acrylamide)-SDS gel. (*Right*) Immunoblot of Fus3-HA and B42-Ste5-HA immunoprecipitates. Duplicate immunoprecipitates from those used in the kinase assay were performed using 12CA5 monoclonal antibody.

Figure 6. Effect of GST-Ste5 on Fus3-HA kinase assay. (A) Kinase assays of Fus3-HA immune complexes. Total protein (200 μ g) of each strain was immunoprecipitated with 12CA5 monoclonal antibody, and Fus3-HA kinase assays were performed as described in Materials and methods. All strains contained Fus3-HA (pYEE1102) except those in lanes 5, 11, and 16, which contained Fus3 lacking the HA epitope (pYEE1100) indicated by (-). Lanes 1-9 are from a *STE5*⁺ strain (EY1095) containing GST (lanes 1-4) or GST-Ste5 (lanes 5-9). Lanes 10-20 are from a *ste5* Δ strain (BY819) containing GST (lane 10), GST-Ste5 (lanes 11-15), or *STE5* CEN (lanes 16-20). Cells were grown in either galactose (G), to induce expression of the GST fusion proteins, or dextrose (D), to repress their expression, and were induced for 1 hr with α -factor, where indicated (+). (B) Purification and thrombin digestion of GST-Ste5 from kinase assays of Fus3-HA immune complexes. A kinase reaction identical to lane 9 of A was solubilized in RIPA buffer and incubated with glutathione-agarose. The GST precipitates were washed, analyzed directly (lane 2), incubated with thrombin (lane 4), or incubated without thrombin (lane 3). (C) Thrombin digestion of purified GST-Ste5. GST-Ste5 was purified with glutathione-agarose from 200 μ g of whole cell extract from the *STE5*⁺ strain (BY786) containing Fus3-HA + GST-STE5. Thrombin was added (lane 3) or not added (lanes 1,2), and the precipitates were incubated at 30°C for 10 min (lanes 2,3). Samples were separated on a 10% SDS-polyacrylamide gel and visualized with Coomassie blue. (D) Immunoblot with α -GST of duplicate samples from C. Note that a GST doublet is typically seen but the smaller species was inadvertently run off this gel.

and Ste5, shows that the vast majority of the phosphorylation is on Ste5, not GST. Cleavage of the ~120-kD phosphoprotein with thrombin produces a single phosphoprotein of ~100 kD equivalent in size to the Ste5 portion of GST–Ste5 (Fig. 6B, lane 4). In contrast, cleavage of purified GST–Ste5 with thrombin produces three major polypeptides, one of ~100 kD, and two of ~30 kD that are detected by GST antiserum (Fig. 6C, D). Thus, Fus3 phosphorylates Ste5 when the two proteins associate, proving they directly contact one another.

Ste5 associates with an additional protein kinase(s)

One explanation for the interaction between Ste5 and Fus3 is that Ste5 mediates the association between Fus3 and its upstream activating kinases. To test this possibility, we examined whether Ste5 associates with additional protein kinases. We first determined whether GST–Ste5 is still phosphorylated *in vitro* when copurified with catalytically inactive Fus3R42–HA. Normally, no Fus3 kinase activity is detected in kinase assays of Fus3R42–HA (Fig. 7A, lane 4; Elion et al. 1993). Nevertheless, GST–Ste5 is still residually phosphorylated in immune complexes with Fus3R42 (Fig. 7A, lanes 5, 6). This phosphorylation could reflect residual Fus3 kinase activity that is now detectable in the presence of excess GST–Ste5. Indeed, an equivalent ERK2 MAP kinase mutant retains 5% activity *in vitro* when assayed with myelin basic protein (Robbins et al. 1993). However, identical Fus3R42–HA/GST–Ste5 immune complexes do not phosphorylate a large excess of MBP (data not shown), a proven Fus3 substrate (Elion et al. 1993). Thus, GST–Ste5 may be phosphorylated by another protein kinase(s) present in the complex whose activity is independent of Fus3 and specific for GST–Ste5. This possibility is strongly supported by the fact that GST–Ste5 purified from both *FUS3* and *fus3Δ* strains is associated with a protein kinase(s) which phosphorylates GST–Ste5 in the kinase assay (Fig. 7B; see Materials and methods). Significantly, only full-length GST–Ste5 is phosphorylated, although immunoblot analysis shows the presence of shorter degradation products (Figs. 3 and 4). Thus, an additional protein kinase(s) that specifically recognizes Ste5 can copurify with both Fus3 and Ste5, suggesting the existence of a multimeric complex.

Discussion

A novel linkage between Ste5 and Fus3

We demonstrate five features of a previously unknown relationship between Ste5 and Fus3, proteins believed to be positioned three steps apart in the signal transduction pathway and not predicted to interact. First, overexpression of Ste5 suppresses *fus3* point mutations in an allele-specific manner. Second, Ste5 is a limiting component for activation of Fus3 kinase. Third, Ste5 associates with Fus3 *in vivo* as demonstrated by the two-hybrid system and by two methods of copurification. Fourth, Ste5 and Fus3 associate prior to pheromone stimulation, when

Figure 7. GST–Ste5 is associated with a Fus3-independent protein kinase(s). (A) Kinase assays of active Fus3–HA (BY786, lanes 1–3) and inactive Fus3R42–HA (JY165, lanes 4–6) from strains harboring GST–Ste5 (pYBS186). Kinase assays were performed as described in Materials and methods. GST–Ste5 was expressed by growth in galactose (G), repressed by growth in dextrose (D). Cells were induced for 1 hr with α -factor where indicated (+). (B) Phosphorylation of GST–Ste5 by an associated protein kinase(s). Strains were grown in galactose to induce expression of GST–Ste5 and then induced for 1 hr with α -factor. GST–Ste5 was purified from *FUS3* (lane 1, EY1462) and *fus3Δ* (lane 2, EY1450) extracts with glutathione–agarose and incubated in a standard kinase assay for 8 min (Elion et al. 1993). Samples were separated on a 10% SDS–polyacrylamide gel.

Fus3 is inactive, and in the absence of Ste7 and Ste11. Fifth, Ste5 is phosphorylated by Fus3 and additional protein kinase(s) in purified complexes. These data strongly suggest that Ste5 and Fus3 directly interact. The simpler explanation for the effects of Ste5 on Fus3, that of overexpressing a limiting component upstream of Fus3 in the signal transduction pathway, is highly unlikely. This purely indirect mechanism for the effects of Ste5 on Fus3 does not explain the allele-specific suppression, the physical interaction, nor the fact that Ste5 can be phosphorylated by Fus3.

Evidence of a direct role for Ste5 in Fus3 activation: a model for Ste5 function

Our results can best be explained by a model in which Ste5 serves a structural role to tether Fus3 to its activating protein kinases, Ste7 and Ste11 (Fig. 8A). In this model, Ste5 positively regulates Fus3 kinase activity by virtue of its association with Fus3; Ste5 overproduction could cause a higher proportion of Fus3 molecules to be associated in an activating complex. The allele-specific suppression of a loss-of-function *fus3* mutation by excess Ste5, coupled with the ability of Ste5 to enhance Fus3 kinase activity, and to associate with Fus3 and an additional protein kinase(s), are all consistent with this

Figure 8. Three models to explain the interaction between Ste5 and Fus3. The three models stem from the proposed linear pathway of signal transduction in the pheromone response pathway, established by genetic experiments using hyperactive forms of *STE11* (Cairns et al. 1992; Gartner et al. 1992; Leberer et al. 1992; Stevenson et al. 1992; Zhou et al. 1993). See text for explanation. (A) Ste5 directly regulates Fus3 by serving as a nucleation site for Fus3 and activating kinases, Ste7, and Ste11, facilitating rapid signal amplification. The phosphorylation reactions follow the established linear pathway (e.g., *STE11* → *STE7* → *FUS3*). Ste20 is positioned directly upstream of Ste5 as hypothesized by Leberer et al. (1992). This model neither precludes nor requires a catalytic role for Ste5. (B) Feedback regulation between Fus3 and Ste5, which could be negative (N) or positive (+). (C) An additional role for Ste5p downstream of Fus3.

model. Moreover, the association of Ste5 with Fus3 in the absence of pheromone induction and catalytically active Fus3 is consistent with a positive regulatory role for Ste5 that would be required prior to pheromone stimulation. Indeed, this interaction contrasts sharply with the interaction between Fus3 and Ste12, a proven Fus3 substrate that appears to associate preferentially with active Fus3 (Elion et al. 1993). Furthermore, a prediction of this model is that Ste5 should also interact with Ste11 and Ste7, and Fus3 should interact with Ste11. Both the two-hybrid analysis and copurification studies suggests that this is the case (B. Satterberg, D. Lyons, and E.A. Elion, in prep.).

Thus, the primary role for Ste5 as a nucleation site for the assembly of protein kinases involved in signal transduction is consistent with the previous epistasis analyses (MacKay 1983; Blinder et al. 1989; Elion et al. 1991a, b; Cairns et al. 1992; Leberer et al. 1992, 1993; Stevenson et al. 1992; Hasson et al. 1994; Ramer and Davis 1993), if we assume that the enzymatic reactions, but not necessarily the physical associations, are in a linear series. The fact that Ste5 is so limiting for Fus3 activation may suggest Ste5 is in low abundance, consistent with a critical role as a potent activator. Furthermore, the existence of a complex between Ste5 and Fus3 also permits feedback regulation between the two proteins, which would explain why Fus3 phosphorylates Ste5.

Alternative interpretations of the Ste5/Fus3 interaction

Alternative explanations for the interaction between Ste5 and Fus3 are based on the premise that Ste5 interacts with Fus3 only as a substrate. The first alternative is that Ste5 and Fus3 associate solely to feedback regulate Ste5, resulting in either down-regulation or activation of

signal transduction (Fig. 8B). The feedback model does not readily explain the allele-specific suppression of *fus3-205*, nor the fact that the association between Fus3 and Ste5 is not enhanced by pheromone-induction as might be expected. However, numerous MAP kinases have been shown to phosphorylate upstream signal transduction components in vitro, including the EGF receptor (for review, see Pelech and Sanghera 1992a) and Raf-1 kinase (Lee et al. 1992). Moreover, Fus3 has been shown to phosphorylate Ste7 in vitro (Zhou et al. 1993), and genetic analysis suggests Fus3 negatively regulates its own activation (Gartner et al. 1992). Thus, it is plausible that Fus3 may feedback regulate Ste5, but perhaps in the context of a multimeric complex.

A second alternative is that Ste5 acts in a second place directly downstream of Fus3 to promote G_1 arrest, transcription, and mating, requiring phosphorylation by Fus3 for function (Fig. 8C). Both the interaction and phosphorylation data are consistent with Ste5 interacting with Fus3 as a substrate. This interpretation could explain the inability of hyperactive alleles of *STE11* to fully suppress a *ste5Δ* mutant (Stevenson et al. 1992), the ability of high copy *STE5* to suppress a *far1* mutant (Leberer et al. 1993), and resemblance of Ste5 to transcription factors (Mukai et al. 1993; Perlman et al. 1993). However, many observations argue against this interpretation. First, overproduced Ste5 is an allele-specific suppressor of a mutant, *fus3-205*, that appears to be defective in activation (B. Satterberg and E.A. Elion, in prep.). Second, Ste5 associates with Fus3 prior to pheromone stimulation regardless of Fus3 activity, as might be expected for a regulatory interaction. Third, a *far1* mutation can be suppressed by increasing the activity of other upstream components besides Ste5 (E.A. Elion, unpubl.). Fourth, Ste5 does not appear to play a direct role in transcription activation, because a *ste5Δ ste12Δ* double mutant ap-

pears no more defective in transcription than a *ste12Δ* single mutant (E.A. Elion, unpubl.).

Precedents for protein complexes mediating signal transduction

The notion of a signal transduction pathway operating through a multimeric protein complex, with at least one protein playing a primarily structural role, is not unprecedented. One example comes from bacterial chemotaxis. Though originally ordered by double mutant studies in a linear pathway [receptors → CheW (a protein of unknown function) → CheA (a histidine kinase) → CheY (a phospho-activated response regulator)] (for review, see Stock et al. 1992), more rigorous biochemical experiments now show that CheW's function is solely as a "coupling protein" between the CheA kinase and the receptors, stimulating CheA activity more than 10-fold (Borkovich and Simon 1990; McNally and Matsumara 1991; Ninfa et al. 1991; Gegner et al. 1992). A second example comes from mammalian signal transduction. Recent reports reveal that the proteins Raf-1 kinase and its regulator Ras:GTP interact to form a complex (Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993), and that MAP kinase kinase can be found in this complex (Moodie et al. 1993; Van Aelst et al. 1993).

Further investigation will determine how Ste5's interaction with Fus3 and other protein kinases contributes to signal transduction. The association of multiple protein kinases into a ternary complex with Ste5 might enable the proper set of protein kinases to interact, assuring specificity. Such specificity might be important in light of the existence of at least three MAP kinase cascades in *S. cerevisiae* (Errede and Levin 1993). The conservation of MAP kinase signal transduction modules across widely divergent organisms raises the question of whether Ste5 is also conserved in other MAP kinase signal transduction cascades. It is tempting to speculate that the Ste5-dependent associations we detect in yeast provide a paradigm for an analogous form of regulation in higher eukaryotes.

Materials and methods

Media, strains, and yeast strain construction

Yeast media, containing either 2% dextrose, 2% raffinose or 2% galactose, were prepared as described (Sherman et al. 1986). All strains (Table 2) except the EGY048 derivatives are isogenic *sst1Δ* derivatives of W303 (EY699, Elion et al. 1991b). *SST1* encodes a protease that degrades α -factor (Ciejek and Thorne 1979). The *fus3* point mutants are described elsewhere (B. Satterberg and E.A. Elion, in prep.). Yeast transformations were performed by the lithium acetate procedure (Ito et al. 1983). Gene replacement (Rothstein 1983) was used to construct *ste* derivatives using pJB220, pJB221, pNC149, pNC202, and pNC163.

Plasmids and plasmid construction

Plasmids are listed in Table 3. Standard methods were used for bacterial transformations, plasmid DNA preparation, and plas-

mid constructions (Sambrook et al. 1989) using restriction and DNA-modifying enzymes from New England Biolabs. pYBS351 was made by cloning the 2-kb *EcoRI* *fus3R42-HA* fragment from pYEE127 (Elion et al. 1993) into pYEE1102. pYBS186 was made by first subcloning the 3-kb *KpnI* *STE5* fragment of pJB215 (gift of J. Brill, MIT) into the *KpnI* site of pUC118, to make pYBS97; then subcloning the 3-kb *BamHI* *STE5* fragment (containing all but the first 23 amino acids of the *STE5* gene) from pYBS97 into pEMBL-GST to make pYBS186. Note that this GST-Ste5 construct complements the mating defect of a *ste5Δ* strain when it is expressed under either repressive (i.e., glucose) or inducing (i.e. galactose) conditions. pYBS138 is YCplac33 (Gietz and Sugino 1988) with *STE5* on a 4-kb *BamHI-XbaI* fragment. pYBS146 contains the 3-kb *BamHI* *STE5* fragment from pYBS97 in-frame after a tripartite fusion protein that contains, in order, an ATG start codon, the SV40 nuclear localization signal, the transcriptional acidic-activation domain B42, and HA, in pJG4-5 (Zervos et al. 1993). pYBS150 (Elion et al. 1993) contains a 1-kb *BamHI-BglII* fragment containing the *CDC28* open reading frame (derived from pGALCDC28, gift of J. Celenza), into the *BamHI* site of pYBS139 (a derivative of pYBS135 in which the *EcoRI* site was filled-in to create the proper reading frame).

α -Factor sensitivity, and β -galactosidase assays

α -factor sensitivity was measured by a halo assay as described (Elion et al. 1993), using 50 μ l of an overnight culture of yeast cells and 5 μ l of 0.1 mM synthetic α -factor in dimethylsulfoxide (synthesized by C. Dahl, Harvard Medical School). All halos were done in duplicate. β -Galactosidase activity was measured as described (Craven et al. 1965), using yeast extracts prepared by glass bead breakage, as described below.

Preparation of yeast extracts

Yeast strains containing plasmids were grown at 30°C in selective (SC) media with 2% dextrose to an A_{600} of 0.4–0.8 and then induced for 1 hr at 30°C with 50 nM α -factor. Genes under control of the *GAL1* promoter were induced by first growing strains overnight in selective media with 2% raffinose to an A_{600} between 0.5 and 1.2, followed by growth for 5–6 hr in 2–3 volumes of selective media containing 2% galactose, and then, where indicated, induced with α -factor. Cells were collected at 4°C, washed once with cold sterile water, then either frozen in dry ice/ethanol or used immediately for protein extract preparation. Whole cell extracts were prepared by lysis with glass beads, as described in (Elion et al. 1993), including 250 mM NaCl in the lysis buffer. Protein concentrations were determined with the Bio-Rad protein assay.

Preparation of immune complexes for kinase assays and for coimmunoprecipitation experiments

All immune complexes and kinase assays were performed exactly as described (Elion et al. 1993), except for the B42 fusion proteins, which were immunoprecipitated in RIPA buffer (Sambrook et al. 1989). BSA (1%) was included in coimmunoprecipitation experiments. Casein and MBP were added as substrates in the kinase assays at a final concentration of 0.5 mg/ml.

Western blotting

Samples were electrophoresed by SDS-PAGE on 7.5%, 8% or 10% polyacrylamide gels (with acrylamide/bis-acrylamide ratios of either 38:2 or 30:0.8), as noted in each figure legend.

Table 2. Yeast strains

Strain	Genotype	Source
All strains are W303 background, except EGY048		
EY957	<i>MATa ade2-1 his3-11,15 leu2-3,112 sst1Δ trp1-1 ura3-1 can1-100 Gal⁺</i>	Elion et al. (1993)
EY940	<i>EY957 fus3-6::LEU2</i>	Elion et al. (1993)
EY959	<i>EY940 + pYEE114</i>	Elion et al. (1993)
EY960	<i>EY940 + pYEE121</i>	Elion et al. (1993)
EY1144	<i>EY940 + pYEE128</i>	Elion et al. (1993)
EY966	<i>EY940 kss1::HIS3</i>	Elion et al. (1993)
BY360	<i>EY966 fus3::LEU2</i> replaced with <i>fus3-205</i>	B. Satterberg and E. Elion (in prep.)
BY366	<i>EY966 fus3::LEU2</i> replaced with <i>fus3-211</i>	B. Satterberg and E. Elion (in prep.)
BY367	<i>EY966 fus3::LEU2</i> replaced with <i>fus3-212</i>	B. Satterberg and E. Elion (in prep.)
BY369	<i>EY966 fus3::LEU2</i> replaced with <i>fus3-2</i>	B. Satterberg and E. Elion (in prep.)
EY1466	<i>BY360 + pJB207 + YEP24</i>	this study
EY1467	<i>BY366 + pJB207 + pJB223</i>	this study
EY1469	<i>BY369 + pJB207 + YEp24</i>	this study
EY1470	<i>BY366 + pJB207 + YEp24</i>	this study
EY1471	<i>BY360 + pJB207 + pJB223</i>	this study
EY1472	<i>BY369 + pJB207 + pJB223</i>	this study
EGY048	<i>MATa LEU2::pLexAop6-LEU2 ura3 his3 trp1 Gal⁺</i>	Zervos et al. (1993)
BY872	<i>EGY048 + pJK103 + LexA202PL + pJG4-5</i>	this study
BY874	<i>EGY048 + pJK103 + LexA202PL + pYBS150</i>	this study
BY873	<i>EGY048 + pJK103 + LexA202PL + pYBS146</i>	this study
BY875	<i>EGY048 + pJK103 + pHMØ + pJG4-5</i>	this study
BY876	<i>EGY048 + pJK103 + pHMØ + pYBS150</i>	this study
BY818	<i>EGY048 + pJK103 + pHMØ + pYBS146</i>	this study
BY716	<i>EGY048 + pJK103 + LexA-FUS3 + pJG4-5</i>	this study
BY719	<i>EGY048 + pJK103 + LexA-FUS3 + pYBS150</i>	this study
BY718	<i>EGY048 + pJK103 + LexA-FUS3 + pYBS146</i>	this study
EY1095	<i>EY957 fus3-8::ADE2</i>	this study
BY783	<i>EY1095 + pYEE1100 + pEMBL-GST</i>	this study
BY784	<i>EY1095 + pYEE1102 + pEMBL-GST</i>	this study
BY785	<i>EY1095 + pYEE1100 + pYBS186</i>	this study
BY786	<i>EY1095 + pYEE1102 + pYBS186</i>	this study
JY165	<i>EY1095 + pYBS351 + pYBS186</i>	this study
EY1450	<i>EY1095 + YCp403 + pYBS186</i>	this study
EY1452	<i>EY1095 + pYEE1102 + pYBS186</i>	this study
BY819	<i>EY1026 with ste5::TRP1</i> replaced for <i>ste5::URA3</i>	this study
BY843	<i>BY819 + pYEE1100 + pEMBL-GST</i>	this study
BY844	<i>BY819 + pYEE1102 + pEMBL-GST</i>	this study
BY845	<i>BY819 + pYEE1100 + pYBS186</i>	this study
BY846	<i>BY819 + pYEE1102 + pYBS186</i>	this study
BY847	<i>BY819 + pYEE1100 + pYBS138</i>	this study
BY848	<i>BY819 + pYEE1102 + pYBS138</i>	this study
JY161	<i>BY819 + pYBS351 + pYBS186</i>	this study
JY106	<i>EY940 + pJB223 + pYEE1100</i>	this study
JY108	<i>EY940 + pJB223 + pYEE1102</i>	this study
JY177	<i>EY940 + YEp24 + pYEE1100</i>	this study
JY147	<i>EY940 + YEp24 + pYEE1102</i>	this study
JY173	<i>EY940 + YEp24 + pYBS351</i>	this study
EY1026	<i>EY940 ste5::URA3</i>	this study
JY179	<i>EY1026 + pYEE1100</i>	this study
JY181	<i>EY1026 + pYEE1102</i>	this study
JY183	<i>EY1026 + pYBS351</i>	this study

Table 3. Plasmids

Plasmid	Markers	Source
pYEE114	<i>URA3 CEN FUS3</i>	Elion et al. (1993)
pYEE121	<i>URA3 CEN FUS3-HA</i>	Elion et al. (1993)
pYEE128	<i>URA3 CEN fus3R42-HA</i>	Elion et al. (1993)
YCp403	<i>HIS3 CEN</i>	Stearns et al. (1990)
pYEE1100	<i>HIS3 CEN FUS3</i>	Elion et al. (1993)
pYEE1102	<i>HIS3 CEN FUS3-HA</i>	Elion et al. (1993)
pYBS351	<i>HIS3 CEN fus3R42-HA</i>	this study
YEp24	<i>URA3 2μ</i>	
pYBS101	<i>URA3 2μ STE12</i>	B. Satterberg and E. Elion (in prep.)
pYBS102	<i>URA3 2μ FAR1</i>	Elion et al. (1993)
pJB223	<i>URA3 2μ STE5</i>	J. Brill (MIT)
pYBS138	<i>URA3 CEN STE5</i>	this study
pSL1655	<i>URA3 STE11-4</i>	Stevenson et al. (1992)
pJB220	<i>ste5 101::TRP1</i>	J. Brill
pJB221	<i>ste5 102::URA3</i>	J. Brill
pNC149	<i>ste7-3::URA3</i>	Company et al. (1988)
pNC202	<i>ste11-4::URA3</i>	B. Errede (University of NC, Chapel Hill)
pNC163	<i>ste12-1::URA3</i>	Company et al. (1988)
pJB207	<i>FUS1-lacZ LEU2 2μ</i>	J. Brill
LexA202PL	<i>HIS3 2μ pADH1-LexA</i>	Zervos et al. (1993)
LexAFUS3	<i>HIS3 2μ pADH1-LexA-FUS3</i>	Zervos et al. (1993)
pHMØ	<i>HIS3 2μ pADH1-LexA-Bicoid</i>	Zervos et al. (1993)
pJG4-5	<i>TRP1 2μ pGAL1-B42-HA</i>	Zervos et al. (1993)
pYBS146	<i>TRP1 2μ pGAL1-B42-HA-STE5</i>	this study
pYBS150	<i>TRP1 2μ pGAL1-B42-HA-CDC28</i>	Elion et al. (1993)
pJK103	<i>URA3 2μ pLexA-lacZ</i>	Kamens et al. (1990)
pEMBL-GST	<i>URA3 2μ pGAL-GST</i>	C. Chan (University of Texas, Austin)
pYBS186	<i>URA3 2μ pGAL-GST-STE5</i>	this study

Western blotting was performed essentially as described (Burnette 1981), with the modifications noted in (Elion et al. 1993). Nitrocellulose filters were blocked in 20 mM Tris at pH 7.5, 150 mM NaCl, 0.05% Tween-20 (TBST) containing 5% nonfat-dry milk, then incubated with primary antibody diluted in TBST–5% milk overnight at 4°C or 2 hr at room temperature. Anti-HA mouse monoclonal antibody 12CA5 tissue culture supernatant was provided by Harvard University antibody facility; anti-GST affinity-purified rabbit antiserum was a gift of R. Van Etten, Harvard Medical School. Immunoblots were washed five times with TBST at room temperature, and then incubated for 1–2 hr at room temperature with secondary antibody (HRP-conjugated goat-anti-mouse IgG or HRP-conjugated goat-anti-rabbit IgG, purchased from Bio-Rad) in TBST–5% milk. After five washes in TBST and two washes in TBS, the blots were developed with the Amersham ECL kit, according to manufacturer's instructions using Fuji RX X-ray film.

Precipitation, purification, and thrombin-cleavage, of GST proteins

GST–Ste5 was purified with glutathione–agarose (Sigma) following the same approach used for immunoprecipitations (Elion et al. 1993), using 200 μ g of whole cell extract in the presence of modified H buffer, 150 mM NaCl, and 1% BSA.

Non-specific aggregates were first pelleted by a 15 min microfuge spin at 4°C. The supernatant was then incubated with 25 μ l of glutathione agarose (at 75 μ g/ μ l) at 4°C for 30–60 min with constant rotation. Glutathione precipitates were pelleted by a 15-sec spin at 1500 rpm in a Sorvall RT6000B centrifuge, washed two times with modified H buffer with 150 mM NaCl, and then three times with modified H buffer. Samples to be analyzed by gel electrophoresis were resuspended in 30 μ l 2 $\times$ SDS-PAGE sample buffer (Sambrook et al. 1989) and boiled for 5 min. Samples to be cleaved with thrombin were washed once in PBS with 1% TX-100, once in 50 mM Tris at pH 7.5 with 150 mM NaCl, and once in TCB (thrombin cleavage buffer: 2.5 mM CaCl₂, 50 mM Tris at pH 7.5, 150 mM NaCl), as described (Ausubel et al. 1992), except that all buffers were cold. The samples were then resuspended in a final volume of ~20 μ l TCB, to which was added either 5 μ l of thrombin (0.1 unit/ μ l in TCB; Sigma T-6759; stored at –20°C) and 2 μ l of heparin (10 units/ μ l in TCB; Sigma H-3393; stored at –20°C), or 7 μ l of TCB. Reactions were carried out for 10 min at 30°C. These cleavage conditions were determined empirically, based on previously described conditions (Chang 1985). Reactions were stopped by addition of 30 μ l of 2 $\times$ SDS-PAGE sample buffer followed by boiling for 5 min.

Phosphorylated GST–Ste5 was purified from Fus3–HA immune complexes as follows: First, 1 ml of RIPA buffer (either at

room temperature or 42°C) was added to Fus3-HA immunoprecipitates after an 8 min kinase reaction, and the sample was mixed by inverting ~10 times over a 5–10 min period at 23°C. The protein-A-Sepharose and associated proteins were then pelleted, and the supernatant was transferred to a tube containing 25 μ l glutathione-agarose. The glutathione precipitation was then done at 4°C in RIPA buffer, and the subsequent thrombin cleavage was performed exactly as described above.

Preparation of GST–Ste5 for kinase assays

GST–Ste5 was purified from 200 μ g of whole cell extract using glutathione agarose exactly as described above. Glutathione precipitates were washed once with modified H buffer containing 150 mM NaCl, twice with modified H buffer and twice with kinase buffer. Kinase reactions were then performed exactly as described [Elion et al. 1993].

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