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Phosphorylation of FAR1 in Response to α-Factor: A Possible Requirement for Cell-Cycle Arrest

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Exposure of yeast **a** cells to α-factor causes cells to arrest in the G1 phase of the cell cycle. The *FAR1* gene is required for this cell-cycle arrest; its product is necessary for the inhibition of a G1 cyclin, CLN2. Earlier work demonstrated that α-factor caused an increase in the transcription of *FAR1* severalfold over a measurable basal level. We now show that transcriptional induction of *FAR1* from a heterologous promoter is not sufficient to inhibit CLN2 in the absence of α-factor. We also show that FAR1 is phosphorylated in response to α-factor and propose that this phosphorylation may be required for FAR1 activity.

INTRODUCTION

α-factor is a peptide-mating factor of the yeast *Saccharomyces cerevisiae* that elicits cell-cycle arrest in G1 and other mating responses in the **a** cell. *FAR1* was identified as a gene required for cell-cycle arrest by α-factor (Chang and Herskowitz, 1990). Genetic evidence indicates that *FAR1* plays a role in cell-cycle arrest by inhibiting a G1 cyclin, CLN2; *FAR1* is necessary for arrest in a $CLN2^+$ cell but is not necessary in a $cln2^-$ cell. FAR1 may inhibit either the activity or synthesis of CLN2 in response to α-factor. These and other studies have led to a model in which α-factor causes cell-cycle arrest in G1 by inhibiting the synthesis or activity of a set of functionally redundant G1 cyclins: CLN1, CLN2, and CLN3 (Richardson *et al.*, 1989; Wittenberg *et al.*, 1990; Chang and Herskowitz, 1990; Elion *et al.*, 1990).

We imagine that FAR1 is inactive in the absence of α-factor and is activated after exposure of cells to α-factor by components of a signal-transduction pathway (see Cross *et al.*, 1988; Marsh *et al.*, 1991). This signal-transduction pathway includes a receptor (STE2), a heterotrimeric G protein, a set of protein kinases (STE7, STE11, KSS1, and FUS3), and a DNA-binding transcriptional activator (STE12). We have previously reported that transcription of *FAR1* is induced by α-factor four- to five-fold in **a** cells (Chang and Herskowitz, 1990). This induction may be mediated by the STE12 transcriptional activator: the upstream regulatory region of *FAR1* contains potential STE12 binding sites (PRE sequences) (Dolan *et al.*, 1989; Errede and Ammerer, 1989) that may be responsible for induction of *FAR1* by α-factor. Both the basal and induced levels of the *FAR1* transcript are dependent on STE12 (Chang and Herskowitz, 1990).

In this paper we present studies on the regulation of FAR1 by the mating-factor-response pathway. One simple possibility is that the increased transcription of *FAR1* induced by α-factor is sufficient to activate FAR1 function and thereby inhibit CLN2. We show here that this simple hypothesis is not true. We report that FAR1 protein is rapidly phosphorylated in response to α-factor and propose that this modification may be necessary for activation of FAR1.

MATERIALS AND METHODS

Strains and Media

All strains used were isogenic to the 15 Dau strain background and have been previously described (Chang and Herskowitz, 1990). Strains were as follows: wild-type, FC279; $far1^-$, FC280; $far1^-$ $cln1^-$ $cln3^-$, FC 319; $far1^-$ $cln3^-$, FC329; $far1^-$ $cln1^-$ $cln2^-$, FC290; $far1^-$ $cln2^-$ $cln3^-$, FC322. α-Factor was obtained from Sigma (St. Louis, MO). Sgal medium is minimal medium containing 2% galactose and 0.1% sucrose, supplemented with the appropriate amino acids. Phosphate-free minimal medium (M. Garabedian, personal communication) was a gift from M. Krstic, University of California, San Francisco. YEPD (rich medium) is described by Chang and Herskowitz (1990).

Assays for Cell-Cycle Arrest and Other Phenotypes

Cell-cycle arrest in response to α-factor was measured by halo assay as described in Chang and Herskowitz (1990). In Figure 2, cells were grown overnight in Sgal-trp liquid medium and then diluted and plated as lawns onto Sgal-trp agar plates. Growth rates of strains were assayed by colony size. Strains were replica printed onto Sgal-trp plates and grown for 1 day at 30°C and then streaked onto fresh Sgal-trp plates and assayed for colony size after 4 days incubation at 30°C. *FUS1-lacZ* expression and cell-cycle arrest were assayed by dose-response

titrations in culture (Chang and Herskowitz, 1990) in FC280 (wild-type) containing pSB234 (p*FUS1-lacZ*) (Trueheart *et al.*, 1987) and either pFC24 (*GAL-FAR1*) or pRS129 (*GAL* vector). Mating and budding assays were performed as described in Chang and Herskowitz (1990).

Plasmid Construction of pGAL-FAR1

pRS129 is a centromere-based *TRP1* plasmid containing a *GAL1* promoter (constructed by R. Sikorski; gift from J. Li, University of California, San Francisco). pFC24 (*GAL-FAR1*) contains a 3.2-kb genomic DNA fragment containing the entire *FAR1* open reading frame and 3′ regions inserted into pFC129. A polymerase chain reaction (PCR) fragment (0.8 kb) containing the 5′ part of *FAR1* was created using an oligonucleotide (CGCTCGAGCAACAGATGCCCAC) that contains an engineered *Xho* I site and a fragment containing 8 bp upstream of the initiator AUG codon of the *FAR1* open reading frame (underlined) and an oligonucleotide encoding a sequence internal to *FAR1* at the *Hin*dIII site (CCTGTGAAGCTTCTCGCCG). This PCR fragment was ligated along with a 2.8-kb *Hin*dIII-*Bgl* II fragment, which contained the rest of the *FAR1* gene, into the polylinker *Xho* I-*Bam*HI sites of RS129 in a directed triple ligation. DNA manipulations and transformations were performed as referenced in Chang and Herskowitz (1990).

FAR1 Antibody

Rabbit antibodies were raised against a TrpE-FAR1 fusion protein (encoded by pFC27) and affinity purified to a GEX-FAR1 fusion protein (encoded by pFC28). pFC27 (TrpE-FAR1) was constructed by ligating a *Xho* I-*Pvu* II fragment of pFC24, which contains the full-length *FAR1* open reading frame into *Sal* I-*Sma* I sites of pATH23 (Koerner *et al.*, 1991). pFC28 was constructed from a triple ligation containing the following fragments: a 0.9-kb PCR-generated fragment encoding the 5′ end of *FAR1* using the oligonucleotides GGAGATCTATGCCCACATTTGG, which encodes a *Bgl* I sites and the initiation AUG codon of *FAR1* (underlined), and CCTGTGTAAGCTTCTCCGCCG, which hybridizes to the internal *Hin*dIII site of *FAR1;* a 1.5-kb *Hin*dIII-*Eco*RI fragment from pFC15 (Chang and Herskowitz, 1990) containing the 3′ end of *FAR1;* and pGEX-2T (Ausubel *et al.*, 1991) cleaved with *Bam*HI and *Eco*RI. The fusion proteins were produced in *Escherichia coli* and extracted as described in Andrews and Herskowitz (1989). Both fusion proteins were found to be largely in the insoluble fraction. The extracts were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% acrylamide). A major 97-Kd degradation product of the 120-Kd TrpE-FAR1 fusion protein was isolated by excision from the gel, and either eluted protein or protein embedded in gel were used to immunize rabbits (BabCo, Berkeley, CA). Three milliliters of FAR1 antisera (third bleed) were affinity purified (C. Peterson, personal communication) by incubating the sera with a nitrocellulose strip containing a 90-Kd glutathione transferase-FAR1 fusion protein. Antibody bound to the strip was then eluted and concentrated to 0.3 ml.

Preparation of Yeast Extracts

Crude SDS yeast extracts were prepared by growing 10-ml cells to OD_{600} of 0.6–1.0. Cells were harvested, washed once in water, and resuspended in 100 μl SDS sample buffer (100 mM tris-(hydroxymethyl)aminomethane [Tris] pH 6.8, 4% SDS, 10% glycerol, 20% β-mercaptoethanol). The suspension was vortexed twice with glass beads for 30 s, boiled for 5 min, incubated on ice for 5 min, vortexed for 10 s, and centrifuged by microcentrifuge for 5 min. Ten to twenty microliters of the supernatants were used per lane for Western blotting.

Native extracts were prepared by a mortar and pestle method (Sorger *et al.*, 1987). 300 ml cells were grown to an approximate OD_{600} 1.0. Cells were washed once in water and once in Buffer D and then resuspended in 2 ml of Buffer D (50 mM Tris, pH 8.0, 20% glycerol, 50 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.3 mg/ml benzamidine, 5 mM EDTA, 50 mM NaF, 50 mM β-glycerol phosphate, 1 mM sodium vanadate). The cell suspension was frozen by dripping into liquid nitrogen. The pellets were ground to a fine powder while frozen using a mortar and pestle. The powder was thawed, and the supernatant was collected following a 5-min centrifugation. The extracts were typically 5–7 mg/ml protein as determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Western Blots

Proteins were electrophoresed by SDS-PAGE (8% acrylamide, 0.21% bis-acrylamide) and were transferred from gel to nitrocellulose by electroblotting. Immunoblots were performed using the ECL Western blotting detection system (Amersham, Arlington Heights, IL) as recommended. For the primary-antibody coupling, either crude FAR1 antiserum (second bleed; 1:1000 dilution) or affinity-purified serum (1:200 dilution) were used. Donkey anti-rabbit Ig-peroxidase (Amersham) was used as a secondary antibody at 1:1000 dilution.

Phosphatase Treatment of FAR1

Before phosphatase treatment, FAR1 was immunoprecipitated to separate it from endogenous yeast phosphatases present in the extracts. Twenty microliters of native yeast extract and 10 μl of affinity-purified FAR1 antibody in 200 μl RIPA buffer (50 mM Tris, pH 8, 0.1% SDS, 0.5% deoxycholate, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors, as listed above) were incubated on ice for 90 min and then incubated with protein A-coupled Sepharose beads (Sigma) for 45–60 min on a nutator at 4°C. The beads were washed three times with RIPA, twice with phosphatase buffer (50 mM Tris, pH 8.0, 10% glycerol, protease inhibitors), and resuspended in 20 μl of phosphatase buffer. The immunoprecipitates were incubated at 37°C for 30 min with 5 μl (25 units/μl) molecular biology grade calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN) or mock treated with 5 μl of phosphatase buffer. Protein was released from the beads by boiling in SDS sample buffer, electrophoresed on SDS-PAGE, and visualized by Western blotting using crude FAR1 antiserum.

In Vivo [^{32}P]Orthophosphate Labeling

Cells (10 ml) were grown to OD_{600} 0.5–1.0 in *Sga* I medium. Cells were washed twice in phosphate-free SD minimal medium containing galactose (M. Garabedian, personal communication), incubated for 30 min at 37°C, and washed twice more in phosphate-free medium. 0.5 mCi of [^{32}P]orthophosphate (Amersham) was added to the culture and incubated at 30° for 70 min. 1 μM α-factor was added to one of the tubes 10 min after addition of the radionucleotide label. After labeling, cells were washed twice in phosphate-buffered saline and disrupted with glass beads and boiling as in the crude SDS extract, except that the cells were lysed in 100 μl of SDS lysis buffer (2% SDS, 100 mM Tris 7.5, 20% glycerol, 1% β-mercaptoethanol, and protease and phosphatase inhibitors). Twenty microliters of extract was immunoprecipitated with affinity-purified FAR1 antisera. Cold 50 mM Na phosphate was added to both the beads and the RIPA mix before the beads were added to reduce background of unincorporated label. The beads were washed twice with 1 ml RIPA and once with Buffer A (2 M urea, 50 mM NaCl, 50 mM Tris, 0.1% deoxycholate, 0.1% Triton X100). Samples were boiled in SDS sample buffer and analyzed by electrophoresis on SDS-PAGE followed by autoradiography. The autoradiograph shown in Figure 5 is a 35-d exposure.

RESULTS

Effect of Transcriptional Induction of FAR1 on Arrest

We first tested whether transcriptional induction of *FAR1* is sufficient for activation of FAR1 and inhibition of CLN2. If this were true, then high levels of *FAR1*

expression from a strong heterologous promoter should inhibit CLN2 function even in the absence of α-factor. The *FAR1* open reading frame was fused to the *GAL1* promoter on a centromere-based plasmid (pFC24; see MATERIALS AND METHODS for details). This construct was shown to produce high levels of FAR1 protein by Western blotting using a polyclonal antibody raised against FAR1 protein (described below). The p*GAL-FAR1* construct produced FAR1 protein at higher levels than the α-factor-induced levels of FAR1 in wild-type cells (Figure 1, lanes 3 and 4). FAR1 produced from the p*GAL-FAR1* construct appeared as a heterogenous collection of bands that run at predominantly slightly faster mobilities than the native FAR1 protein, suggesting that some of the FAR1 protein under these conditions may be cleaved or may have a different translation start site.

We confirmed that the *FAR1* gene carried in the p*GAL-FAR1* plasmid is functional by a complementation test. α-Factor arrest was assayed by the halo assay, in which the growth of a lawn of **a** cells is inhibited around an α-factor source, forming a halo (Sprague, 1991). In Figure 2 *far1*$^-$ mutant cells (carrying a control vector, pRS129) did not form a halo and thus did not exhibit α-factor arrest (upper left); *far1*$^-$ cells carrying a plasmid with p*GAL-FAR1* (pFC24) did exhibit α-factor arrest (lower left), demonstrating that p*GAL-FAR1* encodes a functional FAR1 protein. Neither of these strains formed a halo when grown on glucose medium, indicating that the production of FAR1 by the p*GAL-FAR1* construct is dependent on the *GAL1* promoter. Microscopic examination of cultures confirmed that *far1*$^-$ cells carrying pFC24 arrest as unbudded cells in the presence of α-factor when grown in galactose medium but not in glucose medium. *far1*$^-$ cells carrying a control vector (pRS129) did not arrest in the presence of α-factor either in galactose or glucose media.

Overexpression of FAR1 protein from p*GAL-FAR1* had very little effect on the growth of wild-type cells, mating frequency, arrest in response to α-factor, morphological changes (shmoo formation), or budding pattern (see MATERIALS AND METHODS). In particular, cells with p*GAL-FAR1* were not supersensitive to α-factor for arrest or for induction of an α-factor-inducible construct, *FUS1-lacZ*, as assayed by dose-response curves in culture (see MATERIALS AND METHODS). The only phenotype found for cells expressing p*GAL-FAR1* is that they were slightly larger and more elongated than cells carrying the vector alone. The significance of this altered morphology is not known, but altered morphology can be associated with a slight delay in the cell cycle.

Because FAR1 is thought to affect primarily CLN2, we did not expect FAR1 to have significant cell-cycle effects in a wild-type cell, because the other two CLN products can functionally substitute for an inactive CLN2 (Richardson *et al.*, 1989; Chang and Herskowitz, 1990). We therefore assayed the effect of galactose-induced expression of FAR1 on CLN2 in a strain that is dependent on CLN2 for growth, a *far1*$^-$ *cln1*$^-$ *cln3*$^-$ mutant strain. In-

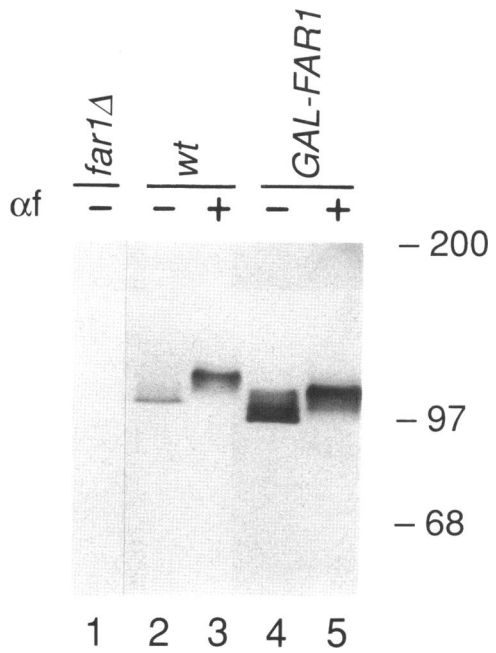

Figure 1. FAR1 protein in wild-type and p*GAL-FAR1* strains in the absence and presence of α-factor. Lane 1, FC280 (*far1::URA3*); lanes 2 and 3, FC279 (*FAR*$^+$); lanes 4 and 5, FC300 (*GAL-FAR1* in *far1::URA3*). Cells were grown Sgal medium with complete amino acids (lanes 1–3) or lacking tryptophan (lanes 4–5) and were treated with 1 μM α-factor for 2 h (lanes 3 and 5). Crude SDS extracts were electrophoresed on SDS-PAGE and immunoblotted using affinity-purified FAR1 antibody. The approximate positions of prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD) are indicated ($M_r \times 10^{-3}$).

activation of *CLN2* in the *cln1*$^-$ *cln3*$^-$ strain causes cells to arrest in G1 and leads to an inability to form colonies (Richardson *et al.*, 1989; Chang, unpublished data). We found that expression of p*GAL-FAR1* in *far1*$^-$ *cln1*$^-$ *cln3*$^-$ cells did not cause cell-cycle arrest in the absence of α-factor, as shown by the ability of these cells to grow at a distance away from the α-factor source (Figure 2; lower left). Thus, galactose-induced expression of FAR1 must not fully inactivate CLN2 in the absence of α-factor. The *far1*$^-$ *cln1*$^-$ *cln3*$^-$ cells carrying p*GAL-FAR1* did exhibit arrest when exposed to α-factor (Figure 2; lower left), demonstrating that transcriptional induction of *FAR1* and α-factor treatment are not equivalent. These observations are not consistent with the simple hypothesis that increased synthesis of *FAR1* is sufficient to inhibit CLN2.

The *GAL-FAR1* construct did exert a small inhibitory effect on the growth of *far1*$^-$ *cln1*$^-$ *cln3*$^-$ cells, as determined by colony size, suggesting that overexpression of *FAR1* may weakly inhibit the activity of CLN2. This weak inhibitory effect was also seen in *far1*$^-$ *cln2*$^-$ *cln3*$^-$ and *far1*$^-$ *cln3*$^-$ mutants but not in *far1*$^-$ *cln1*$^-$ *cln2*$^-$, *far1*$^-$, or wild-type cells. These observations are consistent with the notion that overexpression of FAR1 protein may weakly inhibit the activity of CLN1 and CLN2 but not CLN3.

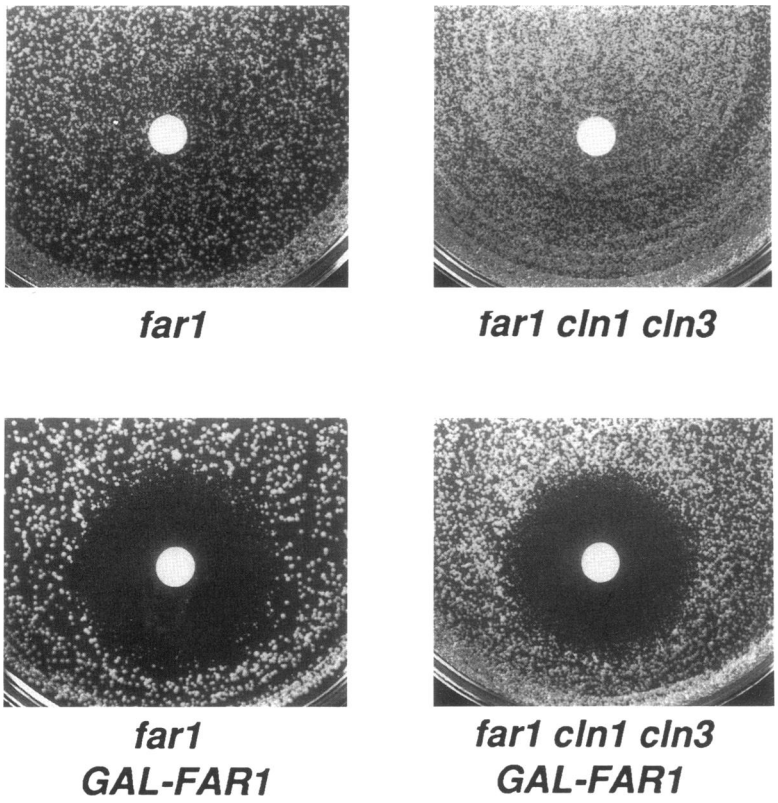

Figure 2. Galactose-induced expression of *FAR1* is not sufficient for inhibition of CLN2. Lawns of **a** strains of different genotypes were spread on Sgal-trp agar plates (containing galactose), and a disc containing 1 μg α-factor was placed on the plate. Plates were incubated for 3 d at 30°C and photographed. FC280 (*far1*) and FC319 (*far1 cln1 cln3*) were transformed with either pRS129 (*GAL* vector; upper) or pFC24 (p*GAL-FAR1;* lower).

Phosphorylation of FAR1

We next determined whether FAR1 is posttranslationally modified in response to α-factor. Polyclonal antibodies were raised in rabbits against a TrpE-FAR1 fusion protein and affinity-purified against another FAR1 fusion protein of a different molecular weight (see MATERIALS AND METHODS). Western blotting with FAR1 antisera demonstrated in wild-type yeast extracts a major band of 97 Kd (Figure 1, lane 2), whose size is consistent with the 780-amino-acid FAR1 open reading frame predicted by the *FAR1* nucleotide sequence (Chang and Herskowitz, 1990). This 97-Kd protein was confirmed to be FAR1 by showing that it was absent in extracts from *far1::URA3* mutant strains (Figure 1, lane 1), overexpressed in a strain containing p*GAL-FAR1* (Figure 1, lane 4), and truncated in strains carrying insertion mutations in the *FAR1* coding region.

Figure 3 shows a time course of the effect of α-factor on the FAR1 protein. First, the amount of FAR1 protein increased in cells treated with α-factor. This increase was apparent by 15 min. Second, the apparent molecular weight of all the FAR1 protein was altered by α-factor treatment. This shift was evident within 5 min after exposure to α-factor.

The shift in mobility was found to be a consequence of α-factor-induced phosphorylation. FAR1 protein from α-factor-treated cells was immunoprecipitated, incubated in vitro with alkaline phosphatase, and then analyzed by Western blotting using FAR1 antibody. Incubation with alkaline phosphatase (CIP) eliminated the α-factor-induced mobility shift so that the phosphatase-treated FAR1 protein migrated at the same mobility as FAR1 from untreated cells (Figure 4, lanes 2 and 5). The effect of alkaline phosphatase was inhibited by a phosphatase inhibitor, β-glycerol phosphate, demonstrating that the effect of the phosphatase was due to phosphatase activity and not, for example, to contaminating proteases (Figure 4, lane 6). These data show that α-factor causes FAR1 to be phosphorylated at one or more sites, which causes a change in mobility. Phosphatase treatment had no effect on the mobility of FAR1 from cells without α-factor (Figure 4, lane 3), suggesting that this form of FAR1 does not contain phosphorylations that alter its mobility.

We further confirmed that FAR1 is a phosphoprotein by in vivo [^{32}P]orthophosphate labeling. FAR1 protein was immunoprecipitated from [^{32}P]orthophosphate-labeled cells carrying p*GAL-FAR1*. Figure 5 shows that FAR1 protein was phosphorylated both in the absence and presence of α-factor (Figure 5, lanes 2 and 3). The labeled 97-Kd band corresponding to FAR1 was not present in a strain deleted for the *FAR1* gene (Figure 5, lane 1), confirming that this phosphoprotein was FAR1. Thus, FAR1 is phosphorylated in the absence of α-factor and acquires additional phosphorylation upon α-factor treatment.

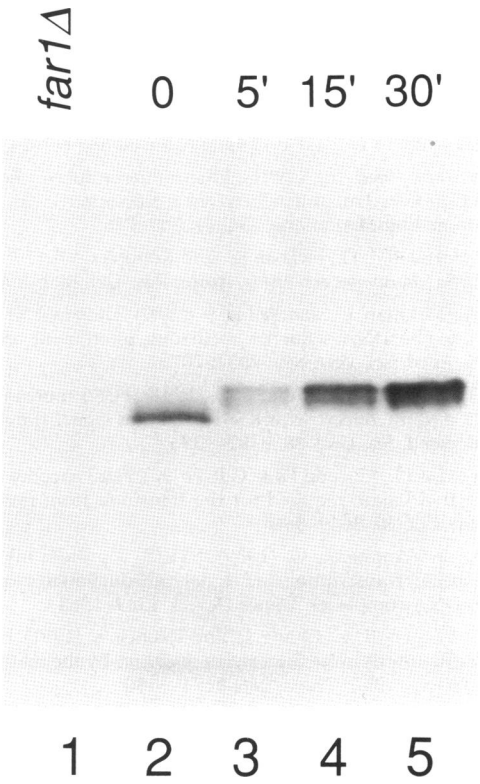

Figure 3. Time course of the effect of α-factor on FAR1 protein. Lane 1, FC280 (*far1::URA3*); lanes 2–5, FC279 (*FAR*$^+$). Cells were treated with α-factor for the times indicated, harvested on ice, and quickly extracted by SDS and boiling. Crude extracts were electrophoresed on SDS-PAGE and immunoblotted using affinity-purified FAR1 antibody.

DISCUSSION

FAR1 is affected by α-factor in at least two ways. First, transcription of *FAR1* is induced approximately four- to fivefold. Second, the FAR1 protein is rapidly phosphorylated.

Transcriptional regulation of *FAR1* is probably mediated at least in part by the DNA binding transcription factor STE12, which may bind at the *FAR1* promoter region at four putative PRE sequences (Kronstad *et al.*, 1987; Chang and Herskowitz, 1990; Dolan *et al.*, 1989). The transcriptional induction of *FAR1* by α-factor presumably contributes to the increase of FAR1 protein levels. Transcriptional induction of *FAR1* is not sufficient to inhibit CLN2 in the absence of α-factor: expression of *FAR1* from a strong constitutive promoter in *cln1*$^-$ *cln3*$^-$ cells did not arrest division in the absence of α-factor but did arrest division in the presence of α-factor. Thus, FAR1 may require posttranslational modifications necessary for its activation. Another possibility is that FAR1 may work with other factors that are dependent on α-factor for their activity.

We have found that FAR1 is a phosphoprotein in the absence of α-factor and that it acquires additional phosphorylation(s) in response to α-factor. We propose that a component of the signal-transduction pathway phosphorylates FAR1 and activates its function. The rapidity of the phosphorylation and the fact that FAR1 protein is not present in a shifted form in a *cdc28-ts* mutant arrested in G1 (Chang, unpublished observations) suggests that this phosphorylation does not arise merely as a consequence of G1 arrest. There is presently no proof that the phosphorylation induced by α-factor affects FAR1 activity. Demonstration of the role of FAR1 phosphorylation will await the mapping and mutating of the phosphorylation site(s).

FAR1 thus joins a group of several proteins that are phosphorylated in response to α-factor. These proteins include STE2, STE4, STE12, and glycogen synthase (Reneke *et al.*, 1988; Cole and Reed, 1991; Francois *et al.*, 1991; Song *et al.*, 1991).

Four protein kinases identified as components of the α-factor response pathway are candidates for the kinase that phosphorylates FAR1: STE7, STE11, KSS1, and FUS3 (Teague *et al.*, 1986; Rhodes *et al.*, 1990; Elion *et al.*, 1990, 1991). Mutants lacking STE7, STE11, or FUS3 are defective in cell-cycle arrest (Hartwell, 1980; Elion *et al.*, 1990). Rhodes *et al.* (1990) have demonstrated that immunoprecipitates of STE11 contain protein kinase activity in vitro. It would be interesting to test if

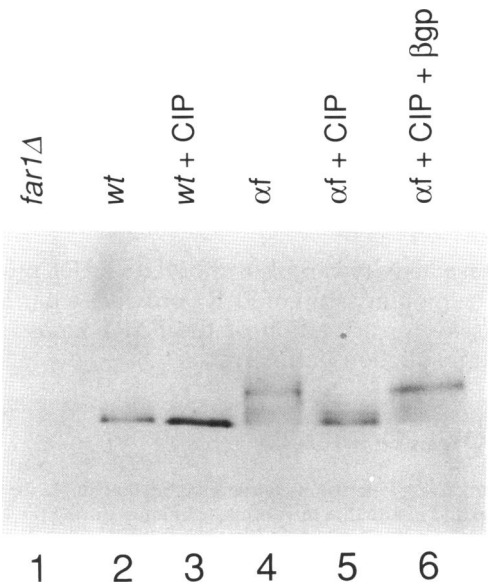

Figure 4. FAR1 is phosphorylated in response to α-factor. Lane 1, FC280 (*far1::URA3*); lanes 2–6, FC279 (*FAR1*$^+$). Native extracts were prepared from cells that had been grown to exponential phase in YEPD and had been treated with 1 μM α-factor for 2 h (lanes 4–6). FAR1 immunoprecipitates were treated with calf intestinal phosphatase (CIP; lanes 3, 5, and 6) or mock treated (lanes 2 and 4). In lane 6, 10 mM β-glycerol phosphate (βgp) was added to the immunoprecipitate before addition of phosphatase. The immunoprecipitates were then boiled in SDS-sample buffer, electrophoresed on SDS-PAGE, and visualized by immunoblotting with FAR1 antiserum.

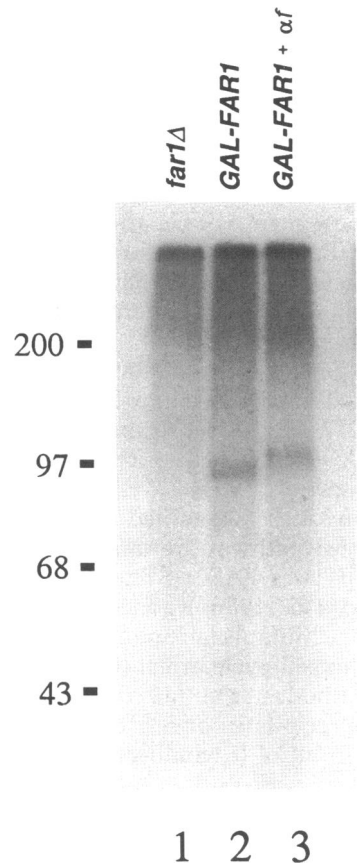

Figure 5. In vivo [^{32}P]orthophosphate labeling of FAR1. Lane 1, FC280 (*far1::URA3*); lane 2, FC300 (*GAL-FAR1*); lane 3, FC300 treated with α-factor. FAR1 was immunoprecipitated from extracts from in vivo [^{32}P]orthophosphate-labeled cultures, electrophoresed on SDS-PAGE, and visualized by autoradiography. See MATERIALS AND METHODS for details.

this kinase activity can phosphorylate FAR1 protein in vitro. The mobility shift of FAR1 provides a particularly facile assay for activation of the FAR1 kinase by the signal-transduction pathway.

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