Tyrosine phosphorylation of a yeast 40 kDa protein occurs in response to mating pheromone

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Communicated by A. Rich

Tyrosine phosphorylation of proteins in the yeast *Saccharomyces cerevisiae* has been examined following exposure to the mating pheromone α -factor. When a cells are treated with α -factor a protein of ~40 kDa molecular weight is tyrosine phosphorylated. This tyrosine phosphorylation response requires an intact signal transduction pathway, is not restricted to a short interval of the cell division cycle, and requires protein synthesis for its maximal accumulation. Mating competent *fus3* deletion strains fail to elaborate the phosphotyrosine response. The possibility that *FUS3* encodes the 40 kDa protein is discussed.

Key words: *FUS3*/mating pathway/phosphotyrosine/signal transduction/yeast

Introduction

Protein tyrosine phosphorylation is an important step in the transduction of many signals from the extracellular milieu (reviewed in Hunter, 1987; Ullrich and Schlessinger, 1990). Although numerous protein tyrosine kinases have been described and analyzed, the function of this modification and how it is regulated is poorly understood. A system which is amenable to genetic analysis and responds to an exogenous signal by phosphorylating tyrosine residues would benefit the study of how phosphotyrosine modifications are controlled and how they exert effects at the molecular level. The *Saccharomyces cerevisiae* response to mating pheromone provides a system in which this modification can be examined.

Yeast mating pheromones are peptides secreted by the two haploid cell types. α cells secrete α -factor that binds to receptors on the surface of α cells, and α cells secrete α -factor that binds to receptors on the surface of α cells. Binding of mating factors to the appropriate receptors initiates a signal that is transduced through a series of intermediaries. The signal results in a block to cell division in the G₁ phase of the cell cycle and the activation of genes that are necessary for cellular and nuclear fusion. The response to mating pheromones culminates in the formation of the α/α diploid cell type (for reviews see Cross *et al.*, 1988; Herskowitz, 1988; Marsh and Herskowitz, 1988; Fields, 1990).

Many of the genes necessary for the response to mating factors have been identified genetically and cloned.

Molecular and genetic analysis has led to the following, generally accepted, model for response to mating factors: *STE2* and *STE3*, which encode receptors for the α and α pheromones respectively, are coupled to a heterotrimeric G protein whose subunits are encoded by *STE4*, *STE18* and *GPA1* (also known as *SCG1*). The *STE5*, *STE7*, *STE11*, *FUS3* and *KSS1* gene products are involved in transducing the signal following G protein activation (reviewed in Fields, 1990; Elion *et al.*, 1991). The combined action of these gene products leads to the activation of the *STE12* encoded transcription factor that binds to *cis*-acting regulatory elements in pheromone responsive genes and activates their expression (Dolan *et al.*, 1989; Errede and Ammerer, 1989; Dolan and Fields, 1990).

The pheromone-mediated block to cell division is carried out by multiple gene products (Chang and Herskowitz, 1990; Elion *et al.*, 1990) that down-regulate G₁ cyclins encoded by *CLN1*, *CLN2* and *CLN3* (Richardson *et al.*, 1989; Wittenberg *et al.*, 1990; reviewed in Reed, 1991). The *CLN* gene products are required for the activity of the *CDC28* protein kinase (Lorincz and Reed, 1984), a homolog of the *Schizosaccharomyces pombe cdc2* encoded p34 (reviewed in Fantes, 1988; Lee and Nurse, 1988; Reed, 1991), which is essential for progression through the G₁ control point of the cell cycle termed 'START' (Hartwell *et al.*, 1974; Pringle and Hartwell, 1981). Down-regulation of the *CLN* gene products is thought to be mediated in part by *FAR1* and *FUS3* (Chang and Herskowitz, 1990; Elion *et al.*, 1990, 1991).

One aspect of the mating response pathway that remains poorly understood concerns the functions of *STE7*, *STE11*, *KSS1* and *FUS3* that allow propagation of the signal and cell cycle arrest. Similarly, the epistatic relationships between these genes are unclear. The deduced amino acid sequences of *STE7*, *STE11*, *KSS1* and *FUS3* are similar to protein kinases (Teague *et al.*, 1986; Courchesne *et al.*, 1989; Elion *et al.*, 1990). *KSS1* and *FUS3* are most similar to the mammalian ERK family of serine and threonine protein kinases whose activities are thought to be regulated by tyrosine phosphorylation (Boulton *et al.* 1990, 1991; see Discussion).

In this report, we describe the tyrosine phosphorylation of a 40 kDa protein in response to mating pheromone. Our results show that this phosphorylation response requires *FUS3*. This phosphotyrosine modification provides a biochemical marker which should prove useful in studying the molecular interactions that enable yeast cells to respond to mating pheromone.

Results

A 40 kDa protein is tyrosine-phosphorylated in response to α -factor

To determine whether there are changes in protein tyrosine phosphate in response to α -factor, yeast strains carrying an

Fig. 1. Immunoblot analysis of yeast strain L3284 grown in the presence (+) or absence (-) of 1 μ M α -factor for 1 h. Phosphotyrosine (P-Y), phosphoserine (P-S), or phosphothreonine (P-T) at 1 mM was included in the primary anti-phosphotyrosine antibody incubation as indicated. The control filter was processed in the absence of the primary anti-phosphotyrosine antibody incubation. The filters used for the experiments shown in panels A and B were prepared from different electrophoretic separations of the same samples. The position where pre-stained molecular weight markers migrated is indicated as is the position of the p40 immunoreactive species. * indicates the position of the constitutive anti-phosphotyrosine immunoreactive species.

inactivating mutation in *SST1* were used. *SST1* (also known as *BARI*) encodes an endopeptidase that specifically degrades α -factor (Ciejek and Thorner, 1979). The use of this strain allows higher effective concentrations of α -factor to be achieved and eliminates a variable that can affect α -factor concentration. Dividing *MATa*, *sst1* cells were treated with 1 μ M α -factor for 1 h and assayed by immunoblot analysis using an anti-phosphotyrosine antibody as described in Materials and methods. As shown in Figure 1, there is an immunoreactive band of ~40 kDa (hereafter referred to as p40) seen in α -factor treated cells that is absent in untreated cultures. The appearance of p40 (hereafter referred to as the p40 response) was not detectable when the primary antibody incubation was carried out in the presence of phosphotyrosine (Figure 1A). In contrast, the presence of phosphoserine or phosphothreonine in the primary antibody incubation had no effect upon p40 immunoreactivity (Figure 1B). We have been unable to detect any other α -factor dependent changes in phosphotyrosine containing proteins using different electrophoretic conditions designed to display either higher or lower molecular weight proteins (data not shown).

There were also several constitutive anti-phosphotyrosine immunoreactive species. The most prominent were a series of closely spaced bands that migrate slightly slower than p40 (see Figure 1). These immunoreactive bands represent *bona fide* phosphotyrosine containing proteins as demonstrated by the ability of phosphotyrosine to compete for immunoreactivity. A number of bands were observed in the immunoblot that were not eliminated by competition with phosphotyrosine. Control experiments show that these species were due to reactivity of the secondary alkaline phosphatase conjugated detecting antibody and were dependent upon the source and lot of detecting antibody used (control filter in Figure 1B).

To examine the dependence of the p40 response on α -factor concentration, dividing *sst1* cells were incubated with increasing concentrations of pheromone. As shown in Figure 2, p40 immunoreactivity in the *sst1* mutant appeared upon incubation with α -factor concentrations of 5 nM and higher. No p40 immunoreactivity was observed at lower

Fig. 2. Concentration dependence of the p40 response in yeast strain L3284. Cells were incubated for 1.5 h in the presence of the indicated concentration of α -factor prior to immunoblot analysis using the anti-phosphotyrosine antibody as described in Materials and methods.

concentrations and the amount of p40 immunoreactivity did not significantly increase at higher α -factor concentrations. Thus, the p40 response reaches a maximum within a narrow range of α -factor concentrations. With the wild type (*SST1*) strains, the same apparent 'all or none' phenomenon was observed but at higher α -factor concentrations of 10–50 μ M (data not shown). In addition, the level of p40 immunoreactivity in *SST1* strains was lower than that seen in *sst1* strains suggesting that constant α -factor stimulation of the receptor is required for its maximal accumulation. In both wild type and *sst1* strains the α -factor concentrations needed to elicit the p40 response correspond to those needed to observe full morphological response (data not shown).

Fig. 3. Time course of the p40 response following α -factor addition in yeast strain L3284. Cells were incubated with 50 nM α -factor for the indicated length of time prior to harvesting, boiling in SDS sample buffer and immunoblot analysis using the anti-phosphotyrosine antibody as described in Materials and methods.

The p40 response is not restricted to a short interval of the cell division cycle

In order to measure the kinetics of the p40 response, a culture of *ss1* cells was made 50 nM with respect to α -factor, aliquots were withdrawn at increasing times, boiled in SDS-containing sample buffer and assayed for the appearance of p40 immunoreactivity. As shown in Figure 3, p40 immunoreactivity was detectable after a 15 min incubation with the pheromone and reached a maximal level within 45 min. These data are not consistent with the p40 response being restricted to a short interval of the cell division cycle such as that termed 'START', since if it was, the maximal level would be reached after a treatment interval equal to that required to complete the cell cycle (~ 90 min under the conditions tested). The kinetics of the p40 response suggest that the response may occur throughout the cell cycle. In order to investigate this possibility further, cells were synchronized by incubation with α -factor for 2 h, the α -factor was then removed and the culture allowed to resume cell division by the addition of fresh media. Under these conditions, immunoreactive p40 is no longer detectable 15 min after the removal of α -factor (Figure 4 and data not shown). Aliquots of the synchronized cells were withdrawn at increasing times and tested for their ability to elaborate the p40 response after a 15 min incubation with α -factor. As shown in Figure 4, the magnitude of the p40 response was similar at all of the cell cycle stages tested. These results suggest the p40 response can occur throughout the cell division cycle.

Fig. 4. The p40 response during cell cycle progression in a synchronized culture of yeast strain L3284. Cells were synchronized in the G₁ phase by addition of 50 nM α -factor for 110 min. Cells were harvested by centrifugation, washed twice, resuspended in fresh media and tested for the p40 response after re-addition of α -factor for 15 min at the indicated times. C, control asynchronous culture. Cells were monitored microscopically throughout the time course to assure proper synchrony had been achieved.

The p40 response requires an intact signal transduction pathway

The p40 response has been examined in several well characterized mutants in the signal transduction pathway (Hartwell, 1980). These mutants include temperature sensitive mutants in *STE2*, which encodes the α -factor receptor and *STE12*, which encodes the positive transcription factor that activates the expression of pheromone responsive genes. Yeast strains carrying temperature sensitive mutations in *STE2* or in *STE12* did not accumulate p40 at the non-permissive temperature in the presence of α -factor concentrations that elicited the p40 response in isogenic control cultures (Figure 5 and data not shown). We were unable to detect any difference in doubling times between the *STE* strains and the isogenic wild-type strain used for comparison. Thus, the inability to detect the p40 response in the *STE* strains is not due to gross decreases in metabolic rates. These results show that an intact signal transduction pathway is required for the p40 response (see Discussion for further comments).

The p40 response was also examined in an *ss12* strain. *SST2* has been implicated as a negative regulator of the signal transduction pathway and is necessary for recovery from pheromone (Dietzel and Kurjan, 1987). In the *ss12* strain, the p40 response was seen only when high ($> 10 \mu\text{M}$) α -factor concentrations were used and not when low (5–50 nM) concentrations, sufficient to elicit the morphological response in this strain, were tested. Thus in *ss12* mutants the morphological response to pheromone can occur in the absence of a detectable p40 response (see Discussion).

The p40 response is inhibited by cycloheximide

In order to determine whether the p40 response is dependent upon protein synthesis, cells were pretreated with cycloheximide for 5 min followed by a 15 min incubation with α -factor. The p40 response in the cycloheximide-treated

cells was subsequently compared with the response in cells not treated with cycloheximide. As seen in Figure 6, cycloheximide inhibited the p40 response. We estimated that the level of inhibition was 80–90% as determined by comparing the intensities of p40 immunoreactivity in 2-fold dilutions of the samples shown in Figure 6 (data not shown). We also noted a modest (~ 10 – 20%) decrease in the level of the constitutive immunoreactive species in the same samples (see Figure 6). In parallel experiments the level of cycloheximide used was shown to inhibit the incorporation of [35 S]methionine into TCA precipitable material by 86%.

***FUS3* null mutants do not elaborate the p40 response**

To determine whether *FUS3* is required for the p40 response, *FUS3* was disrupted in an *sst1* strain and the ability to elaborate the p40 response was determined in the resulting *fus3* null mutant (Elion *et al.*, 1990). It has recently been shown that either *FUS3* or *KSS1* function is required for mating and that some laboratory yeast strains lack functional *KSS1* (E. Elion, personal communication). It was therefore necessary to determine if the starting strain for this analysis was *KSS1*⁺. All of the *fus3* null mutants analyzed (see Materials and methods) were able to mate. We therefore inferred that the genotype of the parent strain was *FUS3*⁺ *KSS1*⁺. The p40 response was subsequently tested in the parent strain relative to the isogenic *fus3* null mutants. In contrast to the wild type *FUS3* strain, we were unable to detect the p40 response in any of the four independently derived *fus3* deletion strains (Figure 7 and data not shown). We have been unable to detect any changes in protein tyrosine phosphate when the *fus3* null mutants were exposed to α -factor concentrations ranging from 10 nM to 50 μ M (data not shown).

Fig. 5. The p40 response in *sst1* strain L3284, wild type (WT) strain 381G and *ste12* strain 59C. The indicated strains were grown at the non-permissive temperature of 34°C for 12 h followed by the addition of α -factor to 50 μ M for 40 min prior to harvesting and immunoblot analysis using anti-phosphotyrosine antibody as described in Materials and methods. The response in *sst1* strain L3284 is shown for comparison.

Discussion

The results presented here show that a 40 kDa anti-phosphotyrosine immunoreactive protein appears when a cells are exposed to α -factor. The 40 kDa immunoreactive species contains *bona fide* phosphotyrosine as shown by the ability of free phosphotyrosine to compete for immunoreactivity. The response requires an intact signal transduction pathway since strains harboring mutations in *STE2* or in *STE12* do not accumulate p40 immunoreactivity. It should be pointed out that the inability to detect the p40 response in *STE12* mutants does not indicate whether the p40 response occurs before or after *STE12* activation in the pathway since *STE12* is required for constitutive transcription of many genes required for signal transduction including the *STE2* encoded receptor (Fields *et al.*, 1988). Further experiments will be required to order the p40 response with other steps in the pathway. The observation that the response is inhibited by cycloheximide suggests that rate limiting components involved in the phosphorylation event are extremely short lived in their active state and/or that it requires *de novo* protein synthesis. The latter interpretation would imply that *STE12* activation is involved in the elaboration of the p40 response (see below). Our results show that *FUS3* is necessary for the p40 response since mating-competent strains harboring null mutations in this gene fail to accumulate immunoreactive p40.

The predicted molecular mass of the *FUS3* encoded protein is ~ 40 kDa. In addition, the amino acid sequence of *FUS3* is similar to the mammalian ERK1 protein (Boulton *et al.*, 1990). ERK1 protein was initially characterized by its ability to phosphorylate serine and threonine residues of

Fig. 6. Effect of cycloheximide on the p40 response. Yeast strain L3284 was incubated in the presence or absence of 0.5 mg/ml cycloheximide for 5 min prior to the addition of α -factor to 50 nM. Cells were incubated for an additional 15 min and analyzed by immunoblot analysis as described in Materials and methods. In parallel experiments this level of cycloheximide reduced the incorporation of [35 S]methionine into TCA precipitable material by 86%. * indicates the position of the constitutive immunoreactive species.

microtubule-associated protein 2 (MAP2) and is also referred to as MAP2 kinase (Ray and Sturgill, 1987, 1988; Hoshi *et al.*, 1988). ERK1 serine and threonine kinase activity is stimulated in response to a variety of extracellular signals that induce cell division and/or differentiation (Hoshi *et al.*, 1988; Boulton *et al.*, 1990; Ely *et al.*, 1990; Tsao *et al.*, 1990). ERK1 is phosphorylated on tyrosine in response to these signals (Ray and Sturgill, 1988; Boulton *et al.*, 1991) and this modification is required for maximal ERK1 kinase activity *in vitro* (Anderson *et al.*, 1990). Recently, a family of ERK genes have been identified whose products are thought to be involved in transducing phosphotyrosine signals into threonine and serine phosphorylation cascades (Boulton *et al.*, 1991). It has been proposed that the mammalian ERKs, *KSS1* and *FUS3* represent a family of genes whose functions in signal transduction have been conserved between yeast and mammals (Boulton *et al.*, 1990, 1991). These results, taken together with those described here, suggest

that p40 is the *FUS3* gene product. That the p40 response requires protein synthesis for its maximal accumulation is consistent with this possibility since *FUS3* gene expression has been shown to be activated by *STE12* in response to pheromone (Elion *et al.*, 1990). However, our results do not prove that p40 is encoded by *FUS3*. *FUS3* may be required for the activation of other functions that are necessary for p40 tyrosine phosphorylation. It is also possible that *FUS3* encodes the kinase that tyrosine phosphorylates p40.

It has recently been shown that either *FUS3* or *KSS1* is necessary for the transcriptional response to pheromone (Elion *et al.*, 1991). In addition to being required for the transcriptional response, *FUS3* is also involved in cell cycle arrest (Elion *et al.*, 1990, 1991). *KSS1* and *FUS3* encode proteins whose sizes differ only by 15 amino acids (Elion *et al.*, 1990; Courchesne *et al.*, 1989). Since a *fus3*, *KSS1* strain did not accumulate any anti-phosphotyrosine immunoreactivity in the 40 kDa range in response to pheromone induction, the possibility exists that *KSS1* is activated in a *fus3* background by a mechanism that does not directly involve its phosphorylation of tyrosine residues. Elion *et al.* (1990) have isolated alleles of *FUS3* that are defective either in the transcriptional response or in the block cell division. It will be of interest to determine whether the p40 response occurs in these genetic backgrounds.

It is noteworthy that the *sst2* strain did not elaborate the p40 response at the low levels of α -factor that were sufficient to cause the morphological response in this strain. In order to detect immunoreactive p40 in the *sst2* strain, it was necessary to use concentrations of α -factor at least 1000-fold higher than that required to induce the morphological response and cell cycle arrest. This result implies that constant α -factor stimulation of receptor is necessary for the p40 response and suggests that *sst2*, which has been implicated as a negative regulator of signal transduction (Dietzel and Kurjan, 1987), functions independently or downstream of the p40 response in the pathway.

It is likely that multiple feedback loops involving genes with overlapping functions are used by yeast in their response to α -factor. The p40 response provides a biochemical marker that will be useful in dissecting these regulatory circuits and elucidating their relationships to one another.

Materials and methods

Yeast strains and media

The *S. cerevisiae* strains used in this study are shown in Table I. Cells were grown at 30°C in YPD (1% Bacto-yeast extract, 1% Bacto-peptone and 2% glucose) unless otherwise indicated. For α -factor treatment, cells were grown to a density of $1-2 \times 10^7$ cells/ml and α -factor was added from a stock of synthetic α -factor (Sigma Chemical Co., USA) which had been

Fig. 7. Comparison of the p40 response between wild type strain AN37-4C-5 and the isogenic *fus3* strain EWY-1114 following treatment with 100 nM α -factor for 1 h. Two-fold dilutions of the wild type α -factor treated samples were analyzed as shown to establish lower levels of p40 detection.

Table I. Yeast strains

Strain	Genotype	Source
L3284	<i>MATa, sst1-3, ura3-52, his6, lys2-Δ201 trp1-Δ1</i>	G.Fink
JB368	<i>MATa, sst2-4, ura1, cyh2, can1</i>	G.Fink
EWY-1002c-1	<i>MATa, his3-Δ200, leu2-3-112, ura3-52, lys2-801a, trp1a</i>	E. Winter
381G	<i>MATa, sup4-3, cry1-1, his4-580a, trp1a, ade2-1o, tyr1o, lys2o, gal1</i>	YGSC
50B	<i>MATa, sup4-3, cry1-1, his4-580a, trp1a, ade2-1o, tyr1o, lys2o, gal1, ste2</i>	YGSC
59C	<i>MATa, sup4-3, cry1-1, his4-580a, trp1a, ade2-1o, tyr1o, lys2o, gal1, ste12</i>	YGSC
AN37-4C-5	<i>MATa, ura3, leu2, trp1, ade2, met1 sst1, HMLa, HMRa</i>	I.Herskowitz
EWY-1114	<i>MATa, fus3-6::LEU2, ura3, leu2, trp1, ade2, met1, sst1, HMLa, HMRa</i>	this work

dissolved in methanol at a concentration of 1 mM and stored at -20°C . When final concentrations of α -factor higher than 1 μM were used, the powder was dissolved in YPD at a concentration of 1 mM and stored at -80°C until use. *FUS3* was disrupted in strain AN37-4C-5 with the *fus3-6::LEU2* allele to generate strain EWY-1114 by the method of Rothstein (1983) as described by Elion *et al.* (1990).

Immunoblot analysis

Cells were pelleted by centrifugation, suspended in SDS sample buffer at a density of 1×10^9 cells/ml and boiled for 3 min. The cell lysate in SDS sample buffer was clarified by a brief centrifugation and 10 μl (the cell lysate obtained from 10^7 cells) was applied to wells of 1.5 mm thick, 10% polyacrylamide gels and electrophoresed at 17 mA for 12 h. The acrylamide:bis ratio of 38:2 was optimal for resolving the p40 immunoreactivity. Following electrophoresis, the gel was washed twice with transfer buffer [20% methanol, 25 mM Tris(hydroxymethyl)aminomethane and 0.2 M glycine] for 10 min each time and transferred to a nitrocellulose membrane (Schleicher and Schuell, 0.45 μm pore size) using the Genie transfer apparatus (Idea Scientific) at a constant 20 V for 1 h. The nitrocellulose replica was rinsed twice with TBST buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% NaN_3 , 0.05% Tween-20), incubated for 2–6 h in TBST containing 1% BSA (ICN ultrapure) and incubated for 4–14 h in TBST containing 1% BSA to which anti-phosphotyrosine monoclonal antibody IgG2b_K (obtained from Upstate Biotechnology) had been added to 1 $\mu\text{g}/\text{ml}$. This antibody has been shown to be specific for phosphotyrosine and not to cross-react with phosphoserine, phosphothreonine, phosphohistidine, or tyrosine sulfate (Kanakura *et al.*, 1991). The filter was then washed 5 times in TBST for 10 min each time, incubated for 1 h in a 1:5000 dilution of rabbit anti-mouse alkaline phosphatase conjugated antibody (Promega) in TBST. The filter was washed 5 times in TBST for 10 min each time and immunoreactivity visualized using 5-bromo-4-chloro-3-inolyl phosphate and nitro-blue tetrazolium as described by Harlow and Lane (1988).

Acknowledgements

We thank Elaine Elion and Gustaf Ammerer for communicating results prior to publication; Alan Christensen, Robert Knowlton and Brian Reardon for comments on the manuscript; and Gail Leone for secretarial assistance. This work was supported in part by a grant from the Lucille P. Markey Charitable Trust, NIH grant R01GM45772-01 to E.W. and NIH grant CA51170-01 to D.H.

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Received on June 18, 1991; revised on August 13, 1991