Cell, Vol. 73, 747–760, May 21, 1993, Copyright © 1993 by Cell Press

FAR1 Links the Signal Transduction Pathway to the Cell Cycle Machinery in Yeast

Matthias Peter,* Anton Gartner,† Joe Horecka,‡ Gustav Ammerer,† and Ira Herskowitz*
*Programs in Genetics and Cell Biology
Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143-0448
†Institut für Biochemie und Molekulare Zellbiologie der Universität Wien
Ludwig Boltzmann Forschungsstelle
A-1030 Vienna
Austria
‡Institute of Molecular Biology
and Department of Biology
University of Oregon
Eugene, Oregon 97403

Summary

α factor induces arrest of yeast a cells in G1 and transcription of genes involved in mating. Prior work indicates that FUS3, a member of the MAP kinase family, and FAR1, whose molecular activity is unknown, contribute to cell cycle arrest by inhibiting G1 cyclins. Here we show that FAR1 is a substrate for FUS3 and that this phosphorylation regulates association of FAR1 with CDC28-CLN2 kinase. We show also that FAR1 is phosphorylated in vitro by the CDC28-CLN2 complex and in vivo in a CDC28-dependent manner. Mutational analysis of FAR1 reveals a correlation between its ability to associate with CDC28-CLN2 and to arrest the cell cycle. These results suggest that FAR1 protein is the link between the signaling pathway and the cell cycle machinery.

Introduction

α factor is a negative growth factor of Saccharomyces cerevisiae that induces differentiation and cell cycle arrest of target cells (reviewed by Reed, 1991; Marsh et al., 1991). Activation of the yeast signal transduction pathway is initiated by binding of α factor to an integral membrane protein receptor that is coupled to a heterotrimeric G protein. Activation causes release of Gα from Gβγ, which then propagates a signal by an unknown mechanism involving the STE20 and STE5 proteins (Leberer et al., 1992; Stevenson et al., 1992; Ramer and Davis, 1993) to a group of serine/threonine protein kinases encoded by the *STE7*, *STE11*, *FUS3*, and *KSS1* genes (Teague et al., 1986; Fields et al., 1988; Courchesne et al., 1989; Elion et al., 1990; Fujimura, 1990; Rhodes et al., 1990). These kinases are thought to function in the order STE11, STE7, FUS3/KSS1 (Cairns et al., 1992; Gartner et al., 1992; Stevenson et al., 1992; Zhou et al., 1993). At the end of the pathway is a transcription factor, STE12, that binds to the upstream regulatory regions of several genes to activate their transcription (Dolan et al., 1989; Errede and Ammerer, 1989). The activity of STE12 is thought to be controlled by phosphorylation, presumably by one or more of the upstream kinases (Song et al., 1991).

Both FUS3 and KSS1 are related to mammalian mitogen-activated protein (MAP) kinases (Boulton et al., 1991; reviewed by Pelech and Sanghera, 1992). Activity of FUS3 requires phosphorylation of threonine and tyrosine residues (Gartner et al., 1992; Errede et al., 1993). This activation is mediated by STE7 (Errede et al., 1993), which shares sequence identity with the kinase responsible for activation of mammalian MAP kinase (Crews et al., 1992a; reviewed by Sprague, 1992). Substrates for FUS3 and KSS1 have not yet been identified, but candidates (STE12 and G1 cyclins; see below) exist. KSS1 and FUS3 are functionally redundant in that either FUS3 or KSS1 is able to propagate the signal to activate transcription by STE12 (Elion et al., 1991). FUS3 and KSS1 differ, however, in that only FUS3 functions for cell cycle arrest: mutants lacking FUS3 do not arrest in response to α factor, whereas mutants lacking KSS1 arrest (Elion et al., 1991).

G1 cyclins are essential components of the CDC28 protein kinase that is necessary for progression from G1 to S phase (Richardson et al., 1989; Wittenberg et al., 1990; for review see Futcher, 1991; Reed, 1992). Cells exposed to α factor arrest at the same point in the cell cycle as strains either deleted for the three cyclin genes, *CLN1*, *CLN2*, and *CLN3* (Richardson et al., 1989; Cross, 1990), or defective in *CDC28* (Reed, 1980). Cell cycle arrest in response to α factor apparently occurs by inhibition of the G1 cyclins (Wittenberg et al., 1990; Chang and Herskowitz, 1990; Elion et al., 1990). Whether this decrease in CLN activity is mediated by inhibiting function or synthesis of CLN proteins is not known and is complicated by the fact that CDC28–CLN stimulates transcription of *CLN1* and *CLN2* (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991).

The FAR1 protein is specifically required for pheromone-induced cell cycle arrest: a strains lacking FAR1 do not arrest when exposed to α factor, but still exhibit pheromone-specific transcriptional induction (Chang and Herskowitz, 1990). Genetic evidence indicates that FAR1 functions mainly by antagonizing CLN2 activity, since strains lacking both CLN2 and FAR1 arrest in response to α factor. Recent evidence suggests that FAR1 may also contribute to the regulation of the other cyclins, CLN1 and CLN3 (Valdivieso et al., 1993; M. Tyers and B. Futcher, submitted; F. Cross, personal communication). Although FAR1 expression increases approximately 3- to 5-fold in pheromone-treated cells (Chang and Herskowitz, 1990, 1992), transcriptional induction of *FAR1* per se is not sufficient for cell cycle arrest (even in strains whose only G1 cyclin is CLN2). Because FAR1 becomes rapidly phosphorylated after addition of α factor, it has been suggested that phosphorylation may be necessary for FAR1 to become active for inhibiting G1 cyclins (Chang and Herskowitz, 1992).

Strains with a defective *FUS3* gene behave similarly to

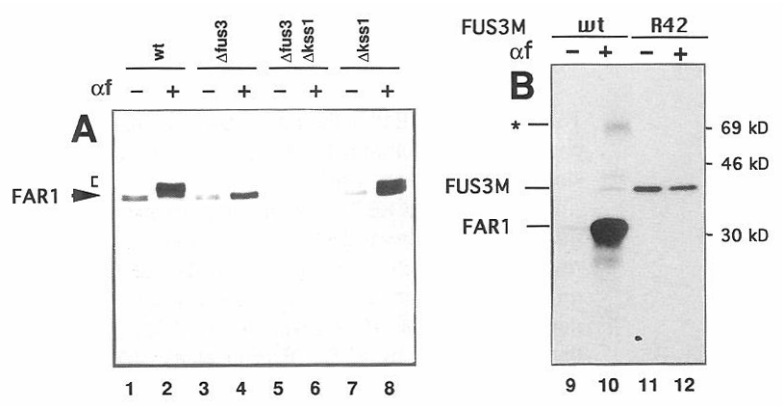

Figure 1. Phosphorylation of FAR1 in Response to α Factor In Vivo and by FUS3 In Vitro

(A) Phosphorylation of FAR1 in vivo was analyzed by immunoblotting of crude extracts prepared from strains lacking various components of the mating pathway. Strains were either exposed (plus sign; lanes 2, 4, 6, and 8) or not exposed (minus sign; lanes 1, 3, 5, and 7) to α factor. The arrowhead points to the position of unphosphorylated FAR1; the bracket marks phosphorylated forms of FAR1. The following strains were analyzed: lanes 1 and 2, wild-type (AN37-4C-S); lanes 3 and 4, *fus3* deletion mutant (AN1015); lanes 5 and 6, *fus3 kss1* deletion mutant (AN42-2A); lanes 7 and 8, *kss1* deletion mutant (AN42-3A).

(B) A purified NH_2-terminal fragment of FAR1 expressed in E. coli was incubated in the presence of [γ-^{32}P]ATP with either wild-type (lanes 9 and 10) or a catalytically inactive mutant FUS3 (R42, lanes 11 and 12) immunoprecipitated from cells (K2702) either treated (plus sign; lanes 10 and 12) or not treated with α factor (minus sign; lanes 9 and 11).

far1$^-$ strains in that they are competent for the transcriptional response to pheromone but are unable to arrest their cell cycle. FUS3 was originally proposed to antagonize CLN3 function specifically, but current evidence indicates a more general role for FUS3 in the inhibition of cyclin activity (Elion et al., 1991; M. Tyers and B. Futcher, submitted; G. A., A. Amon, and K. Nasmyth, unpublished data).

The work presented here provides insights into the arrest-specific functions of FUS3 and FAR1. We show that FAR1 is phosphorylated by FUS3 and that this modification is correlated with activation of FAR1. We also demonstrate that the FAR1 protein physically associates with CDC28–CLN2 kinase in an α factor–dependent manner. Efficient formation of this complex requires prior phosphorylation of FAR1 by active FUS3. We thus provide biochemical evidence that FUS3 contributes to cell cycle arrest by activating FAR1. These observations indicate that FAR1 protein directly links the signal transduction pathway to the machinery needed for cell cycle progression.

Results

Phosphorylation of FAR1 in Response to α Factor Is Dependent on FUS3

FAR1 protein is very rapidly phosphorylated when haploid **a** cells are exposed to α factor (Figure 1A; Chang and Herskowitz, 1992) and is dephosphorylated within minutes after cells are released from α factor arrest (M. P., unpublished data). These results show a correlation between phosphorylation of FAR1 in response to α factor and its ability to arrest the cell cycle. To identify the kinase responsible for this phosphorylation, we analyzed the phosphorylation state of FAR1 in isogenic strains carrying deletions of known kinases involved in the signal transduction pathway.

No α factor–dependent phosphorylation of FAR1 could be detected in *ste5*$^-$, *ste7*$^-$, or *ste11*$^-$ mutants, consistent with the view that FAR1 acts downstream of these proteins in the signal transduction pathway (M. P., unpublished data). The most striking finding is that α factor–dependent phosphorylation of FAR1 did not occur in *fus3* mutants (Figure 1A, lanes 3 and 4). These cells exhibited a normal transcriptional response to α factor, indicated by the increased level of FAR1 protein (Figure 1A, lane 4; see also Elion et al., 1991). In contrast, FAR1 was readily phosphorylated in isogenic wild-type cells (Figure 1A, lanes 1 and 2), in *fus3* mutants carrying a *FUS3* plasmid (M. P., unpublished data), and in cells defective in KSS1 (*kss1*$^-$ FUS3$^+$ strain AN42-3A; Figure 1A, lanes 7 and 8). Since *FUS3*$^+$ kss1$^-$ strains arrest in response to α factor, whereas *fus3*$^-$ KSS1$^+$ strains do not (Elion et al., 1990, 1991), these observations reinforce the correlation between phosphorylation and activation of FAR1 for cell cycle arrest. Strains lacking both *KSS1* and *FUS3* were unable to activate STE12 and exhibited greatly reduced basal levels of FAR1 (Figure 1A, lanes 5 and 6), a behavior previously observed in *ste12* mutants (Chang and Herskowitz, 1990). The basal level of FAR1 protein is thus controlled by the residual activity of the signal transduction pathway in the absence of α factor, as has been described for several other α factor–inducible products (see Marsh et al., 1991).

Mutants of *FUS3* have been obtained that are specifically defective in promoting cell cycle arrest but are able to activate STE12 in response to α factor (G. A., unpublished data). Five different *FUS3* mutants of this type were tested for phosphorylation of FAR1 in response to α factor, and all were defective (M. P., unpublished data).

These observations on the behavior of *fus3* and *kss1* mutant strains indicate that FUS3, or, alternatively, a kinase dependent on *FUS3*, is responsible for phosphorylation of FAR1.

FAR1 Is Phosphorylated In Vitro by Isolated FUS3

We next asked whether FUS3 is able to phosphorylate FAR1 in vitro. To perform in vitro kinase assays, we expressed FUS3 containing a Myc epitope tag in yeast and isolated active FUS3 by immunoprecipitation (Gartner et al., 1992; Errede et al., 1993). As a substrate, we used a purified amino-terminal fragment of FAR1 containing 251 amino acid residues purified from Escherichia coli (Errede et al., 1993). As shown in Figure 1B, FAR1 was strongly

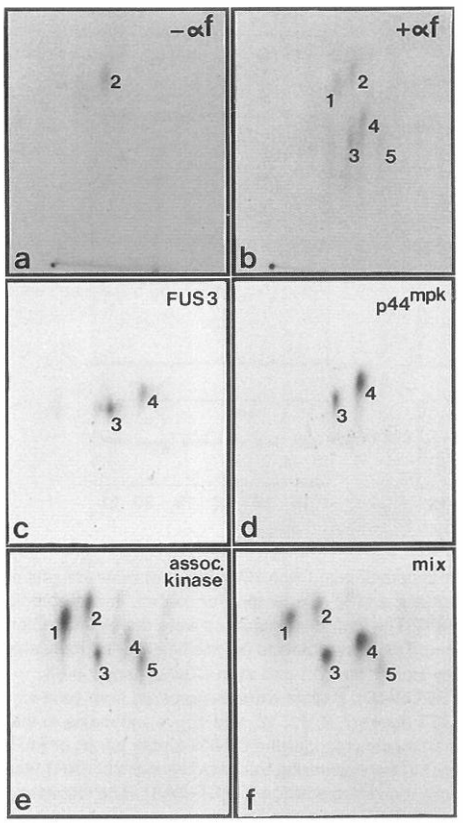

Figure 2. Tryptic Phosphopeptide Analysis of GST–FAR1 after Phosphorylation in the Presence or Absence of α Factor In Vivo or by FUS3, $p44^{mpk}$ Kinase, and a FAR1-Associated Kinase In Vitro

Phosphorylated proteins were eluted from polyacrylamide gels and digested with trypsin. The resulting phosphopeptides were separated by electrophoresis at pH 3.5 (horizontal direction; anode to the right) and ascending chromatography (buffer C; see Experimental Procedures). The analyzed FAR1 proteins were phosphorylated as follows.

(a and b) GST–FAR1 expressed from the *GAL* promoter was immunoprecipitated from ^{32}P-labeled cells without ([a], −αf) or with α factor ([b], +αf).

(c) The NH_2-terminal fragment of FAR1 expressed in E. coli phosphorylated in vitro with immunoprecipitated FUS3 as described in Figure 1B.

(d) The 100 amino acid NH_2-terminal fragment of FAR1 fused to GST was expressed in E. coli and phosphorylated in vitro with $p44^{mpk}$ MAP kinase.

(e) GST–FAR1 immunoprecipitated from yeast cells treated with α factor and phosphorylated in vitro by an associated kinase.

(f) Analysis of phosphopeptides after mixing equal Cerenkov counts of GST–FAR1 phosphorylated in vitro by $p44^{mpk}$ MAP kinase and by the associated kinase.

phosphorylated in these assays if FUS3 was immunoprecipitated from cells treated with α factor (lane 10), but little phosphorylation was observed for immunoprecipitates from uninduced cells (lane 9). Phosphoamino acid analysis revealed that phosphate was incorporated predominantly on serine (A. G., unpublished data). Phosphorylation of FAR1 could not be detected if the immunoprecipitations were carried out with extracts prepared from cells expressing an inactive mutant form of FUS3 in which lysine 42 (the invariant residue conserved in all kinases; Hanks et al., 1988) was changed to alanine (Figure 1B, lanes 11 and 12; Gartner et al., 1992). These results strongly indicate that activated FUS3 is able to phosphorylate FAR1 directly in vitro. Although we cannot exclude the possibility that a *FUS3*-dependent kinase coimmunoprecipitates with FUS3 in these experiments, we note that purified $p44^{mpk}$, a bona fide member of the MAP kinase family (Sanghera et al., 1990), is able to phosphorylate a partially purified 100 amino acid amino-terminal fragment of FAR1 in vitro (M. P., unpublished data; see Figure 2d).

Tryptic Phosphopeptide Analysis of FAR1

To establish the relationship between phosphorylation of FAR1 in vitro and α factor–dependent phosphorylation in vivo, we compared the different phosphoproteins by tryptic peptide mapping (Figure 2). We labeled a yeast strain expressing glutathione S-transferase (GST)–FAR1 fusion protein from the inducible *GAL* promoter with [^{32}P]orthophosphate. Expression of GST-tagged FAR1 was necessary because we were unable to immunoprecipitate FAR1 efficiently with the available polyclonal antiserum. In all biological and biochemical aspects tested, the fusion protein is equivalent to normal FAR1 protein: it is able to complement a *far1* deletion strain fully for both cell cycle arrest as well as for mating (M. P., unpublished data), and migrates slightly slower on SDS gels when immunoprecipitated from cells treated with α factor. The GST moiety remains unphosphorylated under all conditions (M. P., unpublished data).

In the absence of α factor, GST–FAR1 was predominantly phosphorylated on one peptide (peptide 2; Figure 2a); after addition of α factor, it was phosphorylated on three additional phosphopeptides (1, 3, and 4; Figure 2b), an observation consistent with the mobility change of FAR1 on SDS gels (Figure 1A). The phosphorylations observed in the presence and absence of α factor were predominantly on serine residues (M. P., unpublished data). Activated FUS3 phosphorylated FAR1 in vitro on two tryptic peptides that migrate at the same position as phosphopeptides 3 and 4 from the product labeled in vivo (Figure 2c). It therefore appears that FUS3 phosphorylates FAR1 in vitro on two sites that are specifically modified in response to α factor in vivo. The same peptides were also phosphorylated by $p44^{mpk}$ kinase using the GST–FAR1 protein containing the first 100 amino acids of FAR1 as a substrate (Figure 2d). It should be noted that FUS3 did not phosphorylate all of the sites observed in vivo in response to α factor. These sites may be lacking from the substrates used in our in vitro assays or be phosphorylated by a kinase (or kinases) distinct from FUS3 (see below).

Phosphorylation of FAR1 by FUS3 Regulates Formation of a Complex between CDC28–CLN2 and FAR1

Genetic evidence indicates a functional interaction between CLN2 and FAR1 (Chang and Herskowitz, 1990). To investigate whether FAR1 physically interacts with CLN2 or the CDC28–CLN2 complex, we performed coimmunoprecipitation experiments using strains expressing either CDC28 or CLN2 proteins fused to an influenza hemagglutinin (HA) epitope. Both HA fusion genes have been inte-

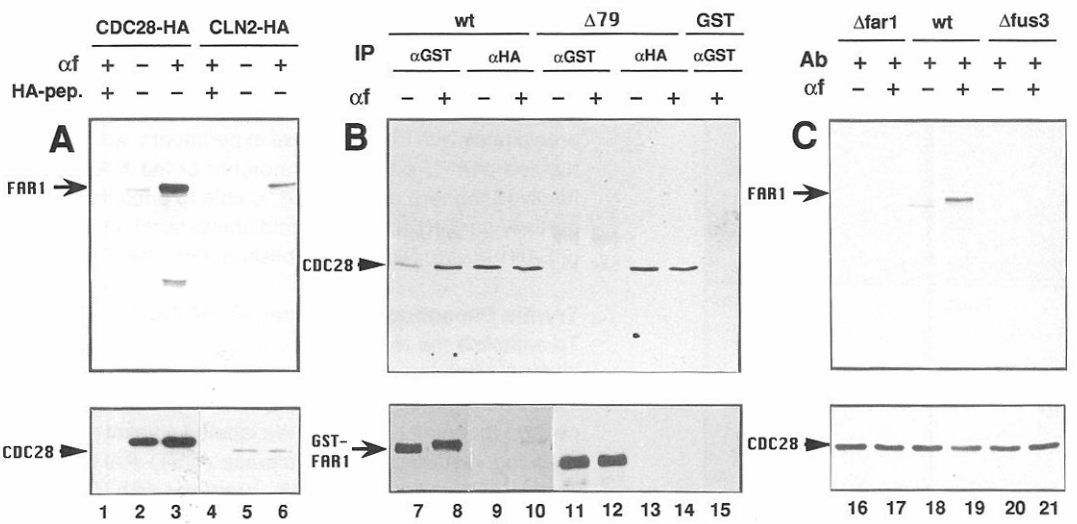

Figure 3. FAR1 Coimmunoprecipitates with CDC28 and CLN2 in an α Factor– and *FUS3*-Dependent Manner

(A) Epitope-tagged CDC28 (lanes 1–3) or epitope-tagged CLN2 (lanes 4–6) was immunoprecipitated with HA antiserum from extracts prepared from strains exposed (plus sign; lanes 1, 3, 4, and 6) or not exposed (minus sign; lanes 2 and 5) to α factor. For control, immunoprecipitations were also carried out in the presence of competing HA peptide (plus sign; lanes 1 and 4). The immunoprecipitates were then immunoblotted with affinity-purified polyclonal antibodies against FAR1 (upper panel) or CDC28 (lower panel). The arrow points to the position of phosphorylated FAR1; the arrowhead marks the position of CDC28 (CDC28–HA [lanes 1–3] migrates slightly slower on SDS gels than CDC28 [lanes 4–6]).

(B) GST–FAR1 was expressed in strains bearing HA epitope–tagged CDC28 (strain RD270-4C). Protein extracts prepared from cells exposed to α factor as indicated (plus sign) were immunoprecipitated with either antibodies to GST (lanes 7, 8, 11, 12, and 15) or antibodies to the HA tag (lanes 9, 10, 13, and 14). Immunoprecipitates were then immunoblotted using specific antibodies recognizing CDC28 (upper panel) or FAR1 (lower panel). Control immunoprecipitations were also carried out from extracts prepared from strains expressing truncated versions of FAR1 (see Figure 6) or GST alone. The arrowhead points to the position of CDC28–HA, whereas the arrow marks the position of GST–FAR1. The following proteins were analyzed: lanes 7–10, wild-type GST–FAR1; lanes 11–14, GST–FAR1Δ79; lane 15, GST alone.

(C) Protein extracts were prepared from wild-type (AN37-4C-S, lanes 18 and 19), *far1* deletion (YMP18, lanes 16 and 17), or *fus3* deletion strains (YMP19, lanes 20 and 21) harboring the CLN2–HA expression plasmid pTP35. CLN2–HA was then immunoprecipitated, and the immunoprecipitates were analyzed for the presence of FAR1 (upper panel) or CDC28 (lower panel) by immunoblotting. The arrowhead points to the position of CDC28; the arrow marks the position of FAR1.

grated at their respective loci by replacement of the endogenous genes and have been shown to be fully functional (Sorger and Murray, 1992; M. Tyers and B. Futcher, submitted).

CDC28 (Figure 3A, lanes 1–3) or CLN2 (Figure 3A, lanes 4–6) proteins were immunoprecipitated using antibodies recognizing the HA epitope from extracts prepared from cells without α factor or incubated with α factor for 10 min (a time too brief for appreciable induction of FAR1 synthesis; Chang and Herskowitz, 1992). Immunoprecipitates were subsequently analyzed by immunoblotting to determine the presence of FAR1 (Figure 3A, upper panel) or CDC28 (Figure 3A, lower panel) proteins. This analysis showed that FAR1 specifically coimmunoprecipitated with both CDC28 and CLN2 and that coimmunoprecipitation was most efficient for cells that were exposed to α factor (Figure 3A, lanes 2, 3, 5, and 6). Coimmunoprecipitated FAR1 protein could not be detected if the immunoprecipitations were carried out in the presence of competing HA peptide (Figure 3A, lanes 1 and 4). Irrespective of whether CDC28 was immunoprecipitated directly or by association with CLN2 (Figure 3A, lower panel, lanes 1–3 and 4–6, respectively), the total amount of CDC28 protein remained constant during the brief incubation of cells with α factor. These results indicate that FAR1 specifically associates with CDC28 and CLN2 in an α factor–dependent manner.

We have also examined association of FAR1 with CLN2 and CDC28 under conditions in which its synthesis was not regulated by α factor. This has been accomplished by expressing GST–FAR1 from the galactose-inducible *GAL* promoter in strains harboring either CDC28–HA (Figure 3B) or CLN2–HA (M. P., unpublished data). We observed that CDC28 protein was present in coimmunoprecipitates selected with antibodies against GST–FAR1 if cells were exposed to α factor (Figure 3B, lane 8); reduced amounts of CDC28 were found if GST–FAR1 was immunoprecipitated from untreated cultures (Figure 3B, lane 7). The lower panel (Figure 3B, lanes 7 and 8) demonstrated that comparable levels of GST–FAR1 were produced in the presence and absence of α factor and that α factor caused the characteristic mobility shift of FAR1 protein. CDC28 protein was not found if the immunoprecipitates were prepared from cells expressing only the GST protein (Figure 3B, lane 15). Moreover, a truncated GST–FAR1 mutant protein that lacks its N-terminal 79 amino acids (GST–FAR1Δ79) was unable to bind to CDC28 (Figure 3B, lanes 11 and 12). This mutant FAR1 protein is unable to arrest the cell cycle (see below).

Reciprocal experiments have been carried out in which CDC28 was precipitated from these same extracts with HA antibodies, and immune complexes were then analyzed for the presence of GST–FAR1 protein. These exper-

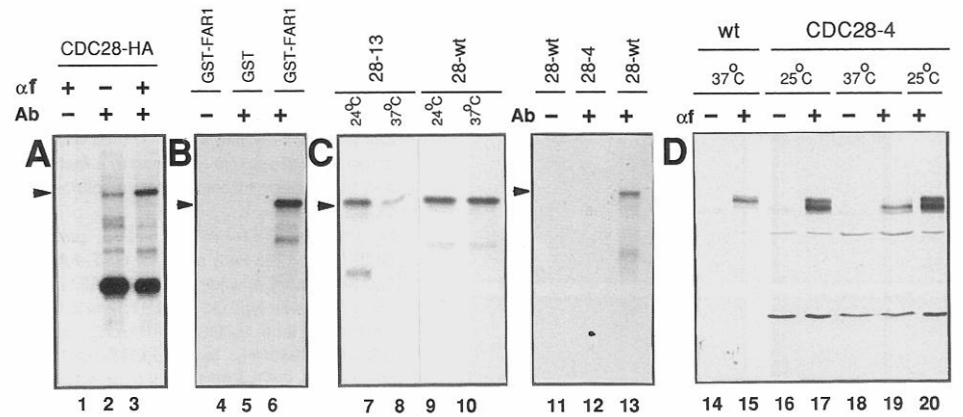

Figure 4. FAR1 Is Phosphorylated by Associated CDC28 Kinase: In Vitro and In Vivo Evidence

(A) CDC28–HA was immunoprecipitated from cells either treated (lane 1 and 3) or not treated (lane 2) with α factor as described in Figure 3. Immunoprecipitates were then assayed for phosphorylation of associated FAR1 (arrowhead) in the presence of [γ-^{32}P]ATP. Control immunoprecipitations were also carried out in the absence of HA antibodies (lane 1).

(B) GST–FAR1 (lanes 4 and 6) or GST alone (lane 5) expressed in wild-type cells (YMP05) was immunoprecipitated with (lanes 5 and 6) or without (lane 4) affinity-purified anti-GST antibodies. The immunoprecipitates were then assayed for kinase activity in the presence of [γ-^{32}P]ATP. The arrowhead marks the position of phosphorylated GST–FAR1.

(C) GST–FAR1 expressed in either wild-type (lanes 9, 10, 11, and 13), *cdc28-13* (lanes 7 and 8), or *cdc28-4* (lane 12) strains was immunoprecipitated from extracts prepared from cells treated with α factor. The immunoprecipitates were divided into equal aliquots and assayed for associated kinase activity in the presence of [γ-^{32}P]ATP at either 24°C (lanes 7 and 9) or at 37°C (lanes 8 and 10–13). Phosphorylation of GST–FAR1 was monitored by SDS–polyacrylamide gel electrophoresis and autoradiography. The arrowhead indicates the position of GST–FAR1. Control immunoprecipitations were carried out in the absence of antibodies against GST (lane 11).

(D) Wild-type (lanes 14 and 15) or *cdc28-4* cells (lanes 16–20) were grown at 25°C and the cultures were divided: one half was further incubated for 1 hr at 25°C (lanes 16, 17, and 20); the other half was incubated for 1 hr at the nonpermissive temperature, 37°C (lanes 14, 15, 18, and 19). Cultures were then divided again, and one half was treated (plus sign; lanes 15, 17, 19, and 20) and the other half was not treated with α factor (minus sign; lanes 14, 16, and 18). Total protein extracts were prepared and immunoblotted with affinity-purified anti-FAR1 antibodies.

iments showed that GST–FAR1 was predominantly coprecipitated with CDC28 if cells were exposed to α factor (Figure 3B, lower panel, lanes 9 and 10). The FAR1 mutant lacking its N-terminal 79 amino acid residues did not associate with CDC28 under these conditions (Figure 3B, lower panel, lanes 13 and 14).

Virtually identical results were obtained if immunoprecipitations were performed against CLN2–HA: GST–FAR1 was readily coimmunoprecipitated with CLN2 in an α factor–dependent manner, whereas GST–FAR1 deletion mutants that were unable to interact with CDC28 were also unable to coimmunoprecipitate with CLN2–HA (M. P., unpublished data).

We conclude from these results that CDC28–CLN2 and FAR1 form a complex after induction with α factor and that the appearance of such a complex is not simply due to increased levels of FAR1 protein. These observations suggest that an α factor–dependent posttranslational modification, most likely phosphorylation, regulates formation of this complex.

The data shown in Figures 1 and 2 establish that FAR1 is phosphorylated by FUS3 in response to α factor. To determine whether complex formation between CDC28–CLN2 and FAR1 is regulated by a *FUS3*-dependent phosphorylation of FAR1, we examined the ability of CLN2 and CDC28 to associate with FAR1 in a *fus3* deletion mutant (Figure 3C). As noted earlier, FAR1 readily coimmunoprecipitated with CLN2 in an α factor–dependent manner in a wild-type strain (Figure 3C, lanes 18 and 19). The cross-reacting material was confirmed to be FAR1 because it was absent from a *far1* deletion strain (Figure 3C, lanes 16 and 17). The amount of FAR1 protein that coimmunoprecipitated with CLN2 was greatly reduced in a *fus3* mutant, whether or not α factor was added (Figure 3C, lanes 20 and 21). The lower panel of Figure 3C shows that the amounts of CDC28 protein that coprecipitated with CLN2 were similar in all immunoprecipitations, regardless of whether cultures were treated with α factor. Similarly, when GST–FAR1 was expressed from the *GAL* regulatory region, complex formation between CDC28 and GST–FAR1 was reduced in a *fus3* mutant strain in comparison with the isogenic wild-type strain (M. P., unpublished data). Taken together, these results indicate that phosphorylation of FAR1 by FUS3 regulates the formation of a complex containing FAR1, CDC28, and CLN2.

FAR1 Immune Complexes Contain CDC28–CLN2 Protein Kinase Activity That Phosphorylates FAR1

We observed that immune complexes isolated against either CDC28 or CLN2 HA-tagged proteins were able to phosphorylate an associated protein with the same mobility as FAR1 (Figure 4A, lanes 2 and 3; M. P., unpublished data). Higher levels of this phosphorylated protein were detected when the kinase was immunoprecipitated from α factor–treated cells (Figure 4A, lanes 2 and 3). We therefore investigated whether FAR1 is able to associate with an active CDC28–CLN kinase by immunoprecipitating GST–

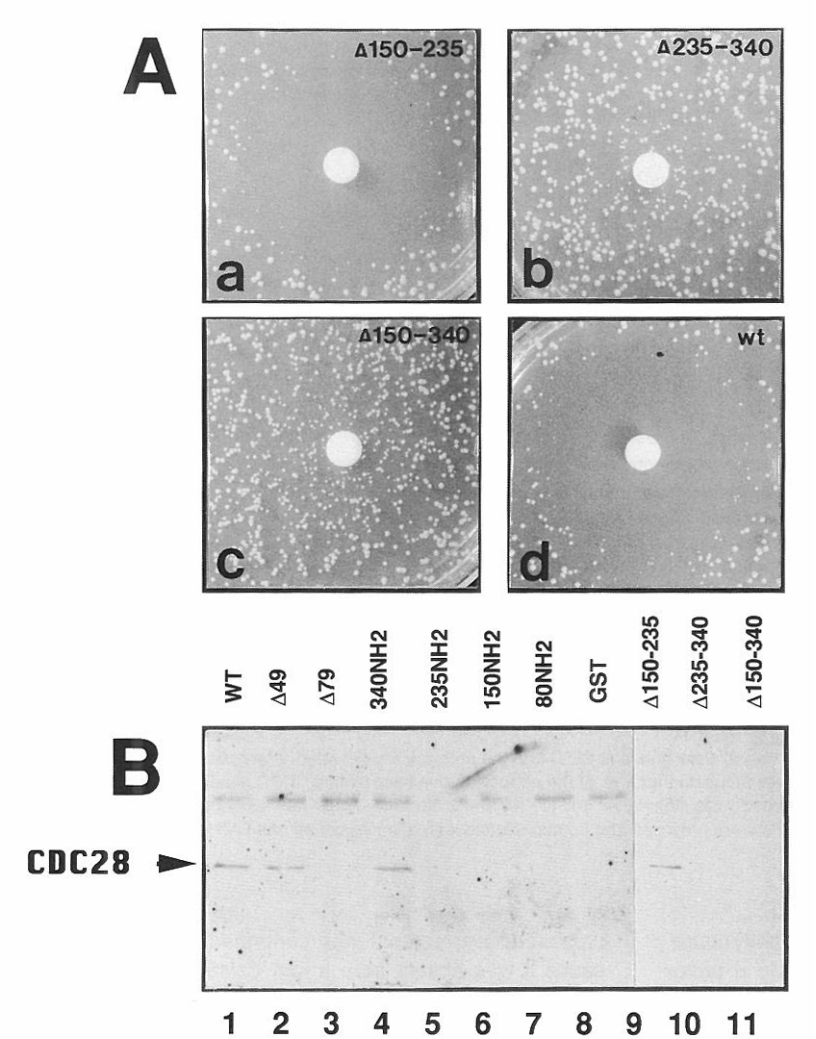

Figure 5. Ability of FAR1 Mutants to Exibit Cell Cycle Arrest in Response to α Factor and to Bind to CDC28

Various deletion mutants of GST–FAR1 were expressed in a strains lacking FAR1 (YMP18) and analyzed by halo assay for their ability to arrest the cell cycle in response to α factor (A) and their ability to coimmunoprecipitate with CDC28 (B).

(A) Panel a, GST–FAR1Δ150-235; panel b, GST–FAR1Δ235-340; panel c, GST–FAR1Δ-150-340; panel d, wild-type GST–FAR1.

(B) Lane 1, wild-type GST–FAR1; lane 2, GST–FAR1Δ49; lane 3, GST–FAR1Δ79; lane 4, GST–FAR1340NH_2; lane 5, GST–FAR1235-NH_2; lane 6, GST–FAR1150NH_2; lane 7, GST–FAR180NH_2; lane 8, GST; lane 9, GST–FAR1-Δ150-235; lane 10, GST–FAR1Δ235-340; lane 11, GST–FAR1Δ150-340.

FAR1 from whole-cell extracts and assaying phosphorylation of FAR1 in vitro as described above. As shown in Figure 4B (lane 6), immunoprecipitated GST–FAR1 was readily phosphorylated. Kinase activity could not be detected in immunoprecipitates containing the GST moiety alone (Figure 4B, lane 5) or in the absence of the primary antibody (Figure 4B, lane 4). Immunoblotting of the gel shown in Figure 4B confirmed that the phosphorylated 120 kd protein is indeed GST–FAR1 (M. P., unpublished data).

Tryptic phosphopeptide mapping of GST–FAR1 phosphorylated in vitro by this associated kinase showed a pattern almost identical to the in vivo pattern from α factor–treated cells (Figure 2e). Figure 2f shows the results of mixing tryptic digests of GST–FAR1 phosphorylated by the associated kinase and by $p44^{mpk}$ in vitro. Interestingly, the kinase associated with FAR1 strongly phosphorylated the α factor–inducible site (phosphopeptide 1), which was not phosphorylated in vitro by FUS3 using the amino-terminal FAR1 protein as a substrate.

To address the identity of the associated protein kinase, we examined phosphorylation of FAR1 in strains bearing a temperature-sensitive mutation of *CDC28*. GST–FAR1 was immunoprecipitated from both a wild-type strain and from an isogenic mutant whose kinase activity is temperature sensitive in vitro (Reed et al., 1985). We observed reduced phosphorylation of FAR1 in the *cdc28-13* mutant when the kinase assays were performed at 37°C (Figure 4C, lanes 7–10), even though both wild-type and mutant CDC28 proteins could be readily coimmunoprecipitated with FAR1 (M. P., unpublished data). Phosphorylation of FAR1 was also reduced using a second *cdc28-ts* mutant, *cdc28-4* (Figure 4C, lanes 11–13). Deletion mutants of GST–FAR1 that are unable to interact with CDC28–CLN2 did not display an associated kinase activity (M. P., unpublished data; see Figure 6). Taken together, these results indicate that the kinase activity associated with FAR1 is due to CDC28 itself or, less likely, is dependent on CDC28 activity to stimulate another kinase. These results suggest that FAR1 is a substrate of the associated CDC28–CLN2 kinase.

To investigate whether CDC28 kinase also contributes to phosphorylation of FAR1 in vivo, we examined the mobility of FAR1 on SDS gels after incubating the *cdc28-4* mutant at the nonpermissive temperature in the presence or absence of α factor. As shown in Figure 4D, the most upshifted (phosphorylated) form of FAR1 was absent in

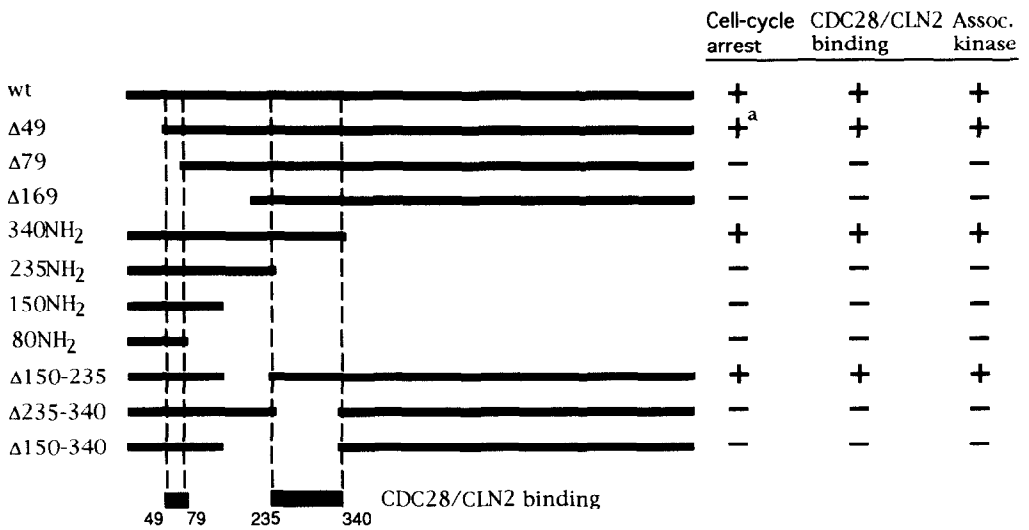

Figure 6. The Ability of Mutant FAR1 Proteins to Bind CDC28 Correlates with Their Ability to Arrest the Cell Cycle in Response to α Factor

A schematic representation of the analyzed GST–FAR1 deletion mutants. Mutant proteins were expressed in *far1*⁻ strain YMP18 as described in Figure 5 and tested for their ability to arrest the cell cycle in response to α factor. These proteins were also analyzed for their ability to coimmunoprecipitate with CDC28–CLN2 and for associated kinase activity as described above.

[a]The halo size of the FAR1Δ49 mutant is slightly reduced compared with wild type.

cdc28-4 cells at 37°C (lane 19) but was present at 25°C (lanes 17 and 20); wild-type cells exibited normal phosphorylation of FAR1 at 37°C (lanes 14 and 15). These results indicate that full phosphorylation of FAR1 in response to α factor also requires CDC28 activity.

The Ability of Mutant FAR1 Proteins to Bind CDC28–CLN2 Correlates with Their Ability to Arrest the Cell Cycle in Response to α Factor

To determine whether formation of a complex between FAR1 and the CDC28–CLN2 kinase is functionally important for cell cycle arrest in response to α factor, we have carried out a deletion analysis of the FAR1 protein. The truncated FAR1 proteins were expressed in a *far1* deletion mutant and tested for their ability to arrest the cell cycle in response to α factor using halo assays (Figure 5A) and growth in liquid culture (M. P., unpublished data). Representative data are shown in Figure 5, demonstrating that deletion of residues 150–235 had no effect on arrest (Figure 5A) whereas deletion of residues 235–340 or 150–340 (Figures 5B and 5C) inactivated FAR1. The constructs were also tested for their ability to bind CDC28 and CLN2 using coimmunoprecipitation experiments (Figures 5B and 6). Figure 5B shows, for example, that FAR1 deleted for residues 150–235 was still able to bind CDC28 (lane 9), whereas other deletions, such as those removing residues 235–340 or 150–340 (lanes 10 and 11), did not bind to CDC28. Finally, the immunoprecipitates were also analyzed for associated kinase activity against GST–FAR1 itself; the results of this analysis are summarized in Figure 6.

We have also characterized a yeast strain (YDH60F3) carrying a mutation in the *FAR1* gene that produces full-length FAR1 protein at normal levels (Figure 7, lanes 3 and 4), but does not arrest in response to α factor (Figure 7A). This mutant FAR1 protein is not fully phosphorylated in response to α factor (Figure 7B, compare lanes 6 and 7) and fails to bind to CLN2 (Figure 7C, lanes 10 and 12). Failure of FAR1 to be fully phosphorylated or to associate with CLN2 is not a consequence of the failure to arrest the cell cycle, since FAR1 protein from an α factor–resistant *CLN3* mutant (YDH60B1; J. Horecka and G. Sprague, unpublished data) was fully phosphorylated (Figure 7B, lanes 5 and 6) and was able to bind CLN2 (Figure 7C, lane 11).

The analysis of *FAR1* mutants demonstrates a correlation between the ability of FAR1 to bind to CDC28–CLN2 and its ability to arrest the cell cycle. We interpret these results to indicate that formation of this complex is required for cell cycle arrest in response to external signals.

Discussion

FAR1 Is a Substrate for FUS3

Several lines of evidence indicate that FAR1 is an in vivo substrate of FUS3. First, we have shown that phosphorylation of FAR1 in response to α factor requires a functional *FUS3* gene. Since *fus3*⁻ strains exhibit normal transcriptional induction by α factor, failure to phosphorylate FAR1 in *fus3*⁻ mutants is not simply due to interruption of the signaling pathway. As expected, phosphorylation of FAR1 requires the products of the *STE5*, *STE7*, and *STE11* genes, which act upstream of *FUS3*. Second, time course experiments indicate that FUS3 is activated within minutes after addition of α factor (Gartner et al., 1992); this rapid activation of FUS3 parallels the rapid phosphorylation of FAR1 (Chang and Herskowitz, 1992). Third, we have observed that FAR1 is phosphorylated in vitro by immunoprecipitated FUS3 from yeast cells on two tryptic peptides that are phosphorylated in response to α factor in vivo. Maximal phosphorylation requires that cells be treated

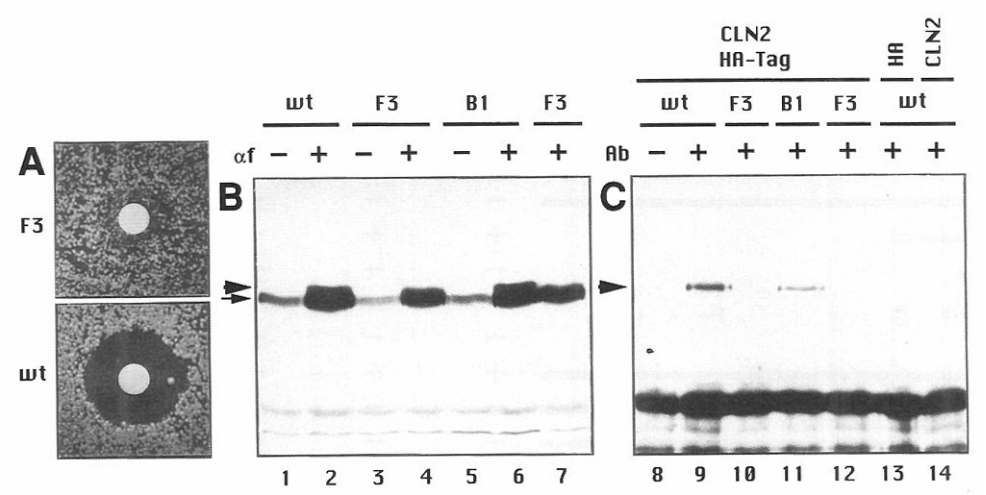

Figure 7. Biochemical Characterization of a FAR1 Mutant Strain That Expresses Full-Length FAR1 Protein Deficient in Binding to CLN2

(A) Strain YDH60F3 carrying a mutation in *FAR1* and the isogenic wild-type strain YDH60 were analyzed for their ability to arrest the cell cycle in response to α factor by halo assay.

(B) Expression and phosphorylation of FAR1 were analyzed by immunoblotting of crude extracts prepared from strains either exposed (plus sign; lanes 2, 4, 6, and 7) or not exposed (minus sign; lanes 1, 3, and 5) to α factor. The arrow marks the position of unphosphorylated FAR1; the arrowhead points to the position of phosphorylated FAR1. The following strains were analyzed: lanes 1 and 2, YDH60; lanes 3, 4, and 7, YDH60F3; lanes 5 and 6, YDH60B1. YDH60B1 is an α factor–resistant strain carrying a dominant allele of *CLN3* (J. H. and G. Sprague, Jr., unpublished data).

(C) Epitope-tagged CLN2 expressed from pTP35 was immunoprecipitated, as described, from extracts prepared from strains exposed to α factor. For control, immunoprecipitations were carried out in the absence of anti-HA antibodies (minus sign, lane 8) and from the YDH60 strain expressing either HA peptide alone (lane 13) or CLN2 protein without the HA tag (lane 14). The following strains were analyzed: lanes 8, 9, 13, and 14, YDH60; lanes 10 and 12, YDH60F3; lane 11, YDH60B1.

with α factor and does not occur with a catalytically inactive FUS3. Recent results also indicate that FUS3 produced in E. coli and activated in vitro is competent to phosphorylate FAR1 (G. A. and B. Errede, unpublished data; Errede et al., 1993). In addition, we have found that a purified MAP kinase, $p44^{mpk}$, phosphorylates FAR1 in vitro on the same tryptic peptides as FUS3. Together, these results indicate that FAR1 is a substrate for FUS3 and make it unlikely that a kinase intervenes between FUS3 and FAR1.

We do not yet know the precise residues on FAR1 that are phosphorylated by FUS3. Four sites in FAR1 conform to the substrate consensus determined for vertebrate MAP kinases, L/PXT/SP (Clark-Lewis et al., 1991; Crews et al., 1992b). Because both FUS3 and $p44^{mpk}$ phosphorylate FAR1 within the amino-terminal 100 amino acids, positions 37 and 64 are good candidates for *FUS3*-dependent phosphorylation.

The α factor–dependent phosphorylation of FAR1 appears to be functionally important. It was noted previously that overexpression of unphosphorylated FAR1 is not sufficient to arrest the cell cycle (Chang and Herskowitz, 1992). We have observed a correlation between phosphorylation of FAR1 and its function in promoting cell cycle arrest. Moreover, the region of FAR1 phosphorylated shortly after pheromone exposure is essential for function: removal of the 79 amino-terminal residues disrupts the ability of FAR1 to arrest the cell cycle in response to α factor. This truncation leaves FAR1 protein otherwise intact and competent for its additional role in conjugation (see Chang and Herskowitz, 1990). Although a definitive proof of the functional importance of *FUS3*-dependent phosphorylation must await the analysis of phosphorylation site mutants, our results provide evidence that these modifications are essential for cell cycle arrest.

FAR1 Associates with CDC28-CLN2 Kinase

Genetic studies have led to the proposal that FAR1 antagonizes CLN2 but have not specified the molecular basis for this inhibition (Chang and Herskowitz, 1990). Here we have shown that FAR1 is physically associated with CDC28–CLN2 and that formation of this complex is regulated by phosphorylation of FAR1 by FUS3. We have demonstrated the presence of CDC28–CLN2 in FAR1 immune complexes in two ways: by the presence of their polypeptides detected by immunoblotting and by the presence of their phosphorylation activity against FAR1 as substrate. This latter result, together with the observation that little monomeric CLN2 is found in cells, strongly argues for the formation of a tripartite complex between FAR1, CDC28, and CLN2. We cannot, however, exclude the possibility that FAR1 binds separately to CLN2 and CDC28.

The importance of the association between FAR1 and CDC28–CLN2 for cell cycle arrest is reinforced by our mutational analysis of FAR1. Without exception, mutants that are unable to arrest the cell cycle in response to α factor are also deficient in their association with CDC28 and CLN2. The analysis further shows that two separated domains of FAR1 (spanning residues 49–79 and 235–340) are required for binding to CDC28–CLN2 kinase: one is a segment in the amino terminus of FAR1 (amino acids 49–79), and the second is a separated region spanning

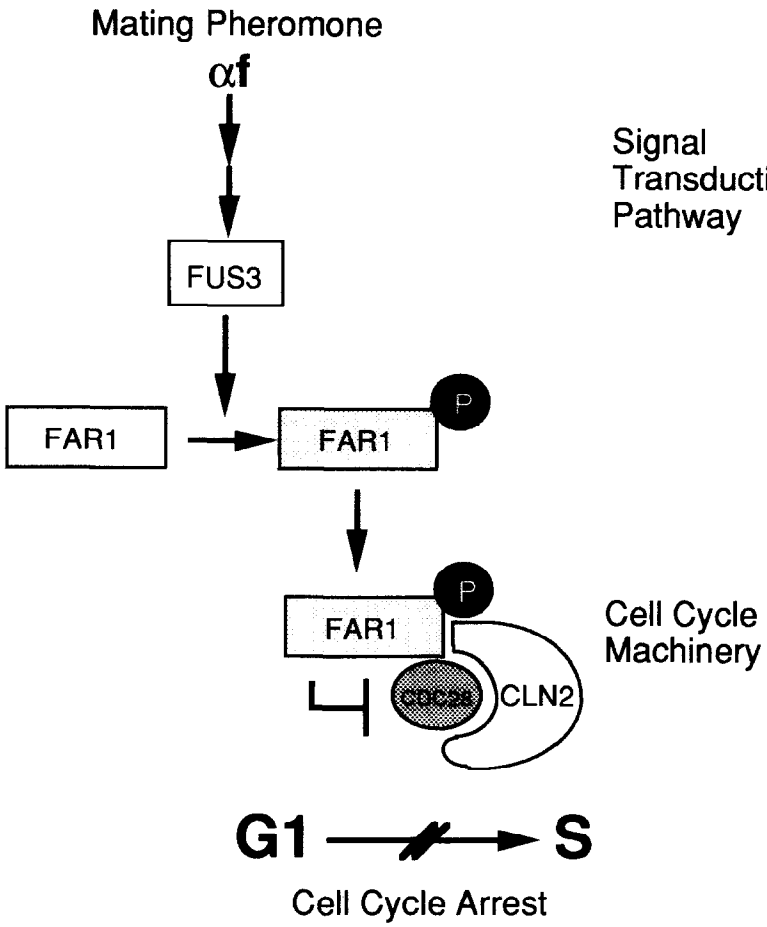

Figure 8. Cell Cycle Inhibition of FAR1 and Its Activation by FUS3

Stimulation of the signal transduction pathway by α factor results in activation of FUS3 and increase of FAR1 protein levels. Activated FUS3 phosphorylates FAR1, which is then able to bind efficiently to the CDC28–CLN2 kinase. Associated CDC28–CLN2 kinase further phosphorylates FAR1 in the complex. We propose that association of FAR1 with the CDC28–CLN2 kinase leads to inhibition of the kinase, perhaps directly or by causing degradation of CLN2, and thus arrest of cells in G1. The functional significance of phosphorylation of FAR1 by CDC28–CLN2 kinase is not known, but may be necessary either for activation of FAR1 to provoke cell cycle arrest or for inactivation of FAR1 to promote recovery from cell cycle arrest.

residues 235–340 (Figure 6). One possibility is that the former segment includes the region that is phosphorylated by FUS3 to govern association with CDC28–CLN2 (see Figures 1 and 2) and that the latter segment spans the actual binding site. We do not know at present whether FAR1 binds the kinase complex by contacting only CDC28 or CLN2 or whether it contacts both proteins simultaneously.

Full phosphorylation of FAR1 in vivo in response to α factor requires not only active FUS3 but also functional CDC28: FAR1 is still phosphorylated under CDC28-deficient conditions in response to α factor (presumably by FUS3), but the slowest migrating form is missing (see Figure 4D). We have also observed that FAR1 is phosphorylated in vitro by an associated kinase that is likely to be CDC28 (see also M. Tyers and B. Futcher, submitted). Although we have no evidence for a physiological role for this *CDC28*-dependent phosphorylation, numerous possibilities can be proposed. For example, phosphorylation might be necessary for FAR1 to inhibit the CDC28–CLN2 kinase (see below), or, alternatively, phosphorylation might inactivate FAR1 and facilitate recovery of cells from α factor arrest.

We propose the following sequence of events necessary for G1 arrest (Figure 8). In response to α factor, FAR1 is first phosphorylated by activated FUS3. This phosphorylation enables FAR1 to bind efficiently to CDC28–CLN2. It is important to note that the cellular activity of FAR1 is increased in response to α factor in two ways: the level of FAR1 protein increases, and FAR1 is phosphorylated by FUS3 and thereby activated. Associated CDC28–CLN2 kinase then further phosphorylates FAR1, with yet unknown consequences for the complex.

Exposure of **a** cells to α factor causes a decrease in transcription of *CLN1* and *CLN2*, as well as a drop in amount of CLN2 protein (Wittenberg et al., 1990; Valdivieso et al., 1993). Our findings indicate that the primary action of FAR1 is to inhibit CDC28–CLN directly and that the decrease in *CLN* transcript levels is an indirect consequence of reduction in CDC28–CLN2 activity. The decrease in transcription would be due to inactivation of the positive feedback loop by which CLN2 stimulates its own synthesis (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991).

There are several possible ways in which FAR1 might control the activity of CDC28–CLN2. For example, binding might directly inhibit kinase activity or prevent the kinase from interacting with key substrates. A similar inhibition mechanism has recently been proposed for the p40 protein, which binds to CDC28 kinase after mitosis (Mendenhall, 1993). Alternatively, binding of FAR1 to CDC28–CLN2 might target the kinase for inactivation, for example,

by changing the phosphorylation state of the CDC28 subunit or by inducing degradation of CLN2.

Do FAR1 and FUS3 Inhibit Multiple G1 Cyclins?

A compendium of arguments has led to the view that arrest of the cell cycle by α factor requires inhibition of CLN1, CLN2, and CLN3 (Wittenberg et al., 1990; Chang and Herskowitz, 1990; Elion et al., 1990; Tyers et al., 1992). First, any of these three cyclins is sufficient to support vegetative growth; all combinations of double mutants are viable and α factor sensitive, whereas the triple mutant is inviable (Richardson et al., 1989; Cross, 1990). Second, mutants that produce hyperactive versions of CLN3 are resistant to arrest by α factor (Nash et al., 1988; Cross, 1988). If full activity of FAR1 is dependent on phosphorylation by FUS3, then *fus3*$^-$ mutants should exhibit phenotypes in common with *far1*$^-$ mutants. Indeed, both *fus3*$^-$ and *far1*$^-$ mutants fail to arrest in response to α factor despite having an intact signal transduction pathway (Elion et al., 1990, 1991; Chang and Herskowitz, 1990).

On the basis of genetic arguments, it has been proposed that independent pathways inhibit CLN1, CLN2, and CLN3 (Chang and Herskowitz, 1990; Elion et al., 1990; see also Nasmyth, 1990). Recent studies, however, make this simple view no longer tenable. First, FAR1 antagonizes both CLN1 and CLN2 (Valdivieso et al., 1993; F. Cross, personal communication; M. Tyers and B. Futcher, submitted). Second, FUS3 inhibits not only CLN3 but also CLN1 and CLN2 (Elion et al., 1991; G. A., G. Amon, and K. Nasmyth, unpublished data; M. Tyers and B. Futcher, submitted; B. Satterberg and E. Elion, personal communication). Third, inactivation of individual G1 cyclins also affects expression of other G1 cyclins, owing to the existence of positive feedback mechanisms (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; see also Tyers et al., 1992). These results suggest that FAR1 and FUS3 antagonize multiple G1 cyclins, and they are consistent with our observation that FUS3 activates FAR1 for cell cycle arrest.

Parallels between FAR1 and Retinoblastoma Proteins

FAR1 and the retinoblastoma protein (Rb) have some formal similarities in that both products appear to restrain cell growth (reviewed by Weinberg, 1990; Ducommon, 1991; Cobrinik et al., 1992). Both FAR1 and Rb associate with cyclin-dependent kinases of the p34^{cdc2} family (see above; Hu et al., 1992), and both associate with G1 cyclins (this paper; Matushime et al., 1992; Hinds et al., 1992; for review see Cobrinik et al., 1992). The initial phosphorylation of Rb after mitogen stimulation (DeCaprio et al., 1992) may be analogous to the phosphorylation of FAR1 by FUS3. The negative growth factors of yeast act by controlling activity of the G1 cyclins, as may also be true for growth factors of mammalian cells (Matsushime et al., 1991). Indeed, Rb associates with cyclin D1 (Matushime et al., 1992; S. F. Dowdy and R. A. Weinberg, personal communication), and recent evidence suggests that Rb may regulate cyclin D1 activity (S. F. Dowdy and R. A. Weinberg, personal communication). Key questions about Rb are what its downstream target is and what governs its activity. Our studies provide answers to both questions for FAR1. Whether the answers for Rb are analogous remain to be determined.

Experimental Procedures

Preparation of Extracts and Immunochemical Techniques

Immunoblots

Total yeast protein extracts used for immunoblotting were prepared as described by Hann and Walter (1991). Cells were resuspended in 0.5 ml of ice-cold buffer A (20 mM Tris [pH 8.0], 50 mM NH_4OAc, 0.5 mM EDTA, 2% trasylol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 2 mM leupeptin) and immediately mixed with 0.5 ml of ice-cold 20% trichloroacetic acid. One volume of acid-washed glass beads was added, and cells were vortexed twice for 30 s using a bead beater (high setting), with 1 min cooling on ice in between. The liquid was removed, and the beads were washed with 0.5 ml of a 1:1 mixture of buffer A and 20% trichloroacetic acid. Then the two supernatants were pooled and the proteins pelleted by centrifugation for 10 min at 15,000 × g at 4°C. The pellet was resuspended in trichloroacetic acid–sample buffer (Hann and Walter, 1991), and extracts were boiled for 10 min. The insoluble cell debris was separated by 10 min centrifugation at 15,000 × g at room temperature. Proteins were separated by SDS–polyacrylamide gel electrophoresis and electroblotted to nitrocellulose (Schleicher & Schuell, Inc.) using the Bio-Rad Minigel system as recommended by the manufacturer. Blots were blocked by incubation for 3–5 hr in Tris-buffered saline–Triton X-100 (TBST; 50 mM Tris [pH 7.5], 500 mM NaCl, 0.1% Triton X-100) containing 10% fat-free milk powder. Antibodies were incubated in TBST containing 2% milk for 3–12 hr before the blots were washed three times for 10 min with TBST. Secondary antibodies were phosphatase-coupled conjugates of either protein A (Boehringer Mannheim), goat anti-rabbit immunoglobulin G (IgG) (Boehringer Mannheim), or goat anti-mouse IgG (Bio-Rad Laboratories) diluted 1:2000 in TBST containing 2% milk. After 60 min of incubation at room temperature, the blots were washed three times for 10 min with TBS containing 0.3% Triton X-100 and three times for 10 min with TBST. Blots were developed using the epichemiluminescence detection reagents provided by Amersham Corporation, as recommended by the manufacturer.

Immunoprecipitations

Extracts for immunoprecipitation experiments were prepared as follows (Harlow and Lane, 1988). Cells were pelleted and resuspended either in buffer N (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 10 mM NaF, 60 mM β-glycerophosphate, 1 μM calyculin A, 1% trasylol, 0.1 mM PMSF, 1 mM benzamidine, 1 mM leupeptin) or in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM sodium phosphate [pH 7.2], 100 mM NaCl, 20 mM NaF, 80 mM β-glycerophosphate, 1 μM calyculin A, 1% trasylol, 0.1 mM PMSF, 1 mM benzamidine, 1 mM leupeptin). One volume of acid-washed glass beads was then added, and cells were lysed in four 30 s cycles using a bead beater (high setting). Cell breakage was routinely checked using a microscope. Extracts were centrifuged (15,000 × g) for 10 min at 4°C, and the supernatant was removed and centrifuged as before for an additional 10 min. In some cases, the extracts were precleared by incubation for 30 min at 4°C with secondary antibodies coupled to agarose or Affigel (see below) to remove proteins that bind nonspecifically. RNAase A and DNAase I (30 μg/ml each) were added, and the extracts were incubated for 1 hr on ice with the primary antibodies. HA–peptide (NH_2–YPYDVPDYA–COOH; Field et al., 1988) was included as appropriate in the immunoprecipitations at a final concentration of 250 μM. The extracts were then centrifuged (15,000 × g) for 10 min at 4°C, and the supernatant was further incubated with gentle shaking at 4°C for 45 min with either goat anti-mouse IgG, anti-rabbit IgG coupled to agarose (Sigma A-6531 and A-8914, respectively), or protein A coupled to Affigel (Bio-Rad Laboratories). Immune complexes were washed four times with ice-cold buffer N or RIPA buffer and twice with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.2]); samples were then boiled in gel sample buffer and centrifuged, and the supernatant was subjected to SDS–polyacrylamide gel electrophoresis.

Metabolic Labeling with [^{32}P]Phosphate

Cells transformed with pTP9 were pregrown overnight at 30°C in selective medium (SD-URA) and transferred into fresh phosphate-depleted YEP medium (Rubin, 1975) supplemented with galactose (2%) and sucrose (0.5%) at an OD_{600} of about 0.1. Cells were grown to an OD_{600} of 0.6, harvested by centrifugation, and resuspended in 10 ml of medium as above containing 500 μCi/ml [^{32}P]orthophosphate (New England Nuclear). α factor (1 μg/ml) was added to half of the culture, and the cells were incubated for 30 min at 30°C. Cells were harvested, washed twice with ice-cold PBS, and resuspended in 1 ml of RIPA buffer supplemented with 50 mM sodium phosphate. Immunoprecipitations were performed as above.

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis

Two-dimensional analysis of tryptic phosphopeptides was carried out as described previously (Peter et al., 1990), except that Kodak 13255 cellulose thin-layer chromatography plates were used. Phosphoamino acid analyses were performed in one dimension as described by Gu et al. (1992) or in two dimensions as described by Peter et al. (1990).

In Vitro Phosphorylation Assays

FUS3 Kinase Assays

Fifty milliliter cell cultures (K2702) were grown in selective medium to an OD_{600} of 0.8–1.2 before α factor was added to 1 μg/ml for 30 min. Cells were harvested by centrifugation at 4°C and washed twice with 5 ml of ice-cold stop mix (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, 50 mM NaF). Cell pellets were disolved in 200 μl of lysis buffer (150 mM NaCl, 15 mM $MgCl_2$, 50 mM Tris–HCl [pH 7.5], 1% Nonidet P-40, 0.1 mM sodium orthovanadate, 15 mM p-4-nitrophenylphosphate, 40 μg/ml aprotinin, 20 μg/ml leupeptin). An equal volume of acid-washed glass beads was added, and lysis was effected by two 3 min bursts of shaking. The extract was clarified by centrifugation (once for 5 min and twice for 15 min in a microfuge), and the protein concentration was determined using the Bio-Rad protein assay. Extract (180 μg) was adjusted to 30 μl with lysis buffer and incubated for 1 hr on ice with 3 μg of anti-Myc antibody 9E10. The immune complexes were then harvested by addition of 20 μl of a 1:1 suspension of protein A beads for 1 hr at 4°C. Immunoprecipitates were washed five times with lysis buffer and twice with 25 mM MOPS (pH 7.2), 0.1 mM sodium orthovanadate, 15 mM p-4-nitrophenylphosphate. Six milliliters of HBII buffer (25 mM MOPS [pH 7.2], 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol [DTT], 0.1 mM sodium orthovanadate, 15 m M p-4-nitrophenylphosphate, 1 mM PMSF, 40 μg/ml aprotinin, 20 μg/ml leupeptin) was then added, and the reactions were prewarmed for 2 min at 30°C.

The kinase reactions were started by addition of 10 ml of reaction cocktail (25 mM MOPS [pH 7.2], 500 μl of FAR1, 3 μCi of [γ-^{32}P]ATP [6000 Ci/mmol]) and incubated for 5 min at 30°C. Reactions were stopped by addition of 40 μl of 3× sample buffer (Laemmli, 1970) and subsequent boiling for 3 min.

Kinase Assays with Purified p44^{mpk}

Sea star oocyte p44^{mpk} purified to homogeneity as described by Sanghera et al. (1990) was obtained from S. Pelech. Kinase assays were performed as described previously (Peter et al., 1992).

Kinase Assays Using Immunoprecipitates

GST–FAR1 or GST–FAR1 deletion mutants as well as CDC28–HA and CLN2–HA were expressed in appropriate yeast strains and then immunoprecipitated as described above. The immunoprecipitates were divided in equal aliquots and prewarmed for 5 min at 30°C. In the case of *cdc-ts* mutants, aliquots were prewarmed for 15 min at either 24°C or 37°C. Reactions were started by addition of 30 ml of prewarmed kinase mix (10 mM Tris [pH 7.5], 10 μM ATP, 10 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP [Amersham Corporation], 1 mM PMSF, 1% aprotinin, 1 mg/ml leupeptin, 1 mM benzamidine) and incubated at 30°C. In the case of *cdc-ts* mutants, reactions were incubated at 24°C or 37°C. One aliquot was routinely immunoblotted to control the efficiency of the immunoprecipitation. When indicated, histone H1 (Boehringer Mannheim) was included in the kinase mix at a final concentration of 0.5 mg/ml. After 10 min the kinase reactions were centrifuged, and 30 μl of 3× sample buffer was added to the supernatants. The pellets were washed three times with ice-cold immunoprecipitation buffer and twice with PBS before phosphorylated proteins were eluted with 60 μl of sample buffer. Samples were boiled for 5 min and centrifuged, and the supernatants were analyzed by SDS–polyacrylamide gel electrophoresis.

Preparation of FAR1 Substrates for In Vitro Phosphorylation Assays

E. coli (TG1-DE3) harboring the *FAR1* expression plasmid pGA1896 was grown in Luria broth containing 2 mg/l ampicillin to an OD_{600} of 0.4–0.6 and induced with 0.6 mM isopropyl β-D-thiogalactopyranoside for 2 hr. Cells were harvested and lysed in 20 ml of lysis buffer (100 mM Tris–HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM PMSF, 2 μg/ml pepstatin and leupeptin, 1 mg/ml lysozyme) for 30 min at 4°C. $MgCl_2$, $MnCl_2$, and DNAase I were then added to final concentrations of 10 mM, 1 mM, and 10 mg/ml, respectively. After 30 min at room temperature, 20 ml of detergent buffer (0.25 M NaCl, 1% deoxycholic acid [w/v], 1% Nonidet P-40 [v/v], 20 mM Tris–HCl [pH 7.5], 2 mM EDTA) was added, and the mixture was vortexed. The extract was spun for 20 min at 15,000 rpm (SS34 rotor), and the inclusion body pellet was washed four times in 0.5% Triton X-100, 1 mM EDTA and dissolved in 2 ml of 8 M urea, 50 mM Tris–HCl (pH 8.0), 1 mM DTT, 1 mM EDTA. Then 2 ml of 2× sample buffer (Laemmli, 1970) was added, and the sample was boiled for 3 min. The proteins were further purified by preparative SDS–polyacrylamide gel electrophoresis. FAR1 was visualized by aqueous Coomassie blue staining (50% methanol, 0.05% Coomassie brilliant blue R), and the band was excised, crushed by pressing through a syringe, and extracted overnight in 50 mM Tris–HCl, 100 mM NaCl, 0.1 mM EDTA, 0.2% SDS. After removal of the acrylamide, the protein was precipitated in 4 vol of acetone at −20°C for 30 min and centrifuged (20,000 rpm, 20 min), and the pellet was dissolved in 50 mM Tris (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 0.05% SDS. Purified FAR1 was then dialyzed against 50 mM MOPS (pH 7.2) using a CENTRICON 10 microconcentrator.

E. coli harboring the expression plasmid pGEX3–*FAR1*–NH_2 was induced as described above. Cells were pelleted, frozen in liquid nitrogen, and lysed at 4°C in 5 vol of PBS containing 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% aprotinin, 2 mg/ml leupeptin, and 1 mg/ml lysozyme. The lysate was centrifuged for 60 min at 10,000 rpm in a Sorvall SS34 rotor. The supernatant was incubated for 30 min at 4°C with glutathione–agarose (Sigma G-451) and then loaded on a column. The column was washed with 50–100 ml of PBS containing 0.1% Tween-20 and 1 mM DTT and 2 column volumes of PBS containing 1 mM DTT. GST–FAR1–NH_2 was eluted with PBS containing 10% glycerol, 100 mg/ml bovine serum albumin, and 10 mM reduced glutathione.

Antibodies

Antibodies against the HA epitope were obtained from Berkeley Antibody Company. Polyclonal antibodies against GST were prepared and affinity purified by Dr. D. Kellogg essentially as described by Kellogg et al. (1992). Polyclonal antibodies against FAR1 have been reported previously and were affinity purified as described (Chang and Herskowitz, 1992). Polyclonal peptide antibodies against CDC28 were used as described by R. Deshaies et al. (unpublished data).

DNA Manipulations

Plasmid Constructions

Standard DNA manipulations were performed according to Sambrook et al. (1989) or Ausubel et al. (1989). DNA fragments were eluted from agarose gels using Quiaex as recommended by the manufacturer (Quiagen Inc.). Polymerase chain reaction (PCR) primers were synthesized using a Millipore oligonucleotide synthesizer. PCRs were performed with Vent polymerase (New England Biolabs) as recommended by the manufacturer (20 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C).

Construction of HA Epitope-Tagged CLN2 (pTP35)

The genomic ClaI–SphI fragment of CLN2 was ligated into pRS316 (Sikorski and Hieter, 1989) to give rise to the plasmid AS100. AS100 was then digested with XhoI and KpnI, and the vector fragment was gel purified and ligated with the XhoI–KpnI fragment containing *CLN2* derived from plasmid pMT104 (M. Tyers and B. Futcher, submitted) to give rise to pTP35. This plasmid is able to complement fully a strain lacking *CLN1*, *CLN2*, and *CLN3* (M. P., unpublished data).

Table 1. Yeast Strains

Strain	Genotype	Source
FC279	**a** *ura3Δ his2 ade1 trp1 leu2 bar1::LEU2*	Chang and Herskowitz, 1990
FC280	**a** *ura3Δ his2 ade1 trp1 leu2 bar1::LEU2 far1::URA3*	Chang and Herskowitz, 1990
YMP18	**a** *ura3Δ his2 ade1 trp1 leu2 bar1::LEU2 far1::TRP1*	This study
BH1001	**a** *ura3-52 his4-519 leu2 trp1 can1-101 FUS1::lacZ (URA3)*	Neiman et al., 1990
BH1005	**a** *ura3-52 his4-519 leu2 trp1 can1-101 FUS1::lacZ (URA3) ste12::TRP1*	Neiman et al., 1990
BH1008	**a** *ura3-52 his4-519 leu2 trp1 can1-101 FUS1::lacZ (URA3) ste11Δ*	Neiman et al., 1990
AN1014	**a** *ura3-52 his4-519 leu2 trp1 can1-101 FUS1::lacZ (URA3) fus3::LEU2*	A. Neiman
AN1002	**a** *ura3-52 his4-519 leu2 trp1 can1-101 FUS1::lacZ (URA3) ste5::LEU2*	A. Neiman
AN1012	**a** *ura3-52 his4-519 leu2 trp1 ste7Δ FUS1::lacZ (LEU2)*	Neiman et al., 1993
AN37-4C-S	**a** *ura3-del leu2 trp1 ade2 met1 HML***a** *HMR***a** *bar1-1*	Neiman et al., 1993
AN1015	**a** *ura3-del leu2 trp1 ade2 met1 HML***a** *HMR***a** *bar1-1 fus3::URA3*	Neiman et al., 1993
YMP19	**a** *ura3-del leu2 trp1 ade2 met1 HML***a** *HMR***a** *bar1-1 fus3::TRP1*	This study
AN42-3A	**a** *his4-519 ura3-52 leu2 trp1 kss1::URA3*	A. Neiman
AN42-2A	**a** *his4-519 ura3-52 leu2 trp1 fus3::URA3 kss1::URA3*	A. Neiman
K2702	**a** *HML***a** *HMR***a** *bar1::HISG fus3::LEU2 kss1::URA3 fus1::lacZ-URA3 hoΔ ura3-52 ade2-1 can1-100 his3 leu2-3,112 trp1-1 met*	Gartner et al., 1992
PS558	**a** *ade1 his2 leu2 trp1 ura3 bar1-1*	P. Sorger
PS560	**a** *ade1 his2 leu2 trp1 ura3 bar1-1 cdc28-13*	P. Sorger
K699	**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL⁺ psi⁺*	P. Sorger
K1989	**a** *cdc28-4 trp1-1 leu2-3,112, his3-11,15 ura3*	P. Sorger
RD270-4C	**a** *CDC28-HA::TRP1 can1-100 leu2 ura3 trp1*	R. Deshaies
MTy263	**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL⁺ psi⁺ cln2::CLN2-HA-LEU2*	M. Tyers
Y57	**a** *ura3-52 trp1Δ63 leu2 his3-Δ1 prb1-1122 pep4-3 prc1-407*	J. Li
YMP05	**a** *ura3-52 trp1Δ63 leu2 his3-Δ1 prb1-1122 pep4-3 prc1-407 bar1::LEU2*	This study
YDH60	**a** *ura3-52 leu2-3,2-112 ade1 trp1-DH1 GAL1::STE4(LYS2) FUS1::HIS3(HIS3) mfa2-Δ1::FUS1::lacZ*	J. Horecka
IH1792	**a** *cry1 lys1*	Laboratory collection
IH1793	α *lys1*	Laboratory collection

Construction of the GST-FAR1 Fusion (pTP9)

pFC24 vector (Chang and Herskowitz, 1992) was first digested with NotI and subsequently filled in using Klenow polymerase. The reaction was further digested with XhoI, and the XhoI–NotI *FAR1* fragment was gel purified. The pRD54 vector (R. Deshaies, unpublished data) was digested with KpnI, blunt ended using T4 DNA polymerase, and further digested with XhoI. The vector fragment was gel purified and ligated with the *FAR1* fragment to give pTP9. This plasmid fully complements a *far1* deletion strain (YMP18) for both cell cycle arrest in response to α factor as well as for the mating defect (M. P., unpublished data).

Construction of FAR1 Deletion Mutants

NH_2-terminal deletion mutants were constructed by PCR using pFC21 as the template. The downstream primer, oFC1 (5′-CCTGTGAAGC-TTCTCGCCG-3′), covers the HindIII site within the *FAR1* coding sequence (Chang and Herskowitz, 1992). The following primers were used:

oTP1: 5′-CGCTCGAG**ATG**AGTTTACTCCGAGAGTCG-3′.
oTP2: 5′-CGCTCGAG**ATG**GTTGTGGAGTCTACGTG-3′.
oTP11: 5′-CCCGCTCGAG**ATG**GCATCTGGTTTTTCG-3′.

PCR products were gel purified and digested with XhoI and HindIII. The XhoI–BamHI vector fragment of pFC24 (Chang and Herskowitz, 1992) was ligated in a triple ligation containing the PCR fragments and the HindIII–BglII–*FAR1* fragment obtained from a HindIII–BglII digest of pFC21 (see Chang and Herskowitz, 1992) to give the plasmids pTP11 (FAR1Δ79), pTP12 (FAR1Δ169), and pTP15 (FAR1Δ49). GST fusions of these NH_2-terminal deletion mutants of *FAR1* were generated by ligation of the XhoI–SphI fragments isolated from pTP11, pTP12, or pTP15, respectively, and the XhoI–SphI vector fragment obtained from pTP9.

Construction of carboxy-terminal deletion mutants of *FAR1* involved use of pFC24 as a PCR template. The upstream PCR primer oFC2 was 5′-CGCTCGAGCAACAG**ATG**CCCA-3′. The following downstream primers containing a stop codon and a KpnI cloning site were used:

oTP15: 5′-CCCGGTACCAGTTTAATTTGTAGCTTTGATG-3′.
oTP16: 5′-CCCGGTACCTTACTCACCTATGTACC-3′.
oTP17: 5′-CCCGGTACCTTACATATTGGATGACGG-3′.
oTP18: 5′-CCCGGTACCCTAAACATGTTCTGGACC-3′.

The PCR products were gel purified, digested with XhoI and KpnI, and ligated into pRD54 or pRD56 vectors (R. Deshaies, unpublished data) digested with XhoI and KpnI to give rise to pTP16 (GST–FAR180NH_2), pTP17 (GST–FAR1150NH_2), pTP18 (GST–FAR1235-NH_2), and pTP19 (GST–FAR1340NH_2). Internal deletion mutants of *FAR1* were constructed by introducing in-frame BglII sites at the deletion junctions. PCR fragments were amplified using pFC24 from either the 5′ end of *FAR1* using the primer oFC2 (see above) or from the unique SphI site located within the *FAR1* coding sequence using the primer oTP23 (5′-CCCATCAGCATGCGACAAATTC-3′). The following PCR oligonucleotides were used:

oTP21: 5′-GAGTGTAAGATCTCTGGCCAAGTG-3′.
oTP22: 5′-GATTAAACAGATCTCACCTATGTACC-3′.
oTP24: 5′-CTGTTAGATCTTGGATGACGG-3′.
oTP25: 5′-GAACAGATCTTGGTCAGTGTG-3′.

PCR products were gel purified and digested with XhoI–BglII and with SphI–BglII, respectively. The fragments were then ligated in a triple ligation into pFC24 digested with XhoI and SphI to give rise to pTP24 (FAR1Δ150-235), pTP25 (FAR1Δ235-340), and pTP26 (FAR1-Δ150-340). GST fusions of these internal deletions of *FAR1* were generated by ligation of the XhoI–SphI fragments into pTP9.

Construction of Myc Epitope-Tagged FUS3

Plasmids pGA1903 and pGA1905 have been described previously (Errede et al., 1993).

Construction of FAR1 Expression Plasmids for E. coli

The coding sequence of the FAR1 amino terminus (amino acids 1–251) was cloned by PCR using the primers Far1–PCR1 (5′-CACCA-GAATTCAAGAAGCAACATATGCCCACATTTGGG-3′) and Far1–PCR2 (5′-GAGGGAATTCACCTGTGAAGCTTCTCGCCG-3′), which intro-

duced an NdeI site at the start codon and a HindIII site, respectively. The amplified product was cloned as an NdeI–HindIII fragment into the T7 polymerase–inducible expression vector pRK171a (Brizuela et al., 1987), giving rise to plasmid pGA1896.

Plasmid pGEX3–FAR1NH_2 was constructed by digesting pFC28 (Chang, 1991) with SpeI and XbaI followed by religation.

Strain Constructions and Yeast Manipulations

YEPD (complete) medium, SD (minimal) medium, and supplements are described by Hicks and Herskowitz (1976). Standard yeast genetic techniques were used (Sherman et al., 1982). Cell cycle arrest in response to α factor was determined by growth in liquid culture and by halo assays as described (Chang and Herskowitz, 1990, 1992). Yeast transformations were performed by the lithium acetate procedure (Ito et al., 1983). Strain YMP05 was constructed by disruption of the *BAR1* gene using the plasmid ZV77 as described by MacKay et al. (1988). Strains YMP18 and YMP19 were constructed by replacing the *URA3* gene of FC280 and AN1015, respectively, with the *TRP1* gene using the plasmid pJO119 digested with KpnI (Ogas, 1992). Strains are as described in Table 1.

Acknowledgments

We thank M. Tyers, V. L. MacKay, P. Sorger, R. Sikorski, J. Ogas, J. W. Dolan, A. Neiman, J. Li, A. Sil, and R. Deshaies for yeast strains and plasmids. Special thanks go to D. Kellogg and R. Deshaies for the kind gift of antibodies against GST and CDC28, respectively. We are also grateful to A. Neiman for construction of the GST–*FAR1* expression plasmid, S. Pelech for the kind gift of purified $p44^{mpk}$ kinase, and Y. Gu for help with the tryptic phosphopeptide maps. We thank M. Tyers, B. Futcher, F. Cross, B. Errede, E. Elion, and S. F. Dowdy for communicating results prior to publication and F. Chang, D. Morgan, R. Deshaies, J. Ogas, A. Neiman, and J. Gray for many helpful discussions.

This work was supported by the Swiss National Science Foundation and by a Program Project Grant from the National Institutes of Health to I. H. (GM31286). A. G. and G. A. were supported by grants from the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung. Work at the University of Oregon was supported by a Research Grant (GM30027) from the National Institutes of Health to George Sprague.

Received December 18, 1992; revised February 22, 1993.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989). Current Protocol in Molecular Biology (New York: Wiley Interscience and Green Publishing Associates).

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinno, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell *65*, 663–676.

Brizuela, L., Draetta, G., and Beach, D. (1987). $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. EMBO J. *6*, 3707–3714.

Cairns, B. R., Ramer, S. W., and Kornberg, R. D. (1992). Order of action of components in the yeast pheromone-response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. Genes Dev. *6*, 1305–1318.

Chang, F. (1991). Regulation of the cell cycle by a negative growth factor in yeast. PhD thesis, University of California, San Francisco, California.

Chang, F., and Herskowitz, I. (1990). Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. Cell *63*, 999–1011.

Chang, F., and Herskowitz, I. (1992). Phosphorylation of FAR1 in response to α-factor: a possible requirement for cell cycle arrest. Mol. Biol. Cell *3*, 445–450.

Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991). Definition of a consensus sequence for peptide substrate recognition by $p44^{mpk}$, the meiosis-activated myelin basic protein kinase. J. Biol. Chem. *266*, 15180–15184.

Cobrinik, D., Dowdy, S. F., Hinds, P. W., Mittnacht, S., and Weinberg, R. A. (1992). The retinoblastoma protein and the regulation of cell cycling. Trends Biol. Sci. *17*, 312–315.

Courchesne, W. E., Kunisawa, R., and Thorner, J. (1989). A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. Cell *50*, 1107–1119.

Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992a). The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science *258*, 478–480.

Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992b). Erks: their fifteen minutes has arrived. Cell Growth Diff. *3*, 135–142.

Cross, F. R. (1988). DAF1, a mutant gene affecting size control, pheromone arrest and cell cycle kinetics of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *8*, 4675–4684.

Cross, F. R. (1990). Cell-cycle arrest caused by CLN gene deficiency in *Saccharomyces cerevisiae* resembles START I arrest and is independent of the mating pheromone signal. Mol. Cell. Biol. *10*, 6482–6490.

Cross, F. R., and Tinkelenberg, A. H. (1991). A potential positive feedback loop controlling CLN1 and CLN2 gene expression at Start of the yeast cell cycle. Cell *65*, 875–883.

DeCaprio, J. A., Furukawa, Y., Ajchenbaum, F., Griffin, J. D., and Livingston, D. M. (1992). The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression. Proc. Natl. Acad. Sci. USA *89*, 1795–1798.

Dirick, L., and Nasmyth, K. (1991). Positive feedback in the activation of G1 cyclins in yeast. Nature *351*, 754–757.

Dolan, J. W., Kirkman, C., and Fields, S. (1989). The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Natl. Acad. Sci. USA *86*, 5703–5707.

Ducommon, B. (1991). From growth to cell cycle control. Sem. Cell Biol. *2*, 233–241.

Elion, E. A., Grisafi, P., and Fink, G. R. (1990). FUS3 encodes a $cdc2^+$/CDC28-related kinase required for the transition from mitosis into conjugation. Cell *60*, 649–664.

Elion, E. A., Brill, J. A., and Fink, G. R. (1991). FUS3 inactivates G1 cyclins and, in concert with KSS1, promotes signal transduction. Proc. Natl. Acad. Sci. USA *88*, 9392–9396.

Errede, B., and Ammerer, G. (1989). STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein–DNA complexes. Genes Dev. *3*, 1349–1361.

Errede, B., Gartner, A., Zhou, Z.-Q., Nasmyth, K., and Ammerer, G. (1993). MAP-kinase related FUS3 from *S. cerevisiae* is activated by STE7 kinase in vitro. Nature *362*, 261–264.

Field, J., Nikawa, J.-I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988). Purification of RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Fields, S., Chaleff, D. T., and Sprague, J. F., Jr. (1988). Yeast STE7, STE11 and STE12 genes are required for expression of cell-type-specific genes. Mol. Cell. Biol. *8*, 551–556.

Fujimura, H. (1990). Molecular cloning of the DAC2/FUS3 gene essential for pheromone-induced G1-arrest of the cell cycle in *Saccharomyces cerevisiae*. Curr. Genet. *18*, 395–400.

Futcher, B. A. (1991). *Saccharomyces cerevisiae* cell cycle: cdc28 and the G1 cyclins. Sem. Cell Biol. *2*, 205–212.

Gartner, A., Nasmyth, K., and Ammerer, G. (1992). Signal transduction in *S. cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Genes Dev. *6*, 1280–1292.

Gu, Y., Rosenblatt, J., and Morgan, D. O. (1992). Cell cycle regulation of cdk2 activity by phosphorylation of Thr160 and Tyr15. EMBO J. *11*, 3995–4005.

Hanks, S. K., Quin, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42–52.

Hann, B., and Walter, P. (1991). The signal recognition particle in *S. cerevisiae*. Cell *67*, 131–144.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Hicks, J. B., and Herskowitz, I. (1976). Interconversion of yeast mating types. Direct observations of the action of the homothallism (HO) gene. Genetics *83*, 245–258.

Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell *70*, 993–1006.

Hu, Q., Lees, J. A., Buchkovitch, K., and Harlow, E. (1992). The retinoblastoma protein physically associates with the human cdc2 kinase. Mol. Cell. Biol. *12*, 971–978.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Kellogg, D. R., and Alberts, B. M. (1992). Purification of a multiprotein complex containing centrosomal proteins from the *Drosophila* embryo by chromatography with low-affinity polyclonal antibodies. Mol. Biol. Cell *3*, 1–11.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Leberer, E., Dignard, D., Harcus, D., Thomas, D. Y., and Whiteway, M. (1992). The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein βγ subunits to downstream signalling components. EMBO J. *11*, 4815–4824.

MacKay, V. L., Welch, S. K., Insley, M. Y., Manney, T. R., Holly, J., Saari, G. C., and Parker, M. L. (1988). The *Saccharomyces cerevisiae BAR1* gene encodes an exported protein with homology to pepsin. Proc. Natl. Acad. Sci. USA *85*, 55–59.

Marsh, L., Neiman, A. M., and Herskowitz, I. (1991). Signal transduction during pheromone response in yeast. Annu. Rev. Cell Biol. *7*, 699–728.

Matsushime, H., Roussel, M. F., Ashmun, R. A., and Sherr, C. J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell *65*, 701–713.

Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J.-Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. (1992). Identification and properties of an atypical catalytic subunit ($p34^{PSK}$/cdk4) for mammalian D type cyclins. Cell *71*, 323–334.

Mendenhall, M. D. (1993). An inhibitor of $p34^{CDC28}$ protein kinase activity from *Saccharomyces cerevisiae*. Science *259*, 216–219.

Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, B. (1988). The *WHI1*$^+$ gene in *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. *7*, 4335–4346.

Nasmyth, K. A. (1990). FAR-reaching discoveries about the regulation of START. Cell *63*, 1117–1120.

Neiman, A. M., Chang, F., Komachi, K., and Herskowitz, I. (1990). CDC36 and CDC39 are negative elements in the signal transduction pathway of yeast. Cell Reg. *1*, 391–401.

Neiman, A. M., Stevenson, B. J., Xu, H.-P., Sprague, G. F., Jr., Herskowitz, I., Wigler, M., and Marcus, S. (1993). Functional homology of protein kinases required for sexual differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* suggests a conserved signal transduction module in eukaryotic organisms. Mol. Biol. Cell *4*, 107–120.

Ogas, J. P. (1992). Identification and analysis of regulators of G1 progression in *Saccharomyces cerevisiae*. PhD thesis, University of California, San Francisco, California.

Pelech, S. L., and Sanghera, J. S. (1992). Mitogen-activated protein kinases: versatile transducers for cell signaling. Trends Biochem. Sci. *17*, 233–238.

Peter, M., Nakagawa, J., Labbé, J.-C., Dorée, M., and Nigg, E. A. (1990). In vitro disassembly of the nuclear lamina and M-phase specific phosphorylation of lamins by cdc2 kinase. Cell *61*, 591–602.

Peter, M., Sanghera, J. S., Pelech, S., and Nigg, E. A. (1992). MAP kinases phosphorylate nuclear lamins and display sequence specificity overlapping with cdc2 kinase. Eur. J. Biochem. *205*, 287–294.

Ramer, S. W., and Davis, R. W. (1993). A dominant truncation allele identifies a gene, STE20, that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *90*, 452–456.

Reed, S. I. (1980). The selection of *S. cerevisiae* mutants defective in the START event of cell division. Genetics *95*, 561–567.

Reed, S. I. (1991). Pheromone signaling pathways in yeast. Curr. Opin. Genet. Dev. *1*, 391–396.

Reed, S. I. (1992). The role of p34 kinases in the G1 to S-phase transition. Annu. Rev. Cell Biol. *8*, 529–561.

Reed, S. I., Hadwinger, J. A., and Lorinz, A. (1985). Protein kinase activity associated with the product of the yeast cell division cycle gene *CDC28*. Proc. Natl. Acad. Sci. USA *82*, 4055–4059.

Rhodes, N., Connell, L., and Errede, B. (1990). STE11 is a protein kinase required for cell-type-specific transcription and signal transduction in yeast. Genes Dev. *4*, 1862–1874.

Richardson, H. E., Wittenberg, C., Cross, F., and Reed, S. I. (1989). An essential G_1 function for cyclin-like proteins in yeast. Cell *59*, 1127–1133.

Rubin, G. (1975). Methods in Cell Biology (New York: Academic Press), pp. 45–64.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sanghera, J. S., Paddon, H. B., Bader, S. A., and Pelech, S. L. (1990). Purification and characterization of a maturation-activated myelin basic protein kinase from sea star oocytes. J. Biol. Chem. *266*, 6700–6707.

Sherman, F., Fink, G. R., and Hicks, J. B. (1982). Methods in Yeast Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. Genetics *122*, 19–27.

Song, O., Dolan, J. W., Yuan, Y. O., and Fields, S. (1991). Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation. Genes Dev. *5*, 741–750.

Sorger, P. K., and Murray, A. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of $p34^{cdc28}$. Nature *355*, 365–368.

Sprague, G. F., Jr. (1992). Kinase cascade conserved. Curr. Biol. *2*, 587–589.

Stevenson, B. J., Rhodes, N., Errede, B., and Sprague, G. F., Jr. (1992). Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G-protein. Genes Dev. *6*, 1293–1304.

Teague, M. A., Chaleff, D. T., and Errede, B. (1986). Nucleotide sequence of the yeast regulatory gene STE7 predicts a protein homologous to protein kinases. Proc. Natl. Acad. Sci. USA *83*, 7371–7375.

Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992). The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. *11*, 1773–1784.

Valdivieso, H. M., Sugimoto, K., Jahng, K.-Y., Fernandes, P. M. B., and Wittenberg, C. (1993). *FAR1* is required for post-transcriptional regulation of *CLN2* gene expression in response to mating pheromone. Mol. Cell. Biol. *13*, 1013–1022.

Weinberg, R. A. (1990). The retinoblastoma gene and cell growth control. Trends Biochem. Sci. *15*, 100–103.

Wittenberg, C., Sugimoto, K., and Reed, S. I. (1990). G1 specific cyclins of S. cerevisiae: cell cycle periodicity, regulation by mating pheromone, and association with the $p34^{cdc28}$ protein kinase. Cell *62*, 225–237.

Zhou, Z., Gartner, A., Cade, R., Ammerer, G., and Errede, B. (1993). Pheromone induced signal transduction requires the sequential function of three protein kinases. Mol. Cell. Biol. *13*, 2069–2080.