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FUS3 Phosphorylates Multiple Components of the Mating Signal Transduction Cascade: Evidence for STE12 and FAR1

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The mitogen-activated protein (MAP) kinase homologue FUS3 mediates both transcription and G1 arrest in a pheromone-induced signal transduction cascade in *Saccharomyces cerevisiae*. We report an in vitro kinase assay for FUS3 and its use in identifying candidate substrates. The assay requires catalytically active FUS3 and pheromone induction. STE7, a MAP kinase kinase homologue, is needed for maximal activity. At least seven proteins that specifically associate with FUS3 are phosphorylated in the assay. Many of these substrates are physiologically relevant and are affected by in vivo levels of numerous signal transduction components. One substrate is likely to be the transcription factor STE12. A second is likely to be FAR1, a protein required for G1 arrest. FAR1 was isolated as a multicopy suppressor of a nonarresting *fus3* mutant and interacts with FUS3 in a two hybrid system. Consistent with this FAR1 is a good substrate in vitro and generates a FUS3-associated substrate of expected size. These data support a model in which FUS3 mediates transcription and G1 arrest by direct activation of STE12 and FAR1 and phosphorylates many other proteins involved in the response to pheromone.

INTRODUCTION

The initiation of conjugation in *Saccharomyces cerevisiae* is a simple model for both negative growth control and differentiation in higher cells. Extracellular peptide pheromones bind to receptors on the surface of haploid cells, which in turn stimulate a G protein–coupled signal transduction cascade. Activation of the cascade causes cell-cycle arrest at Start in G1 phase (termed G1 arrest) and the induction of specialized functions required for cell attachment and fusion (reviewed in Cross *et al.*, 1988; Marsh *et al.*, 1991; Kurjan, 1992). The diverse responses to pheromone depend on the simultaneous inhibition of a conserved cell-cycle apparatus that regulates the mitotic cycle transition from G1 into S (reviewed in Cross *et al.*, 1988) and activation of a transcription factor, STE12 (Dolan *et al.*, 1989; Errede and Ammerer, 1989), that regulates the transcription of many genes required for signal transduction, G1 arrest, and mating.

FUS3, a mitogen-activated protein (MAP) kinase homologue (Boulton *et al.*, 1990), is required for both G1 arrest (Elion *et al.*, 1990, 1991a; Fujimura, 1990) and the activation of mating functions (Elion *et al.*, 1990; 1991a). FUS3 acts in parallel with a second MAP kinase homologue, KSS1 (Courchesne *et al.*, 1989), to promote the expression of STE12-dependent genes (Elion *et al.*, 1991a) but mediates G1 arrest through an additional mechanism that does not overlap with KSS1 (Elion *et al.*, 1991a,b). FUS3 and KSS1 are both phosphorylated in vivo on the same threonine and tyrosine residues as MAP kinases (reviewed in Cobb *et al.*, 1991; Pelech and Sanghera, 1992; Posada and Cooper, 1992a) and require these residues for function, suggesting aspects of their regulation are highly conserved (Gartner *et al.*, 1992).

Genetic evidence suggests FUS3 functions redundantly with KSS1 at one point in the signal transduction cascade that is after the G protein and before the STE12 transcription factor (Elion *et al.*, 1991a). Three other putative protein kinases, STE7 (Teague *et al.*, 1986), STE11 (Rhodes *et al.*, 1990), and STE20 (Leberer *et al.*, 1992), also function in the cascade. Epistasis studies with hyperactive forms of STE11 suggest the kinases operate in the order G protein → STE20 → STE11 → STE7 →

Table 1. Yeast strains

Strain	Genotype[a]	Source
EY957	*MATa sst1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
BY58	*MATa sst1Δ fus3-2 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	B. Satterberg
EY940	*MATa sst1Δ fus3-6::LEU2 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY941	*MATa sst1Δ fus3-7::HIS3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY946	*MATa sst1Δ fus3-6::LEU2 kss1::URA3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY948	*MATa sst1Δ fus3-6::LEU2 ste7::URA3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY949	*MATa sst1Δ fus3-6::LEU2 ste11::URA3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY950	*MATa sst1Δ fus3-6::LEU2 ste12Δ1::URA3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY1013	*MATa sst1Δ far1::URA3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
EY1014	*MATa sst1Δ fus3-6::LEU2 far1::URA3 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion
BY380	*MATa sst1Δ fus3-6::LEU2 far1Δ100 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	B. Satterberg
EY1093	*MATa sst1Δ fus3-8::ADE2 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 Gal+*	E. Elion

[a] Isogenic derivatives of EY699; Elion *et al.*, 1991a.

FUS3/KSS1 → STE12 (Cairns *et al.*, 1992; Gartner *et al.*, 1992; Leberer *et al.*, 1992; Stevenson *et al.*, 1992), with FUS3 and KSS1 nearest to STE12 (Gartner *et al.*, 1992). STE7 is likely to be the kinase that activates FUS3, as it is structurally related to a dual specificity MAP kinase kinase (also termed MAPK/Erk kinase [MEK] kinase) (Crews and Erickson, 1992) that activates MAP kinase in vitro (Alessandrini *et al.*, 1992; Kosako *et al.*, 1992; Matsuda *et al.*, 1992; Nakielny *et al.*, 1992). STE12 is an obvious candidate substrate for both FUS3 and KSS1 (Elion *et al.*, 1991a,b), because it is likely to be activated by phosphorylation (Song *et al.*, 1991; Yuan and Fields, 1991).

FUS3 also appears to function in a branch of the signal transduction cascade that promotes G1 arrest by a mechanism that is independent of STE12 activation (Elion *et al.*, 1990, 1991a; Satterberg and Elion, unpublished data). G1 arrest involves inhibition of CDC28 kinase (Mendenhall *et al.*, 1987) and three functionally redundant G1 cyclins (CLN1, CLN2, CLN3) (Cross, 1988; Nash *et al.*, 1988; Richardson *et al.*, 1989) that together promote the G1-Start cell-cycle transition. It has been hypothesized that G1 arrest occurs by simultaneous inactivation of the G1 cyclins by specific inhibitors (Chang and Herskowitz, 1990). Inactivation of the G1 cyclins blocks CDC28 kinase activity, presumably because they are regulatory subunits of CDC28 (Wittenberg *et al.*, 1990; Tyers *et al.*, 1992). Initial studies suggested that FUS3 specifically inhibits CLN3 (Elion *et al.*, 1990); however, recent work shows that FUS3 also regulates CLN1 and CLN2 (Elion *et al.*, 1991a,b; Satterberg and Elion, unpublished data). It is not known whether FUS3 regulates the G1 cyclins directly or indirectly (or both). FAR1, a protein of unknown function, is a candidate effector of G1 arrest and functions independently of STE12 activation (Chang and Herskowitz, 1990). Although initial work suggested FAR1 specifically inhibits one G1 cyclin, CLN2 (Chang and Herskowitz, 1990), FAR1 may also regulate CLN1 (Valdivieso *et al.*, 1993). FAR1 is hyperphosphorylated in α factor–treated cells, suggesting that it is activated by a protein kinase (Chang and Herskowitz, 1992).

How does FUS3 regulate both transcription and G1 arrest? Although genetic analysis suggests several FUS3 substrates, an in vitro assay for FUS3 kinase to determine its in vivo substrates has not yet been described. In this report, we describe a FUS3- and pheromone-dependent kinase activity that phosphorylates multiple proteins associated with FUS3 in vitro. Several of these substrates are components of the signal transduction apparatus and include the STE12 transcription factor. In addition, we provide genetic and biochemical evidence that efficient G1 arrest requires phosphorylation of FAR1 by FUS3.

MATERIALS AND METHODS

Strains, Media, and Microbiological Techniques

Yeast strains are listed in Table 1. All strains used in this study are isogenic *sst1Δ* derivatives of EY699 (Elion *et al.*, 1991a), a W303-derived strain. *SST1* encodes a protease that degrades α factor (Ciejek and Thorner, 1979). Yeast media were prepared as described in Sherman *et al.* (1986) and contained either 2% dextrose, 2% raffinose, or 2% galactose. Yeast was transformed by the lithium acetate method of Ito *et al.* (1983). Genomic yeast DNA used for Southern analysis and plasmid DNA recovery by transformation into *Escherichia coli* were isolated according to Hoffman and Winston (1987). *E. coli* transformations were performed using either HB101 or DH5α. Plasmid DNA was isolated as described in Sambrook *et al.* (1989). A *dut1-ung1-E. coli* strain [*HfrkL16 PO/45 [lysA(61–62)] dut1 ung1 thi1 relA1 Zb0-279::Tn10 supE 44*] was used for oligonucleotide mutagenesis.

Yeast Strain Construction

fus3Δ null strains were constructed using *fus3-7::LEU2*, *fus3-8::HIS3*, and deletion derivatives of pYEE98 as described previously (Elion *et al.*, 1990). The *fus3-9::ADE2* derivative was made with pYEE129. *kss1Δ* strains were constructed as described (Courchesne *et al.*, 1989). *sst1Δ* derivatives were constructed using pJGSST1 (gift of W. Courchesne, University of Nevada) that contains a *URA3*-marked *sst1* deletion. This construct contains redundant *E. coli hisg* sequences on either side of the *URA3* gene, allowing loss of *URA3* by recombination. *sst1Δ*

ura3- derivatives were isolated after passage of *sst1::URA3* transformants over 5-fluoroorotic acid as described (Boeke *et al.*, 1984). The *ste7::URA3*, *ste11::URA3*, and *ste12::URA3* deletion mutants were constructed by gene replacement (Rothstein, 1983) using plasmids pNC149, pNC202, and pNC163 (gift of B. Errede, University of North Carolina, Chapel Hill). The *far1::URA3* deletion mutant was constructed using plasmid pFC15 (Chang and Herskowitz, 1990); the unmarked *far1Δ100* mutant was made with pYBS94 after passage over 5-fluoroorotic acid.

Plasmid Construction

The *FUS3*, FUS3-hemagglutinin (HA), and *fus3-HA* (R42 and F182) genes were each cloned on a 2.7-kb *Eco*RI-*Eco*RI fragment into pRS316 (URA3, CEN6, ARSH4) (Sikorski and Hieter, 1989) to create pYEE114, pYEE121, pYEE127, and pYEE128, respectively. The *ADE2* gene was then subcloned into pYEE114 and pYEE121 to create pYEE1106 and pYEE1108. FUS3 and FUS3-HA were also subcloned into pRS313 (HIS3, CEN6, ARSH4) (Sikorski and Hieter, 1989) to create pYEE1100 and pYEE1102 and into a YEp13 derivative (Broach *et al.*, 1979) that contains the *ADE2* gene to create pYEE1116 and pYEE1118. pYBS94 is YIp5 containing FAR1 with a *Hin*dIII-*Hin*dIII deletion that removes nucleotides −170 to +740 where +1 is the FAR1 ATG. pYBS102 (FAR1 2μ) was cloned by suppression of the arrest defect of BY58. pYBS161 was created by digestion of the FAR1 *Bam*HI site in pYBS102 followed by a Klenow fill-in reaction and religation. pYBS165 contains the MYC sequence inserted into FAR1 by polymerase chain reaction (PCR) using FAR1 3′ sequence from pYBS102 and the following primers: 5′CGGAATTCTGGAACAAAAGCTTATTTCTGAAGAAGACTTGTAGTAGTT CGGGAATCGAGG, 5′-CGCGATCATGTCGACCACACC. A 4-kilobase *Eco*RI FAR1 fragment was then ligated to the PCR product into the *Eco*RI-*Sal*I sites of YEplac195. pYBS177 was made by inserting the FAR1 coding sequence into a derivative of pJG4-5 (pYBS139, which carries a *Bam*HI site in the appropriate reading frame) by PCR using the following primers: 5′-TGCAGATCTTTGATGCCCACATTTGGG, 5-ATAAGATCTCTAGA GGTT GGGAACT.

Oligonucleotide Mutagenesis

FUS3 was epitope tagged at sites predicted to be antigenic but not within an extended α-helical or β-sheet structure, using the PREDICT '89 computer program of B. Stroud (University of California, San Francisco). The 12CA5 epitope (YPYDVPDYA) (Field *et al.*, 1988) was inserted into FUS3 by oligonucleotide mutagenesis by the method of Kunkel (1985). Single-stranded DNA was prepared from *dut1 ung1* strain EB150 (gift of M. Christman, University of California, San Francisco) containing pYEE114 as follows: M13KO7 phage harboring the kanamycin gene was added to a final concentration of $\sim 8 \times 10^8$ pfu/ml to 2 ml of logarithmically growing cells (A_{600} of 0.5) grown in 2XYT, 0.001% vitamin B1, 50 μg/ml ampicillin. After incubating 75 min at 37°C, 3 ml prewarmed medium containing 70 μg/ml kanamycin was added and cells were shaken overnight at 37°C. Phage particles were pelleted by centrifugation after adding 1.1 ml 20% polyethylene glycol 6000, 2.5 M NaCl to 4.4 ml of culture supernatant and incubating 50 min on ice. The single-stranded DNA was precipitated after resuspending the phage particles in 0.5 ml 10 mM (Tris-HCl, 1 mM EDTA (TE) and extracting twice with phenol and chloroform. The conditions outlined in the BIORAD MUT A-GENE kit were then followed to anneal the mutagenic primer to the single-stranded DNA and synthesize the second strand with T4 DNA polymerase (New England Biolabs, Beverly, MA). The epitope was inserted after amino acid residues 1 (HA#1), 269 (HA#5), 291 (HA#6), 312 (HA#7), 322 (HA#8), and 352 (HA#2) using the following sequence for the epitope: TAC CCA TAC GAC GTC CCA GAC TAC GCT. Fifteen additional nucleotides homologous to FUS3 flanked each side of the epitope sequence.

Preparation of Anti-FUS3 Peptide Antisera

A synthetic peptide corresponding to residues 340–353 of FUS3 was coupled to keyhole limpet haemocyanin with maleimidobenzoyl-N-hydroxysuccinimide ester following manufacturer's instructions (Pierce Chemical, Rockford, IL). Rabbit antisera (raised by East Acres Biologicals, Southbridge, MA) was affinity purified to the same peptide coupled to rabbit albumin (Sigma, St. Louis, MO) that was covalently attached to AffiGel 10 as per manufacturer's instructions (Bio-Rad, Richmond, CA). The affinity purified antiserum recognizes a single ~40-kDa band in *FUS3* strains but not *fus3Δ* strains.

Preparation of Yeast Extracts

Yeast cells were grown to an A_{600} of 0.6–0.8 and induced for 1 h with α factor at a final concentration of 50 nM. Approximately 150 ml of cells were pelleted, washed once with ice water, and then frozen in dry ice/ethanol. Pellets were thawed on ice, and then resuspended in 0.5 ml of a modified version of H buffer (Booher *et al.*, 1989) containing 10% glycerol (25 mM tris(hydroxymethyl) aminomethane [Tris]-Cl pH 7.4, 15 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid [EGTA], 15 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.1% Triton-X-100, 1 mM NaN_3, 0.25 mM each meta- and ortho-vanadate, 0.1 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml each of pepstatin A, chymostatin, leupeptin, peptin). Cells were broken by adding acid-washed glass beads (Sigma) to the meniscus and vortexing five times in 30-s pulses. Fresh buffer (0.3 ml) was then added, and the samples were vortexed 30 s more. Cellular debris was removed by centrifugation at 3000 rpm for 10 min. The supernatant was slightly clarified by a second centrifugation at 11 000 × *g* for 10 min in a microcentrifuge. Samples were immediately aliquoted and frozen at −80°C. Protein concentrations were determined with the Bio-Rad protein assay.

Preparation of Immune Complexes and Kinase Assay

FUS3-HA was immunoprecipitated from yeast extracts as follows: 200 μg of extract was aliquoted to eppendorf tubes containing modified H buffer without glycerol, to a final volume of 0.5 ml. Fifteen microliters of 5 M NaCl and ~0.5 mg 12CA5 (ascites fluid provided by Harvard University antibody facility) were added, and the samples were incubated for 90 min on ice with occasional mixing. Aggregates were pelleted by a 10-min centrifugation in a microcentrifuge. Twenty-five microliters of protein A-sepharose (at 0.1 mg/ml in modified H buffer, Sigma or Pharmacia, Piscataway, NJ) was added to the supernatant, and samples were then rotated at 4°C for 30 min. Immune complexes were pelleted by a 15-s centrifugation at 1500 and washed four times with cold-modified H buffer. Samples to be analyzed by gel electrophoresis were resuspended in 40 μl Laemmli buffer (Sambrook *et al.*, 1989), heated for 5 min in a boiling water bath. Samples to be assayed for kinase activity were washed twice more with kinase buffer (50 mM Tris-Cl, pH 7.4, 20 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 0.5 mM sodium vanadate) and then resuspended in ~20 μl kinase buffer. Reactions were begun by the addition of 1 μl of 20 μM γ-[^{32}P]-ATP (10 μCi of 4500 Ci/mmol, ICN Biochemicals), incubated for 8 min at 30°C, and then stopped by the addition of 25 μl 2× Laemmli buffer. Samples were electrophoresed on sodium dodecyl sulfate (SDS) polyacrylamide gels. Gels were then soaked for a minimum of 1 h in 10% acetic acid, 10% methanol, 0.5% phosphoric acid, 10 mM KPO4 and then 30 min in 10% acetic acid, 10% methanol before being dried onto paper.

Immunoblotting

Unless noted, samples were electrophoresed by SDS polyacrylamide gel electrophoresis PAGE on 8–12% polyacrylamide gels (acrylamide: bis-acrylamide at a ratio of either 38:2 or 30:0.8). Approximately 25–50 μg of whole cell extract or immune complexes from 100 to 200 μg of protein extracts were analyzed. Proteins were transferred to nitrocellulose at 0.3 mA for 50 min following standard procedures. Immunoblots were incubated either overnight at 4°C or 2 h at room temperature with primary antibody (12CA5 culture supernatant, α-P-Tyr monoclonal antibody [gift of T. Roberts, Dana Farber Cancer

Institute], α-*Drosophila* tubulin monoclonal antibody [4A1] that recognizes yeast α and β tubulin [gift of L. Goldstein, Harvard University], Myc1-9E10 culture supernatant [gift of F. McKeon, Harvard Medical School]) in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST) containing either 5% nonfat milk (for both 12CA5 and α tubulin monoclonals) or 5% bovine serum albumin (for α-P-Tyr monoclonal). Immunoblots were then washed with TBST and incubated for 90 min with secondary antibody in TBST-5% milk (goat anti-mouse Ig-HRP) (Bio-Rad). In cases where background was a problem (Myc1-9E10 and α-P-Tyr monoclonal antibodies), the immunoblots were first incubated with rabbit anti-mouse whole Ig (Jackson Laboratories, Jackson Immune Research Laboratories, West Grove, PA). Immunoblots were developed with the Amersham (Arlington Heights, IL) ECL kit.

Isolation of High Copy Suppressors

Strain BY58 (*MATa sst1Δ fus3-2 KSS1 ura3-1*) was transformed with a *URA3* 2 μ high copy library (Carlson and Botstein, 1982) and plated at low density. Ura$^+$ transformants were then replica plated to selective plates with or without 5 μg α factor and scored for the absence of growth on the α factor plate. Candidates that passed retesting were retrieved in *E. coli* by the method of Hoffman and Winston (1987). *FAR1* was recovered three times out of ~40 000 transformants screened as shown by hybridization with a fragment of *FAR1* from plasmid pFC1 (Chang and Herskowitz, 1990) and subsequent restriction analysis. The details of this screen are to be published elsewhere (Satterberg and Elion, unpublished data).

α Factor Sensitivity, Mating, and β-Galactosidase Assays

α factor sensitivity was measured by a halo assay done as previously described (Elion *et al.*, 1990), using 50 μl of an overnight culture of yeast cells. All halo assays were performed in duplicate, using synthetic complete medium and 5 μl of 0.1 mM synthetic α factor in dimethyl sulfoxide (synthesized by Dr. C. Dahl, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA). Mating was measured by a qualitative patch mating test using a *MAT α lys9* strain as described (Elion *et al.*, 1990). Because *sst1Δ* mutants exhibit a severe mating defect (mating at ~10% of *SST1* levels), all *sst1Δ* strains were mated overnight at 30°C rather than for 4 h. β-galactosidase activity was measured in yeast extracts prepared by glass bead breakage (Craven *et al.*, 1965).

RESULTS

Isolation of FAR1 as a Multicopy Suppressor of Arrest Defective fus3 Mutants

One hypothesis that explains how FUS3 mediates G1 arrest is that it activates, by phosphorylation, a protein(s) that subsequently inhibits the G1 cyclins and/or CDC28 kinase. If this were the case, then overexpression of such downstream targets might bypass the requirement for FUS3 in G1 arrest. We therefore screened two yeast libraries for high copy clones that restore α factor sensitivity to an α factor–resistant *MATa fus3-2* strain with a partially functional FUS3 protein (see MATERIALS AND METHODS). The *FAR1* gene was identified three times out of 40 000 transformants tested as one of the most potent high copy suppressors. Overproduction of FAR1 efficiently restores α factor sensitivity to a *MATa fus3-2 KSS1* strain that is α factor resistant (Figure 1A). This suppression is not the consequence of a restored level of *FAR1* expression, because transcription of *FAR1* is normal in this strain (Elion *et al.*, 1990; Satterberg and Elion, unpublished data). FAR1 also suppresses a *fus3* null mutant, although less efficiently, showing that it can still function in the absence of FUS3 (Figure 1A). The reverse is not the case; overproduction of FUS3 does not suppress a *far1Δ* mutant. These results suggest FUS3 and FAR1 function in the same branch of the pheromone response pathway that inhibits multiple G1 cyclins and that FAR1 acts after FUS3.

A far1 Truncation Mutant Requires FUS3 to Promote G1 Arrest

To gain evidence of a possible dependence of FAR1 function on FUS3, we determined the ability of a *far1-589* mutant allele (frameshift mutation introduced at amino acid 588) to promote G1 arrest in strains lacking FUS3. Deletion of the carboxyl-terminus of FAR1 is not thought to affect its ability to promote G1 arrest, although it inhibits a mating function (Chang and Herskowitz, 1990). As expected, a *FUS3 far1Δ* strain harboring *far1-589* on a multicopy plasmid undergoes G1 arrest in the presence of α factor (Figure 1B). By contrast, a *fus3Δ far1Δ* double mutant carrying the same plasmid is α factor resistant, suggesting the truncated derivative of FAR1 requires FUS3 for function. The absence of suppression is not due to increased α factor resistance in the *fus3Δ far1Δ* double mutant, because this strain is not more α factor resistant than a *FUS3 far1Δ* single mutant. Moreover, far1-589 also functions poorly in a nearly wild-type strain containing FUS3-HA (Figure 1B), implying the two proteins fail to interact. These results are consistent with a model in which FAR1 promotes G1 arrest as a consequence of a direct interaction with FUS3.

FUS3 Interacts with FAR1 in a Two Hybrid System

The simplest interpretation of the genetic suppression tests is that FUS3 activates FAR1 by phosphorylation. If this is the case, then it should be possible to detect a physical interaction between the two proteins. Initial attempts to detect FAR1 in FUS3 immune complexes using a functional epitope-tagged derivative of FAR1 (FAR1-MYC, Figure 1B) were unsuccessful. We therefore used a variation of the two hybrid system of Fields and Song (1989) devised by Zervos *et al.* (1993) as a more sensitive way to test whether FUS3 and FAR1 physically interact. We note that this approach does not distinguish between an association that is direct or via an intermediary protein(s). In this assay, FUS3 was fused to a Lex A DNA-binding domain (residues 1–202) that is capable of dimerizing and binding to a Lex A operator within the promoter of a reporter β-galactosidase gene (Brent and Ptashne, 1985). FAR1 was fused to an acidic transcriptional activation domain that does not bind DNA (B42) (Ma

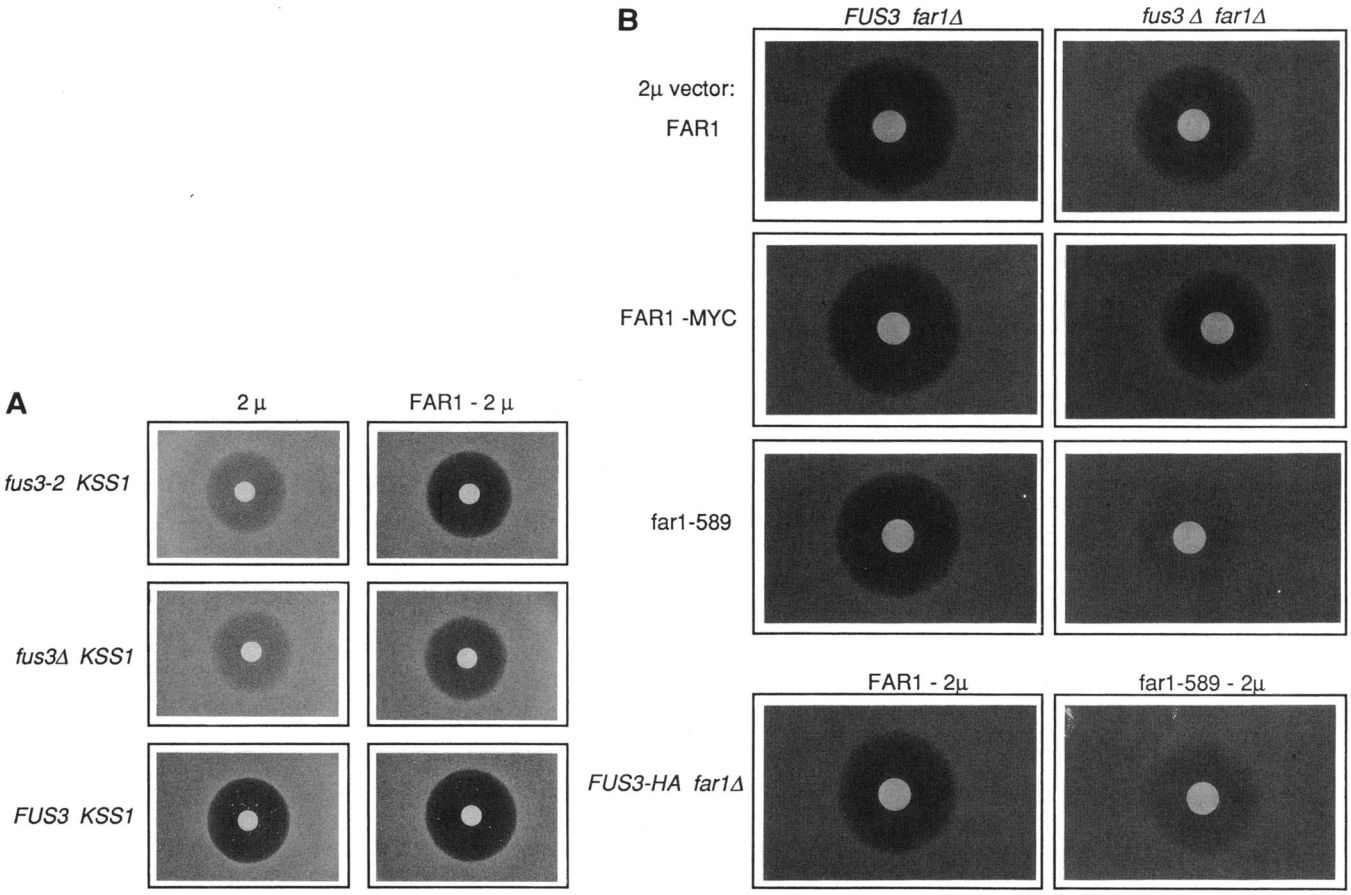

Figure 1. (A) Effect of *FAR1* overexpression on α factor sensitivity of *fus3* mutants. Lawns of *MATa sst1Δ* strains BY58, EY941, or EY957, bearing either YEp24 (URA3 2μ) or pYBS102 (FAR1 URA3 2μ) plasmids were tested for α factor sensitivity on solid media by a halo assay as described (Elion *et al.*, 1990). Strains were grown in SC media lacking uracil to select for the plasmids. The filter disks contain 5 μl of 0.1 mM α factor dissolved in dimethylsulfonate. Strains: *fus3-2 KSS1* (BY58), *fus3Δ KSS1* (EY941), *FUS3 KSS1* (EY957). (B) α factor sensitivity of strains containing *far1-589* on a multicopy plasmid. Halo assays were performed as described in A. Top six panels: Strains BY371 (*sst1Δ FUS3 far1Δ*) and BY380 (*sst1Δ fus3Δ far1Δ*) each bearing one of three multicopy (2μ) *URA3* plasmids: pYBS102 (*FAR1*), pYBS165 (FAR1-MYC), or pYBS161 (*far1-589*). Bottom two panels: Strain BY380 (*sst1Δ fus3Δ far1Δ* containing both pYEE1102 (*FUS3-HA#5 HIS3 CEN4*) and either pYBS102 or pYBS161. All halo assays were done twice on two transformants grown in selective medium.

and Ptashne, 1987). Little expression of β-galactosidase occurs in strains with either fusion protein (Table 2). By contrast, strains containing both fusion proteins exhibit a reproducible eightfold increase in β-galactosidase activity, a similar magnitude as that observed for an interaction between Mxi1 and Max proteins using the same two hybrid system (Zervos *et al.*, 1993). The increase in β-galactosidase activity requires both FUS3 and FAR1, because LEXA202-FUS3 in combination with either B42 or a B42-CDC28 fusion does not show this effect, nor does B42-FAR1 in combination with a LEXA202-Bicoid fusion. Thus, FAR1 and FUS3 are capable of physically associating in vivo, reinforcing the possibility that FAR1 is activated by a direct interaction with FUS3.

Construction of a Functional Epitope-tagged FUS3 That Is Regulated like Native FUS3

To determine whether FUS3 phosphorylates FAR1 or other proteins in the signal transduction cascade, we devised a kinase assay for FUS3. FUS3 was marked with a nine amino acid HA peptide (Field *et al.*, 1988) that is specifically recognized by monoclonal antibody 12CA5 (Green *et al.*, 1987). The HA peptide was inserted at the amino and carboxyl termini of FUS3 and at four internal sites (MATERIALS AND METHODS). Four FUS3-HA constructs complemented both *fus3Δ KSS1* and *fus3Δ kss1Δ* strains for G1 arrest and mating when present on a centromeric plasmid. A polypeptide of M_r ~ 42 kDa consistent with the predicted molecular weight of FUS3-HA was detected in these strains (Figure 2A). Only two of the functional FUS3-HA proteins

Table 2. Two hybrid FUS3 FAR1 interaction

Plasmids[a]	Units β-galactosidase activity[b]
1. LEXA202-FUS3-HIS3-2μ	10
2. LEXA202-FUS3-HIS3-2μ; B42-TRP1-2μ	15
3. B42-FAR1-TRP1-2μ	2
4. LEXA202-FUS3-HIS3-2μ; B42-FAR1-TRP1-2μ	98
5. LEXA202-FUS3-HIS3-2μ; B42-CDC28-TRP1-2μ	16
6. LEXA-BICOID-HIS3-2μ; B42-FAR1-TRP1-2μ	12

[a] Plasmids are maintained in strain BY704 that contains a β-galactosidase reporter gene with a LEXA-dependent UAS on a URA3 2μ plasmid (pJK103). LEXA202-FUS3 is constitutively expressed by the ADH promoter (pLEXFUS3). All B42 fusion proteins are expressed by the GAL1 promoter (pYBS177).

[b] Strains containing the various plasmids were grown selectively in SC-2% glucose medium to an A_{600} of 0.5, pelleted, and then grown for 6 h more in selective SC-2% galactose medium. Samples were then pelleted, washed with ice cold water, and frozen in dry ice/ethanol before preparing whole cell extracts and assaying for β-galactosidase activity as described (Craven *et al.*, 1965). Units represent millimole O-Nitrophenyl B-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram protein, calculated using the relationship $(OD_{420} \times 1.7/0.0045)$/time (min) × volume extract (ml) × mg/ml protein. Each number is the average of two experiments in which two independent plasmid-bearing transformants were assayed.

could be detected easily (#5 and #8). FUS3-HA#5 is present at levels essentially identical to that of wild-type FUS3 (Figure 2, B and C) and was therefore used in the subsequent studies. It will be referred to as FUS3-HA.

The abundance of FUS3-HA increases severalfold after a 1-h induction with α factor, consistent with both the pattern of FUS3 transcription (Elion *et al.*, 1990) and the abundance of native FUS3 protein (Figure 2C). Induction by α factor also increases the amount of tyrosine-phosphorylated FUS3-HA (Figure 3). Mutation of the conserved tyrosine residue 182 in FUS3-HA abolishes both the presence of phosphotyrosine and ability to complement a *fus3Δ*. By contrast, a nonfunctional catalytic mutant, fus3R42-HA, is still phosphorylated on tyrosine (Figure 3). These results confirm that FUS3-HA is regulated in a manner that parallels native FUS3 (Gartner *et al.*, 1992) and will provide a reliable tool to study FUS3 kinase activity.

FUS3-HA Immunoprecipitates Contain a Pheromone-dependent Kinase Activity That Phosphorylates a Spectrum of Polypeptides

An immune complex kinase assay was developed using FUS3-HA. FUS3 kinase activity is likely to be pheromone dependent, because it is required for activation of the pheromone response pathway (Elion *et al.*, 1990, 1991a) and is rapidly phosphorylated on tyrosine 182 and threonine 180 in response to α factor (Gartner *et al.*, 1992). Conditions were therefore sought in which FUS3-HA could support a FUS3- and α factor–dependent kinase activity (MATERIALS AND METHODS). Nondenaturing conditions similar to those of Booher *et al.* (1989) were used to allow optimal kinase activity and coimmunoprecipitation of polypeptides that physically associate with FUS3.

FUS3-HA immune complexes prepared from α factor–treated cells contain a striking kinase activity that phosphorylates ≥7–10 polypeptides of ~18–150 kDa present in the immune complex (indicated by the dots in Figure 4). Almost no activity is found in immune complexes prepared from non-α factor–treated cells. Importantly, all of the kinase activity requires FUS3-HA in the immune complex. No activity is detected in immune complexes prepared from FUS3 extracts that lack the 12CA5 epitope (Figure 4A, compare lanes 1 and 2 with lanes 3 and 4). Furthermore, the dramatic α factor–dependent stimulation of kinase activity parallels the increase in the amount of tyrosine-phosphorylated FUS3-HA present in the reaction (Figure 3), showing a correlation between kinase activity and FUS3 regulation. All of the FUS3-HA–associated kinase activity is still present in strains lacking KSS1 (Figure 4A, lanes 5, 6, 13, and 14), eliminating the possibility that KSS1 is a fortuitous protein kinase present in the assay.

FUS3 Function Is Required for Associated Kinase Activity

To determine whether the FUS3-HA–associated kinase activity is the result of the FUS3 kinase, we examined the effects of the arginine 42 and phenylalanine 182 mutations that block FUS3 function in vivo and are predicted to abolish FUS3 catalytic activity. As shown in Figure 4A, both mutants (fus3R42HA and fus3F182HA) are essentially inactive in vitro, making a compelling argument that the kinase activity we detect is due to FUS3. Longer exposure of the autoradiogram suggests a small amount of kinase activity is still present in immune complexes prepared from the phenylalanine 182 mutant (Figure 4A, lane 10). This activity could be due either to another protein kinase or residual activity in the mutants. However, the complete absence of kinase activity in the arginine 42 mutant implicates FUS3 as the protein kinase responsible for the measured kinase activity. This interpretation is supported by other experiments showing that the FUS3-HA–associated kinase activity copurifies with monomeric FUS3-HA in a glycerol gradient (Kranz and Elion, unpublished data). Taken together, these results argue strongly that FUS3 is the active kinase in the assay, although it is possible that FUS3 is activating another kinase. Therefore, for simplicity, we will henceforth refer to the FUS3-HA–associated kinase activity as FUS3 kinase activity.

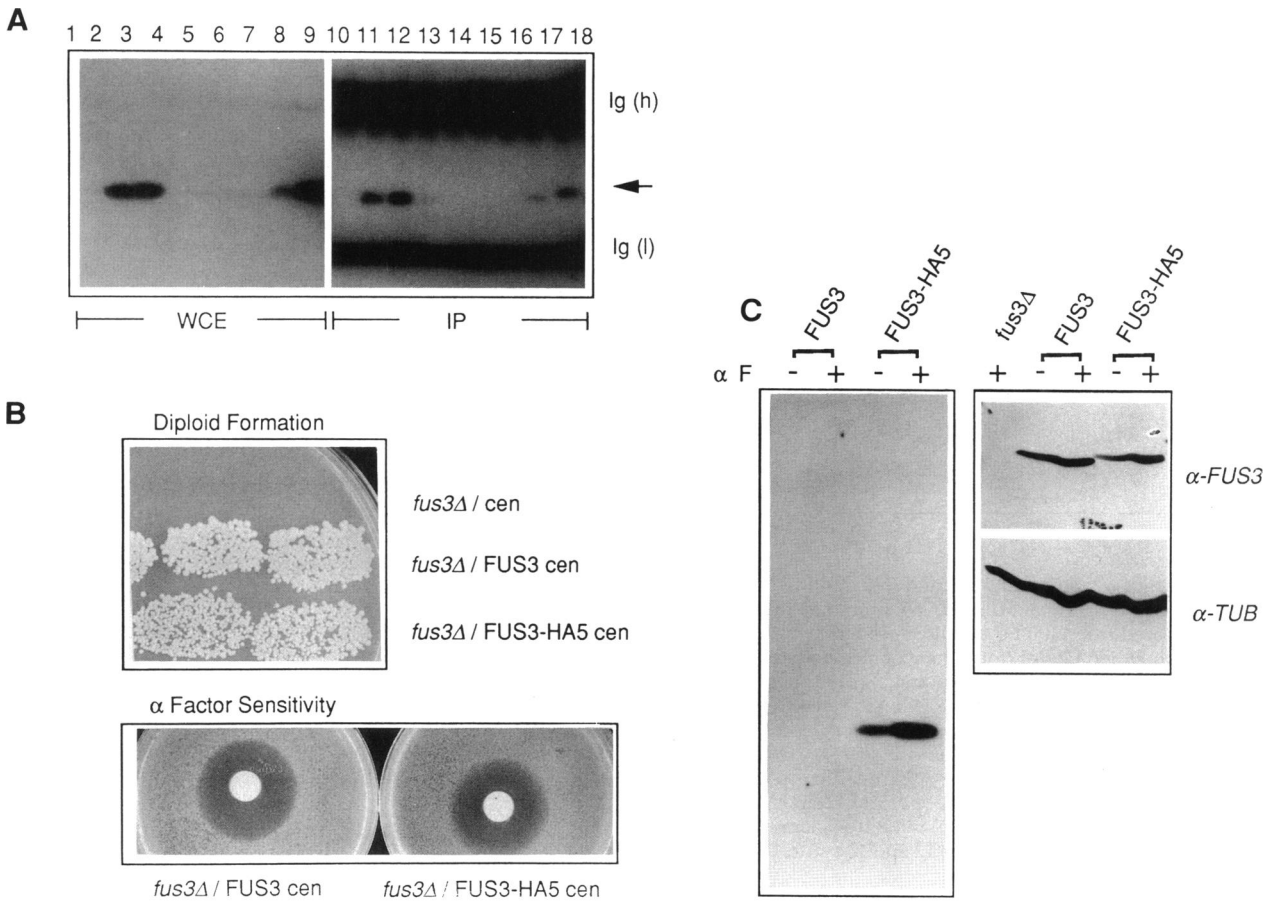

Figure 2. Analysis of epitope-tagged FUS3 protein. (A) Immunoblot of whole cell extracts and immune complexes prepared from a *MATa sst1Δ fus3-6::LEU2* strain containing epitope-tagged derivatives of FUS3 using monoclonal antibody 12CA5. The preparation of whole cell extracts and immune complexes are described in MATERIALS AND METHODS. Lanes 1–9 contain 50 μg extract; lanes 10–18 contain immune complexes from 200 μg of extract. Antibody binding was detected with [^{125}I]-protein A, which also binds 12CA5 heavy and light chains present in the immune complexes (indicated in lanes 10–18). Samples: Lanes 1 and 10 = FUS3 (pYEE114), lanes 2, 3, 11, and 12 = FUS3-HA#5 (pYEE121), lanes 4 and 13 = FUS3-HA#1 (pYEE119), lanes 5, 6, 14, and 15 = FUS3-HA#2 (pYEE120), lanes 7 and 16 = FUS3-HA#6 (pYEE122), lanes 8 and 17 = FUS3-HA#7 (pYEE123), lanes 9 and 18 = FUS3-HA#8 (pYEE124). Strain (EY940). (B) Complementation of mating and arrest defects of a *MATa sst1Δ fus3Δ kss1Δ* strain (EY966) by FUS3-HA#5. Patches of EY966 containing either YCp50, pYEE114, or pYEE121 were pregrown on SC plates lacking uracil overnight at 30°C, mated to a lawn of opposite mating type for 6 h at 30°C, and then replicated onto medium on which only diploids could grow. α factor sensitivity was measured by the halo assay described in Figure 1. (C) Detection of FUS3 and FUS3-HA#5 proteins in uninduced and α factor–treated cells. Left, Immunoblot containing 25 μg of whole cell extracts prepared from strain EY940 containing either pYEE114 or pYEE121 probed with 12CA5. Right, Immunoblots of 50 μg extract prepared from strain EY941 containing either FUS3 or FUS3-HA on multicopy plasmids (pYEE92 and pYEE1112) probed with either FUS3 antiserum or a monoclonal antibody to tubulin. + indicates cells were induced for 1 h with α factor. Antibody binding was detected using goat anti mouse Ig-HRP (Bio-Rad) and a chemiluminescence system (Amersham).

FUS3 Kinase Activity Phosphorylates a MAP Kinase Substrate and Increases with FUS3 Dosage

The substrate specificity of the FUS3-HA–associated kinase activity overlaps that of MAP kinases, a further argument that the in vitro activity is due to FUS3. FUS3-HA immune complexes phosphorylate myelin basic protein (MBP), a preferred MAP kinase substrate, at an equivalent concentration of approximately 50 μM (Figure 4C). The small amount of MBP phosphorylation detected with the nonepitope-tagged FUS3 control (Figure 4C, lane 1) reflects a residual amount of kinase activity present in the MBP preparation (see Figure 5A). MBP is not phosphorylated by the arginine 42 mutant (Figure 4C, lane 2) as seen for the associated substrates. In addition, as found for MAP kinases (Cobb *et al.*, 1991), FUS3-HA immune complexes do not phosphorylate histone H1, H2, H3, or H4, ruling out the possibility that the activity is due to CDC28 kinase.

The amount of FUS3-HA kinase activity parallels the amount of FUS3-HA as expected. Immune complexes prepared from cells harboring FUS3-HA on a multicopy plasmid contain more kinase activity, as measured both by an increase in the amount of radioactivity incorporated into the endogenous substrates and an increase

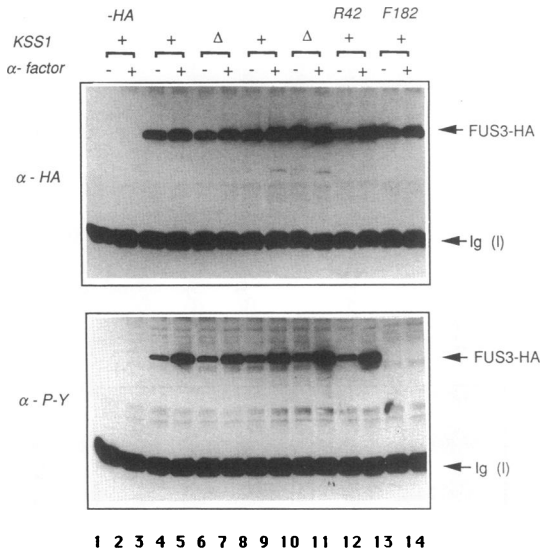

Figure 3. Effect of α factor treatment and R42 and F182 mutations on tyrosine phosphorylation of FUS3-HA#5. Immunoblot of immune complexes prepared from 200 μg of whole-cell extract using 12CA5 and probed with either 12CA5 (top) or anti-phosphotyrosine monoclonal antibody (bottom). Antibody binding was detected with goat anti-mouse Ig-HRP and the Amersham ECL kit. Cells were induced for 1 h with α factor before extract preparation where indicated (+). Lanes 1 and 2 = FUS3 (pYEE114), lanes 3 and 4 = FUS3-HA#5 (pYEE121) in EY940, lanes 5 and 6 = FUS3-HA#5 (pYEE121) in EY966, lanes 7 and 8 = FUS3-HA#5 2μ (pYEE1112) in EY941, lanes 9 and 10 = FUS3-HA#5 2μ (pYEE1112) in EY1040, lanes 11 and 12 = fus3R42-HA#5 (pYEE127), and lanes 12 and 13 = fus3F182-HA#5 (pYEE128) in EY940.

in the amount of phosphorylation of MBP (Figure 3, A and C).

FUS3 Is Likely to Be the ~42-kDa Substrate

FUS3 might be expected to autophosphorylate in vitro, because MAP kinases weakly autophosphorylate on the equivalent of tyrosine 182 (Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991). Several observations suggested that the 42-kDa substrate (Figure 4) is an autophosphorylated form of FUS3-HA: it is the expected size for FUS3-HA: it is absent from both fus3R42-HA and fus3F182-HA immune complexes, and it copurifies with FUS3-HA in a glycerol gradient (Kranz and Elion, unpublished data). If the 42-kDa substrate is an autophosphorylated form of FUS3-HA, it should contain phosphotyrosine. To test this possibility, a gel containing the products of a FUS3-HA kinase assay was treated with 1 M KOH under conditions that hydrolyze serine/threonine- but not tyrosine-phosphoester linkages in MAP kinase (Ray and Sturgill, 1988). Almost all of the incorporated ^{32}P disappears after this treatment with the exception of ^{32}P incorporated into p42 (Figure 4B). Because FUS3 is the only protein of this size that is phosphorylated on tyrosine in α factor–treated cells (Ballard *et al.*, 1991), this result suggests both that p42 is FUS3-HA and that FUS3 autophosphorylates on tyrosine 182 (Figure 4). Moreover, because the vast majority of ^{32}P incorporated into all other substrates is base labile, FUS3 has a preferred specificity for serine and threonine residues, consistent with the properties of MAP kinases (Cobb *et al.*, 1991; Pelech and Sanghera, 1992).

Effects of FUS3-HA Dosage, Salt, and Exogenous Substrates

Two observations suggest that the substrates in the kinase assay associate with FUS3-HA in vivo. First, increasing the in vivo level of FUS3-HA in yeast cells changes the pattern of phosphorylated proteins in vitro (Figure 4A). When FUS3-HA is maintained on a multicopy plasmid, additional proteins are phosphorylated, and several substrates prominent in extracts prepared from cells with low copy FUS3-HA are less apparent. This finding suggests the substrates are binding to FUS3-HA rather than to either immunoglobulin or protein A-sepharose. Second, the profile of substrates is unchanged after immunoprecipitating in relatively high concentrations of salt (e.g., 0.5 M NaCl, Figure 5A). Thus, the substrates may be coprecipitating via an interaction with either FUS3-HA or a protein associated with FUS3-HA.

The coprecipitated substrates are also phosphorylated more efficiently than two nonphysiological substrates, myelin basic protein (MBP) and casein. Although both MBP and casein are readily phosphorylated by FUS3-HA when present at ~50-μM concentrations, a 10- to 100-fold dilution of either protein diminishes their ability to be phosphorylated (Figure 5B). By contrast, the coprecipitated substrates are present in far lower concentrations than FUS3-HA as judged by the inability to detect them in FUS3-HA immune complexes from ^{35}S-methionine-labeled cells. Because the concentration of FUS3-HA is ≤0.1 μM (e.g., the concentration of immunoglobulin), the substrates are probably present at ≤0.01 μM. Thus, FUS3 prefers the associated substrates, suggesting that they are relevant.

STE12 Overproduction Alters the Pattern of Substrates

Overproduction of STE12 causes increased expression of many mating-specific genes (Dolan and Fields, 1990). STE12 was therefore overproduced to determine whether any of the FUS3 substrates are under its control. Induction of a *GAL1-STE12* gene (*STE12-688M*) (Errede and Ammerer, 1989) by growth in galactose causes a time-dependent alteration in the profile of proteins phosphorylated by FUS3. A 4-h induction both increases the amount of a ~80-kDa phosphoprotein and decreases the amount of two ~65-kDa phosphoproteins (Figure 6A, lanes 2 and 6, bands b and c). More FUS3

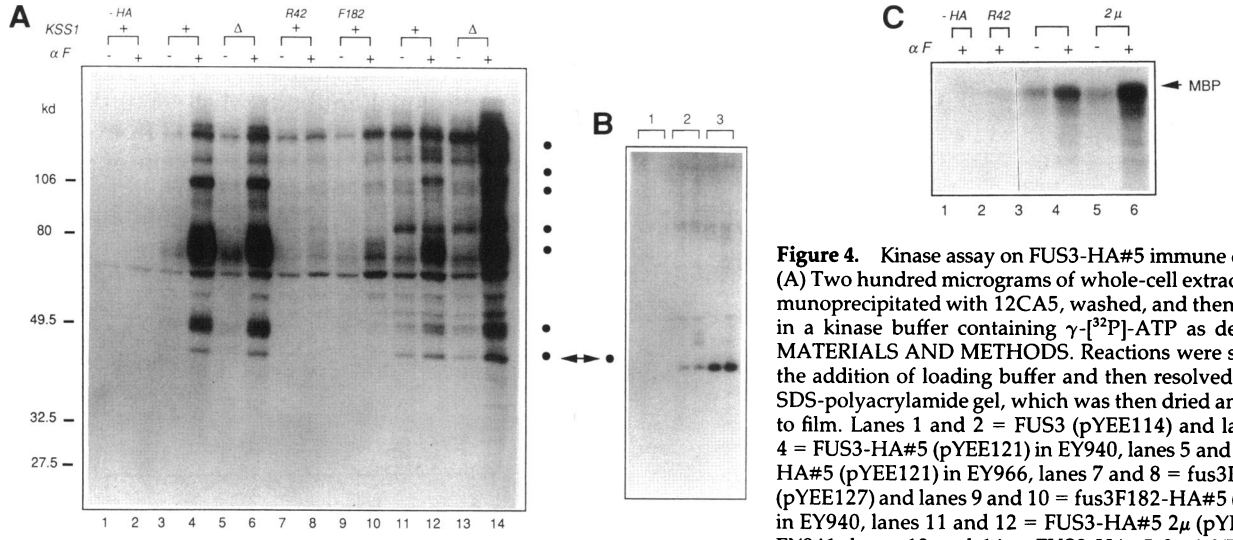

Figure 4. Kinase assay on FUS3-HA#5 immune complexes. (A) Two hundred micrograms of whole-cell extract were immunoprecipitated with 12CA5, washed, and then incubated in a kinase buffer containing γ-[^{32}P]-ATP as described in MATERIALS AND METHODS. Reactions were stopped by the addition of loading buffer and then resolved on an 8% SDS-polyacrylamide gel, which was then dried and exposed to film. Lanes 1 and 2 = FUS3 (pYEE114) and lanes 3 and 4 = FUS3-HA#5 (pYEE121) in EY940, lanes 5 and 6 = FUS3-HA#5 (pYEE121) in EY966, lanes 7 and 8 = fus3R42-HA#5 (pYEE127) and lanes 9 and 10 = fus3F182-HA#5 (pYEE128) in EY940, lanes 11 and 12 = FUS3-HA#5 2μ (pYEE1112) in EY941, lanes 13 and 14 = FUS3-HA#5 2μ (pYEE1112) in EY1040. Cells were induced for 1 h with α factor before extract preparation where indicated (+). (B) Effect of KOH treatment. Duplicate kinase reactions were performed exactly as described in A except that the gel was incubated for 2 h at 55°C in 1 M KOH before being dried and subjected to autoradiography. Lane 1 = FUS3 = pYEE114, lane 2 = FUS3-HA#5 = pYEE121 in EY940, and lane 3 = FUS3-HA#5 2μ = pYEE1112 in EY941. All samples were from α factor–treated cells. (C) Phosphorylation of MBP by FUS3-HA immune complexes. Kinase reactions were performed as in A except that the reactions contained 1 μg/ml MBP and were separated on a 12% gel. Lane 1 = FUS3 (pYEE114) and lane 2 = fus3R42-HA#5 in EY940, lanes 3 and 4 = FUS3-HA#5 (pYEE114) and lanes 5 and 6 = FUS3-HA#5 2μ (pYEE1112) in EY1040.

kinase activity is detected in the non-α factor–treated cells when STE12 is expressed for 6 h, consistent with increased activation of the signal transduction cascade (Dolan and Fields, 1990). By contrast, little phosphorylation of the p65-p80 proteins is detected in the α factor–treated cells (Figure 6A, lanes 3 and 4). The fact that in vivo levels of STE12 affect the profile of substrates in vitro strongly suggests that several of the substrates are components in the signal transduction cascade. It is curious that STE12 overproduction leads to the disappearance of certain substrates. This could be due to their prior phosphorylation by FUS3 or the expression of a phosphatase of restricted activity.

STE7 and STE11 Are Required for FUS3 Kinase Activity and Several Substrates

We examined whether mutation of either STE7 or STE11 protein kinases affects FUS3 kinase activity. Null mutations in STE4 (G_β), STE5, STE7, and STE11 block equally the phosphorylation of FUS3 in vivo (Gartner *et al.*, 1992). However, because STE12-dependent transcription of many signal transduction components requires an intact signal transduction cascade, these *ste* mutations can affect FUS3 activity indirectly. STE12 was therefore overproduced in *fus3*Δ *ste*Δ double mutants harboring FUS3-HA to bypass each *ste* block in transcription. Under these conditions, deletion of either *STE7* or *STE11* drastically reduces FUS3-HA kinase activity, as shown by the decrease in the intensities of the phosphorylated proteins (Figure 6A). Because this decrease is not due to differences in the amount of FUS3-HA precipitated (Figure 6B), it is likely to be due to an effect on kinase activity.

STE7 and STE11 do not contribute equally to optimal FUS3 kinase activity. *ste11*Δ mutants have considerably more kinase activity than do *ste7*Δ mutants (compare lanes 6 and 8, Figure 6A), suggesting that STE7 plays a more critical role in regulating FUS3 kinase activity in vivo. This finding is consistent with the potential role of STE7 as a direct activator of FUS3 (Crews and Erickson, 1992; Gartner *et al.*, 1992). If STE7 is solely responsible for activating FUS3, then the residual amount of kinase activity in *ste7*Δ strains may be the consequence of autoactivation, because the activity depends on FUS3. Furthermore, the fact that *ste11*Δ strains still contain a fairly significant amount of α factor–inducible kinase activity suggests that FUS3 can be activated by α factor in the absence of STE11.

STE7 and STE11 are also critical for the presence of the most prominent substrates in the profile of ~65–80 kDa (Figure 6A, b and c). Whereas three less prominent phosphoproteins (Figure 6A, a, d, and e) are detected in both *ste7*Δ and *ste11*Δ mutants, the p80 phosphoprotein is not detected in the *ste11*Δ mutant; neither p65 nor p80 phosphoproteins are detected in the *ste7*Δ mutant. The fact that specific substrates disappear from strains with mutations in STE7 and STE11 provides further evidence that the coprecipitated substrates are physiologically relevent. Moreover, it is tempting to speculate that p65 and p80 substrates are phosphorylated forms of STE7 and STE11 (respectively), because their sizes are similar to the phosphorylated forms of

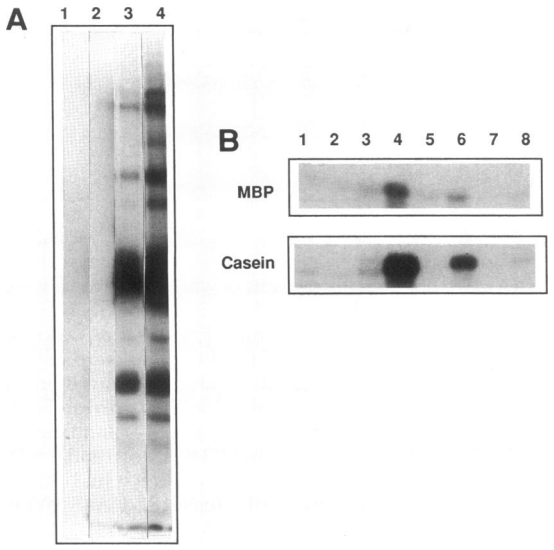

Figure 5. Effect of NaCl on associated substrates and concentration dependence of MBP and casein phosphorylation. (A) Kinase assays were performed as in Figure 4 except that 0.5 M NaCl was present during the immunoprecipitation with 12CA5, and samples were washed two extra times with kinase buffer before incubating with ATP. All samples were from α factor–treated cells. Lane 1: FUS3 extracts (pYEE114); lane 2: fus3R42-HA#5 (pYEE127) extracts; lane 3: FUS3-HA#5 (pYEE121) extracts; lane 4: same as lane 3 except immunoprecipitation was done in 0.15 M NaCl. (B) Kinase assays were performed as in Figure 4 except that immune complexes were incubated with either MBP or casein during incubation with ATP. Lane 1 contains no extract. Lane 2 contains immune complexes from a strain containing untagged FUS3 (pYEE114). Lanes 3–8 contain FUS3-HA#5 (pYEE121). Lanes 3 and 4 = 500 μg/ml MBP or casein, lanes 5 and 6 = 50 μg/ml MBP or casein, lanes 7 and 8 = 5 μg/ml MBP or casein. Lanes 3, 5, and 7 contain extracts from uninduced cells; lanes 4, 6, and 8 contain extracts from cells treated 1 h with α factor.

STE7 and STE11 (Cairns *et al.*, 1992; Rhodes *et al.*, 1990), although other interpretations are possible.

Evidence That STE12 Is Phosphorylated by FUS3

FUS3 is required for optimal STE12 activity in vivo, suggesting that it may activate STE12 by phosphorylation (Elion *et al.*, 1991a,b). Three observations suggest that the ~106-kDa substrate in FUS3-HA immune complexes is STE12: 1) phosphorylated forms of STE12 migrate on SDS-polyacrylamide gels with an apparent molecular weight of ~106 kDa; 2) overexpression of STE12 leads to an increase in the relative amount of p106 that is phosphorylated by FUS3 (Figure 6A, lanes 3 and 4); and 3) FUS3-HA immune complexes prepared from a *ste12* null mutant lack solely the ~106-kDa substrate found in identical *STE12* immune complexes (Figure 7A).

An alternative explanation for the absence of the p106 substrate in *ste12*Δ strains is that STE12 is needed to transcribe the gene encoding p106. FUS3-HA immune complexes were therefore examined for the presence of STE12 protein. The *GAL1-STE12-688* gene contains a 10 amino acid peptide from human c-*myc* at amino acid position 668 (Errede and Ammerer, 1989) and is recognized by monoclonal antibody Mycl-9E10 (Evan *et al.*, 1985). FUS3-HA immune complexes from strains that express STE12-MYC contain a protein of the expected molecular weight that is recognized by Myc1-9E10. The presence of STE12-MYC in these immune complexes must be due to an association between STE12-MYC and FUS3-HA, because STE12-MYC is not detected in immune complexes from strains containing wild-type FUS3, which lacks the 12CA5 epitope (Figure 7B, compare lanes 1 and 2 with 4–6). STE12-MYC is

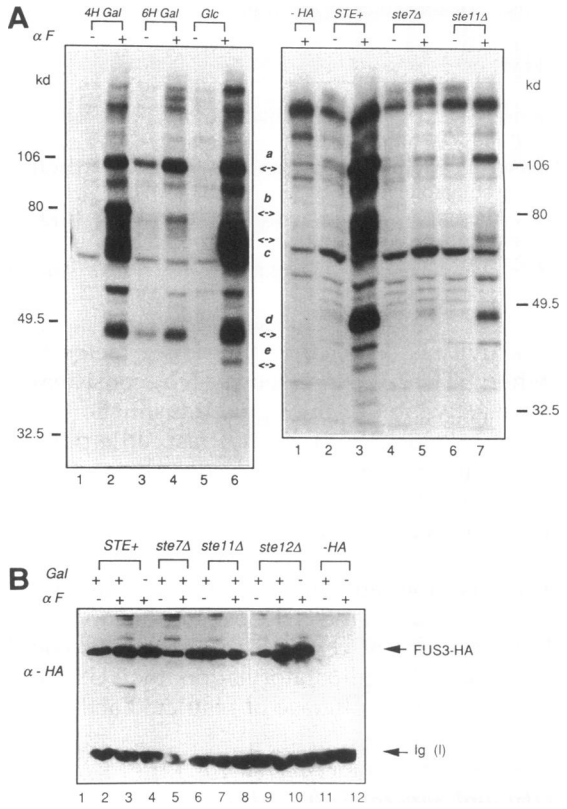

Figure 6. Effect of STE12 overproduction and mutations in STE7 and STE11 on FUS3-HA#5-associated kinase activity. (A) Left: Lanes 1–4 = strain EY966 (*MATa sst1*Δ *fus3*Δ *kss1*Δ) bearing both pYEE121 (*FUS3-HA#5 URA3 CEN4*) and pYEE117 (*GAL1-STE12-MYC HIS3 CEN4*) grown in selective medium containing galactose for 4 or 6 h before extract preparation. Cells were treated for 1 h with α factor where indicated. Lanes 5 and 6 = same strain as in lanes 1–4 grown in selective medium containing glucose before α factor treatment. Right: Strains EY966 (*fus3*Δ *kss1*Δ) EY948 (*ste7*Δ *fus3*Δ), and EY949 (*ste11*Δ *fus3*Δ) bearing pYEE117 (GAL1-STE12-MYC) and either pYEE114 (FUS3) or pYEE121 (FUS3-HA#5) were grown in selective medium containing galactose for 4 h before a 1-h α factor induction where indicated (+). Lane 1 = *FUS3 kss1*Δ, lanes 2 and 3 = *FUS3-HA#5 kss1*Δ, lanes 4 and 5 = *FUS3-HA#5 ste7*Δ, Lanes 6 and 7 = *FUS3-HA#5 ste11*Δ. The arrows (↔) indicate the positions of FUS3- and α factor-dependent phosphoproteins in both panels. (B) Immunoblot of FUS3-HA#5 immune complexes from strains used in Figures 5 and 6, done as described in Figure 3.

detected in FUS3-HA immune complexes from α factor–treated cells after 4 h of galactose induction and in immune complexes prepared from both α factor–treated and nontreated cells after 6 h of induction. Thus, the ability to detect STE12-MYC correlates with the increase in FUS3 kinase activity and the relative amount of the 106-kDa substrate (Figure 6A). Together these data suggest both that STE12 is a target of the FUS3 kinase and that the association of STE12 with FUS3 in vivo depends on an active FUS3 kinase.

Evidence That FAR1 Is Phosphorylated by FUS3

If FAR1 is phosphorylated by FUS3 in vivo, then it should be an efficient substrate in the FUS3 kinase assay. We therefore determined whether exogenously added FAR1 could be phosphorylated by FUS3 in vitro, using the B42-FAR1 fusion protein that physically associates with FUS3 in vivo (Table 2). The FAR1 fusion protein was expressed in vegetatively growing cells and immunoprecipitated from whole cell extracts under stringent conditions, using the 12CA5 antibody that recognizes a HA epitope inserted between the junctions of B42 and FAR1. Under these conditions, the fusion protein is very weakly phosphorylated by an associated kinase activity that is independent of pheromone (Figure 8A, lane 8). By contrast, the FAR1 fusion protein is dramatically hyperphosphorylated when incubated with FUS3 immune complexes from α factor–treated cells, as shown by the appearance of a broad band >106 kDa in the substrate profile (Figure 8A, compare lanes 5 and 6 with lane 7). The hyperphosphorylation requires catalytically active FUS3 (Figure 8A, lanes 12 and 15) and is independent of KSS1 (Figure 8A, lane 14). In addition, it is specific to residues in FAR1, because other B42 fusion proteins are not phosphorylated in this assay. Thus, as expected for a FUS3 substrate, FAR1 is readily phosphorylated in vitro under conditions in which FUS3 is catalytically active.

We also examined whether FAR1 was among the proteins phosphorylated in FUS3-HA immune complexes. FUS3-HA immune complexes prepared from *FAR1* and *far1*Δ strains show no obvious difference in the profile of associated substrates. By contrast, strains containing the *FAR1* multicopy plasmid reveal a novel substrate of ~110 kDa (Figure 8B, indicated by arrow). It is unlikely that the effect of FAR1 on the substrate profile would be indirect, because FAR1 is not involved in either signal transduction or gene expression (Chang and Herskowitz, 1990). Therefore, this substrate is likely to be FAR1, because its apparent molecular weight is similar to the hyperphosphorylated form of FAR1 that accumulates in α factor–treated cells (Chang and Herskowitz, 1992).

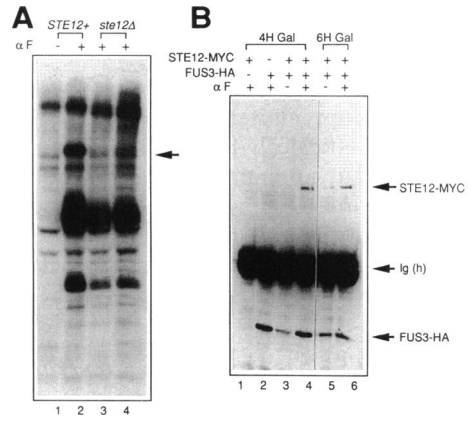

Figure 7. Effect of a deletion in STE12 on FUS3-HA#5–associated kinase activity and detection of STE12-MYC in FUS3-HA#5 immune complexes. (A) Kinase assays performed on extracts from *STE12+* (EY940, lanes 1 and 2) and *ste12*Δ (EY998, lanes 3 and 4) strains containing FUS3-HA#5 (pYEE121) as described in Figure 4. Two independent *ste12*Δ *FUS3-HA#5* transformants were assayed in lanes 3 and 4. + indicates 1-h induction with α factor. The arrow indicates the position of the ~106-kDa phosphoprotein found in *STE12*$^+$ extracts. (B) Immunoblot analysis of STE12-MYC in FUS3-HA#5 immune complexes using Myc1-9E10. The immunoblot was first probed with Myc1-9E10 to detect STE12-MYC and then with 12CA5 to detect FUS3-HA#5. The secondary antibody (goat anti-mouse Ig-HRP) binds to the 12CA5 Ig present in the immune complexes. FUS3 (pYEE114) and FUS3-HA#5 (pYEE121) were maintained in a *STE12*$^+$ (EY966) strain containing GAL1-STE12-MYC (pYEE117) as indicated. Cells were grown either in glucose medium or in galactose medium for 4 or 6 h before a 1-h α factor induction. Lane 1 = FUS3 grown in galactose. Lane 2 = FUS3-HA#5 grown in glucose. Lanes 4–6 = FUS3-HA#5 grown in galactose.

DISCUSSION

A FUS3 Kinase Assay That Faithfully Reflects FUS3 Function In Vivo

The experiments presented demonstrate a FUS3- and α factor–dependent kinase activity that phosphorylates many proteins that coimmunoprecipitate with FUS3. The dependence of kinase activity on two amino acid residues critical for FUS3 function in vivo (lysine 42 and tyrosine 182, Figure 4) implicates FUS3 as the active kinase in the assay. Moreover, the kinase activity is remarkably similar to that of MAP kinases, which are homologous to FUS3 (Cobb *et al.*, 1991; Pelech and Sanghera, 1992). Kinase activity correlates with phosphorylation of tyrosine 182 in response to an extracellular signal (α factor), whereas deletion of STE7, predicted to phosphorylate FUS3 at this residue (Crews and Erickson, 1992; Gartner *et al.*, 1992), causes the most profound decrease in kinase activity (Figures 3 and 5). Like MAP kinases, FUS3 appears to autophosphorylate on tyrosine and to phosphorylate MBP but not histones. Finally, the requirement of prior α factor stimulation and intact STE7 and STE11 shows that the assay responds to biological signals predicted to affect FUS3 function. Thus, the activity is more than likely due to FUS3 kinase, and the assay will be a reliable tool to study both the targets and regulation of FUS3 kinase.

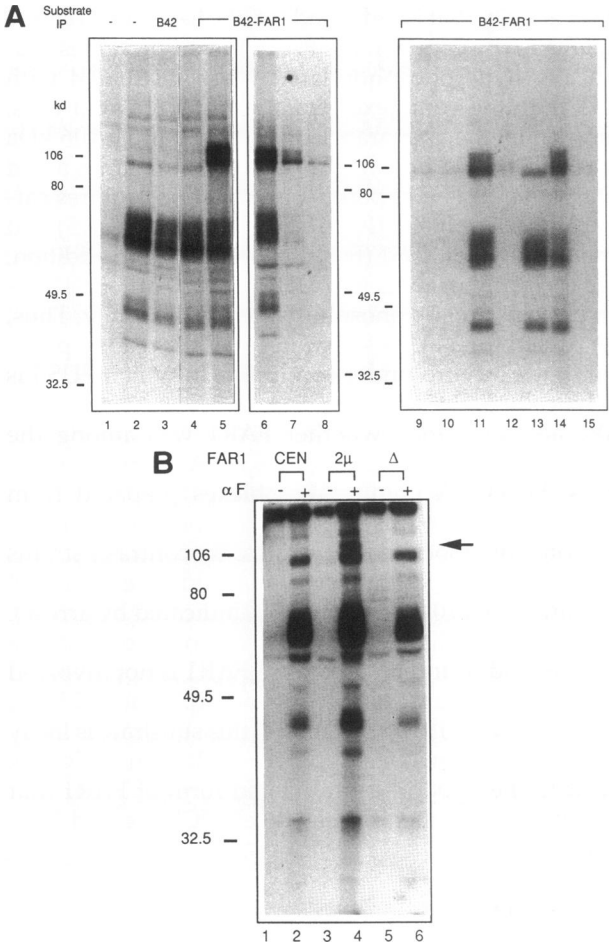

Figure 8. (A) Phosphorylation of an immunoprecipitated FAR1 fusion protein by FUS3-HA#5 immune complexes. Kinase assays were performed on B42-FAR1 and B42 immune complexes in the presence or absence of FUS3-HA#5 immune complexes as described in Figure 4. FUS3-HA#5 and fus3R42-HA#5 were prepared from cells induced for 1 h with α factor (unless noted otherwise by the − symbol below) and immunoprecipitated with 12CA5 in nonstringent modified H buffer (MATERIALS AND METHODS). B42-FAR1 (and B42) was expressed by growing cells in inducing medium containing galactose (unless noted as noninducing glucose medium) before extract preparation and immunoprecipitated with 12CA5 in more stringent RIPA buffer (Sambrook *et al.*, 1989). B42-FAR1 was prepared from both *FUS3 KSS1* (lanes 1–8) and *fus3Δ kss1Δ* (lanes 9–15) strains. FUS3-HA#5 and fus3R42-HA#5 were prepared from both *KSS1* (lanes 9–12) and *kss1Δ* (lanes 5–7) strains. Results are shown from three separate experiments, as indicated by the boxed-in areas. Lane 1: FUS3; lane 2: FUS3-HA#5; lane 3: B42 + FUS3-HA#5; lane 4: B42-FAR1 glucose + FUS3-HA#5; lanes 5 and 6: B42-FAR1 + FUS3-HA#5; lane 7: B42-FAR1 + FUS3-HA#5 (−); lane 8: B42-FAR1; lane 9: B42-FAR1; lane 10: B42-FAR1 + FUS3-HA#5 (−); lane 11: B42-FAR1 + FUS3-HA#5; lane 12: B42-FAR1 + fus3R42-HA#5; lane 13: B42-FAR1 (glucose) + FUS3-HA#5 (*kss1Δ*); lane 14: B42-FAR1 + FUS3-HA#5 (*kss1Δ*); lane 15: B42-FAR1 + fus3R42-HA#5 (*kss1Δ*). (B) FAR1 overproduction in vivo produces a new FUS3-HA#5–associated substrate. Kinase assays were performed on FUS3-HA#5 immune complexes as described in Figure 4. All strains contained FUS3-HA#5 (pYEE1102) and were treated with α factor for 1 h where indicated (+). Lanes 1 and 2 = *fus3Δ FAR1* (EY940) + YCp50, lanes 3 and 4 = *fus3Δ FAR1* (EY940) + FAR1 2μ (pYBS102), lanes 5 and 6 = *fus3Δ far1Δ* (EY1014). The ~110-kd phosphoprotein indicated by the arrow.

The Effects of STE7 and STE11 on FUS3 Kinase Activity Agree with Their Order in the Signal Transduction Cascade

The fact that STE7 is more critical than STE11 for FUS3 kinase activity in vitro implicates STE7 as the kinase activator of FUS3. Although genetic evidence suggested that STE11 acts before both STE7 and FUS3 (Cairns *et al.*, 1992; Stevenson *et al.*, 1992), it is not known whether STE7 or STE11 is the activator of FUS3 (Cairns *et al.*, 1992; Gartner *et al.*, 1992; Stevenson *et al.*, 1992). Our findings are consistent with a linear model in which the kinases act in the order STE11 → STE7 → FUS3 to activate FUS3 (Figure 9). Moreover, the detection of FUS3 kinase activity in *ste11* mutants suggests that an additional STE11-independent mechanism may also lead to activation of FUS3.

Many of the Substrates Associated with FUS3 Are Physiologically Relevant

It is striking that FUS3 phosphorylates so many associated proteins in a manner that depends on all of the signals that regulate its activity (Figures 3–8). The fact that the pattern of proteins phosphorylated by FUS3 in vitro are affected by both overexpression and underexpression of known components of the mating signal transduction cascade in vivo (e.g., FUS3, STE12, FAR1, STE7, STE11, STE12; Figures 3–8) provides compelling evidence that these substrates are physiologically relevant. This point of view is strengthened by the observation that the coprecipitated substrates are utilized ≥1000-fold more efficiently than MBP (Figure 5B). MBP is phosphorylated at comparable concentrations by both FUS3 and MAP kinases, suggesting it is an equally good substrate for these enzymes (Boulton *et al.*, 1991). Thus, many of the substrates that coprecipitate with FUS3 in vitro are likely to be its substrates in vivo.

Evidence That STE12 Is One of the Substrates That Coprecipitate with FUS3

Several lines of evidence suggest that the ~106-kDa substrate associated with FUS3 in vitro is STE12 (Figure 9). Deletion of STE12 causes the disappearance of the ~106-kDa substrate in the FUS3 kinase assay, whereas its overproduction increases the relative amount of this substrate (Figures 5 and 6). The size of this substrate matches that of hyperphosphorylated STE12 found in α factor–treated cells (Song *et al.*, 1991). Moreover, STE12 is present in enzymatically active FUS3 immune complexes in amounts that correlate with the in vivo amount of STE12 (Figure 7). Although these data do not demonstrate that FUS3 directly phosphorylates STE12, they do argue that STE12 is the ~106-kDa substrate associated with catalytically active FUS3.

Previous work is consistent with FUS3 directly activating STE12. FUS3 is essential for optimal STE12 ac-

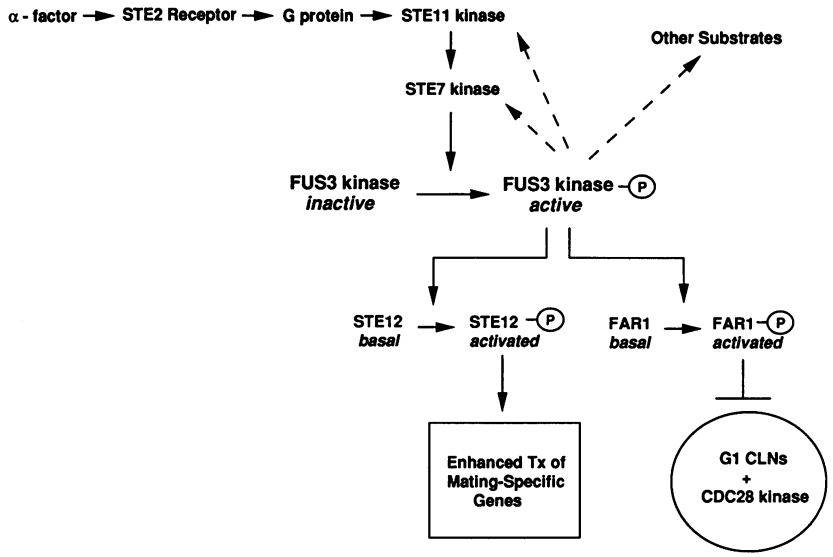

Figure 9. Model for FUS3 function. In the presence of α factor, FUS3 kinase is converted to an enzymatically active form by STE7 kinase, by phosphorylation of residues threonine 180 and tyrosine 182 in FUS3 (Gartner *et al.*, 1992). Activated FUS3 phosphorylates a number of substrates including STE12 and FAR1. Phosphorylation of STE12 and FAR1 by FUS3 converts both proteins from less active (basal) to more active (activated) forms. Activation of STE12 causes heightened expression of mating-specific genes, including many of the signal transduction components (e.g., the receptor, G protein, STE7, FUS3, and FAR1), thereby enhancing signal transduction, G1 arrest, and cell fusion. Activation of FAR1 increases its ability to promote G1 arrest, presumably through inhibition of the G1 cyclins (Chang and Herskowitz, 1990; Valdivieso *et al.*, 1993). A low level of FUS3 kinase activity is also found in uninduced cells. This activity may be responsible either for the maintenance of basal levels of signal transduction (Elion *et al.*, 1990) or for inhibition of certain signal transduction components (Gartner *et al.*, 1992). FUS3 may phosphorylate STE7 and STE11, although the effect of these phosphorylation events is unknown. KSS1 is not shown for simplicity, although it acts in parallel with FUS3 between STE7 and STE12 (Elion *et al.*, 1991a; Stevenson *et al.*, 1992) and may phosphorylate some of the same substrates as FUS3. → indicates positive regulation, ⊣ indicates negative regulation. Tx is an abbreviation for transcription.

tivity for the following reasons: 1) STE12 has 50% activity in the absence of FUS3 (Elion *et al.*, 1991a); 2) in either naturally occurring or genetically engineered *kss1*$^-$ strains, STE12 is completely dependent on FUS3 for activity (Elion *et al.*, 1990; Elion *et al.*, 1991a); and 3) overproduction of STE12 more efficiently bypasses *ste* mutants with complete blocks in α factor–dependent activation of mating-specific transcription than it does *fus3* mutants with intact signal transduction (Elion *et al.*, 1991a). The dependence of STE12 function on FUS3 also agrees with its placement nearest to STE12 in the protein kinase cascade (Gartner *et al.*, 1992). Thus, it seems likely that FUS3 converts STE12 from a less active to a more active state. FUS3 may affect transcriptional activation of STE12 rather than DNA binding, because phosphorylation of STE12 may be required for full activity of a transcriptional activation domain (Yuan and Fields, 1991). Finally, the fact that STE12 has no activity in a strain that lacks both FUS3 and KSS1 may indicate that KSS1 also phosphorylates STE12.

Genetic and Biochemical Evidence Suggests FUS3 Directly Activates FAR1

Genetic evidence presented here supports a model in which FUS3 phosphorylates FAR1 and converts it to a form that more efficiently promotes G1 arrest (Figure 9). The suppression of the arrest defects of *fus3* mutants by excess wild-type FAR1, but not vice versa, suggests that FAR1 acts after FUS3 in a common G1 arrest pathway. Three results indicate that FAR1 is not simply bypassing the *fus3* mutants. First, FAR1 suppresses the *fus3-2* point mutant better than the *fus3* null mutant. Second, a truncated form of FAR1, far1-589, is absolutely dependent on wild-type FUS3 for activity and does not function properly in strains that either lack FUS3 or have an epitope-tagged version of FUS3 (Figure 1B). Third, FAR1 can associate with FUS3 in vivo (Table 2).

Two pieces of biochemical evidence also support the possibility that FAR1 is a FUS3 substrate. First, catalytically active FUS3 immune complexes hyperphosphorylate a FAR1 fusion protein (Figure 8). This reaction is specific, because other substrates are not phosphorylated by FUS3 (e.g., histones, B42). Second, overexpression of FAR1 in vivo produces a new FUS3-associated substrate (Figure 9) that is close in size to the hyperphosphorylated form of FAR1 (Chang and Herskowitz, 1990). The effect of FAR1 on the substrate profile is unlikely to be indirect, because FAR1 is not implicated in the expression of

other proteins (Chang and Herskowitz, 1990). Although we do not demonstrate the presence of FAR1 in FUS3 immune complexes, the ability of FAR1 to associate with FUS3 in vivo shows that the two proteins are capable of coprecipitating. Finally, the ability of FUS3 to hyperphosphorylate FAR1 in vitro matches the requirement of FUS3 for in vivo hyperphosphorylation of FAR1 (Peters and Herskowitz, personal communication).

FUS3 and FAR1 Regulate the Same G1 Cyclins

FUS3 and FAR1 were initially thought to function in parallel pathways to effect G1 arrest, with FUS3 inhibiting CLN3 and FAR1 inhibiting CLN2 (Chang and Herskowitz, 1990; Elion *et al.*, 1990). Our results support a new model in which FAR1 is activated by FUS3 to regulate the G1 cyclins. This interpretation fits with more recent experiments that indicate FUS3 and FAR1 regulate multiple G1 cyclins (Elion *et al.*, 1991a,b; Valdivieso *et al.*, 1993). Further support for this view comes from the observation that overexpression of FAR1 restores α factor sensitivity to *fus3* point mutants that are not suppressed by mutations in individual *CLN* genes (Satterberg and Elion, unpublished data). However, FUS3 may have additional functions required for G1 arrest that are distinct from those that overlap with FAR1, because a *cln1*Δ *cln2*Δ *cln3*Δ strain driven by a *Drosophila* cylin (Lahue *et al.*, 1991) is more dependent on FUS3 than FAR1 for efficient G1 arrest (Elion, unpublished data). Further work is needed to determine whether FUS3 also phosphorylates the G1 cyclins and/or CDC28.

FUS3 Has Additional In Vivo Substrates

Positive regulation of STE12 and FAR1 by FUS3 does not fully explain all of the functions of FUS3. FUS3 is also required for the maintenance of an active signal transduction cascade (Elion *et al.*, 1990, 1991a), efficient cell fusion (Elion *et al.*, 1990), and downregulation of its own phosphorylation (Gartner *et al.*, 1992). Our detection of many physiologically relevant substrates in FUS3 immune complexes suggests that FUS3 phosphorylates proteins involved in these processes. It is interesting to note that STE7 and STE11 may be two of FUS3's targets, because mutations in STE7 and STE11 lead to the disappearance of FUS3-associated substrates whose sizes match the phosphorylated forms of STE7 and STE11 (Rhodes *et al.*, 1990; Cairns *et al.*, 1992). Indeed, this finding correlates with recent work showing that phosphorylation of STE7 is blocked by mutations in FUS3 and KSS1 (Stevenson *et al.*, 1992) and suggests the existence of a feedback loop of regulation.

The data presented support a previous hypothesis that FUS3 acts at a branch point in the signal transduction cascade to phosphorylate multiple proteins involved in different aspects of the response to pheromone (Elion *et al.*, 1990, 1991a,b). Further work is needed to determine whether FUS3 associates with these proteins individually or in multimeric complexes. STE12 and FAR1 are implicated as two of FUS3's targets, providing an explanation for how FUS3 mediates both transcription and G1 arrest. Because KSS1 has redundant signal transduction functions with FUS3, it is likely to share a subset of these substrates.

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