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Pheromone-Induced Phosphorylation of a G Protein β Subunit in S. cerevisiae Is Associated with an Adaptive Response to Mating Pheromone

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Summary

The mating pheromone response in S. cerevisiae is activated by a G protein–mediated signaling pathway in which $G_{\beta\gamma}$ is the active transducer of the signal. When exogenous pheromone is added to vegetatively growing cells, G_β is rapidly phosphorylated at several sites; phosphorylation does not require de novo protein synthesis. A mutation in G_β was constructed that eliminates signal-induced phosphorylation. This mutation leads to enhanced sensitivity to and impaired ability to recover from pheromone, but does not affect the ability of $G_{\beta\gamma}$ to transmit the mating signal. These phenotypes suggest that G protein phosphorylation mediates an adaptive response to pheromone-induced signaling. G_β phosphorylation does not require either the pheromone receptor C-terminus or the product of the *SST2* gene, both of which mediate separate adaptive responses to pheromone. However, G_β phosphorylation is greatly facilitated by the presence of the G_α subunit, which has also been shown to participate in an adaptation to pheromone.

Introduction

Haploid Saccharomyces cerevisiae cells of opposite mating type conjugate to form diploids. Preparation for conjugation is achieved via a pheromone signaling system utilizing G protein–coupled membrane receptors structurally and functionally homologous to the β-adrenergic/rhodopsin family in mammalian cells (reviewed in Cross et al., 1988). *MAT*α cells secrete the tridecapeptide α factor, which is selectively bound by a receptor specific to *MAT***a** cells, the product of the *STE2* gene (Burkholder and Hartwell, 1985; Jenness et al., 1983; Blumer et al., 1988). Similarly, *MAT***a** cells secrete the farnesylated dodecapeptide **a** factor, which is thought to interact with a receptor specific to *MAT*α cells, the product of the *STE3* gene (Hagen et al., 1986). Binding of peptide pheromone to its cognate receptor induces a number of metabolic changes in the cell: the cell cycle is arrested in G1 (Bucking-Throm et al., 1973; Wilkinson and Pringle, 1974), the transcription pattern of a number of genes is rapidly altered (Stetler and Thorner, 1984; Nasmyth and Shore, 1987; Wittenberg et al., 1990), and the cell surface and nucleus undergo physiological and morphological changes necessary for conjugation (Lipke et al., 1976; Trueheart et al., 1987; McCaffrey et al., 1987; Rose et al., 1986). In both haploid mating types, these diverse responses are mediated by three genes encoding products homologous to the subunits of metazoan heterotrimeric G proteins. *GPA1* (or *SCG1*) encodes a G_α subunit (Dietzel and Kurjan, 1987a; Miyajima et al., 1987; Jahng et al., 1988), while *STE4* and *STE18* specify G_β and G_γ subunits (Whiteway et al., 1989).

In mammalian cells, stimulation of the transmembrane receptor leads to a conformational shift that is transmitted to the G_α subunit on the cytoplasmic face of the membrane. The G_α subunit, in turn, undergoes a conformational shift itself to displace bound GDP for GTP (reviewed in Stryer and Bourne, 1986; Gilman, 1987). GTP-bound G_α is thought to dissociate from $G_{\beta\gamma}$; subsequently, either or both moieties (depending on the system) are then able to interact with effector molecules to generate intracellular second messengers (reviewed by Neer and Clapham, 1988). G_α then hydrolyzes GTP to return to an inactive GDP-bound form that reassociates with $G_{\beta\gamma}$ to form the inactive $G_{\alpha\beta\gamma}$ trimer.

In addition to this intrinsic short-term instability of the signal mediated by GTP hydrolysis, several molecular mechanisms for desensitization to an ongoing signal have been described in G protein–dependent signaling systems in vertebrates (Sibley and Lefkowitz, 1985). Most of these involve the receptor. Both the β_2-adrenergic receptor and rhodopsin are phosphorylated on their carboxyl termini (Shichi and Somers, 1978; Benovic et al., 1986). In the case of rhodopsin, a protein, arrestin, has been shown to associate with the phosphorylated receptor to prevent further stimulation of G_α (Wilden et al., 1986). Additionally, ligand-asociated receptor can be selectively internalized and degraded (Sibley and Lefkowitz, 1985).

In S. cerevisiae, several mechanisms for adaptation to the pheromone response have been identified. *MAT***a** cells produce an extracellular protease, encoded by the *BAR1* locus, that specifically degrades α factor pheromone (Hicks and Herskowitz, 1976; Ciejek and Thorner, 1979; Sprague and Herskowitz, 1981). As in mammalian cells, the carboxyl terminus of the pheromone receptor is hyperphosphorylated in response to ligand and has been shown to mediate an adaptive response to pheromone (Reneke et al., 1988; Konopka et al., 1988). Additionally, ligand–receptor complexes are internalized and degraded (Jenness and Spatrick, 1986; Chvatchko et al., 1986). The product of the *SST2* gene, which encodes an as yet unknown function, is also required for adaptation to pheromone, since *sst2* mutant cells are adaptation defective (Chan and Otte, 1982; Dietzel and Kurjan, 1987b). Remarkably, it appears that in yeast the activated G_α subunit stimulates an adaptive response antagonistic to the mating signal generated by the $G_{\beta\gamma}$ subunit (Miyajima et al., 1989; Cole et al., 1990; Stone and Reed, 1990). We report here that this activity may in part be carried out by a signal resulting in the phosphorylation of G_β.

Results

The Yeast Mating Response G_β Subunit Is Rapidly Phosphorylated upon Exposure to Pheromone

To characterize the G protein β subunit encoded by the

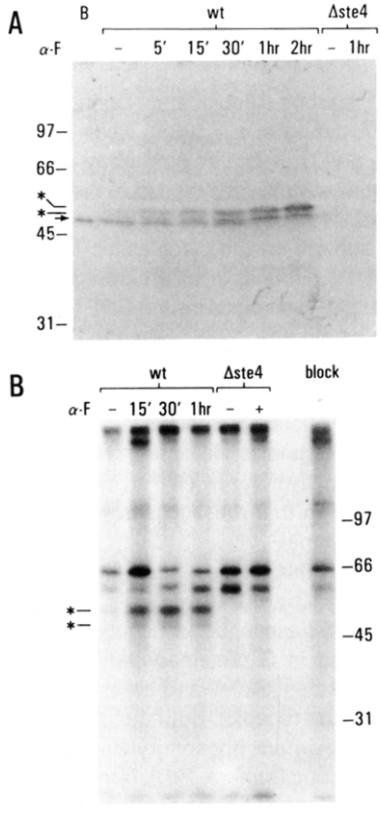

Figure 1. The Ste4 Protein Is Rapidly Phosphorylated in Response to Mating Pheromone

(A) An immunoblot of total yeast proteins prepared with affinity-purified polyclonal antisera to a Ste4 peptide shows the rapid mobility shift of Ste4 to at least two slower migrating species in *MAT***a** haploid cells treated with 2 μg/ml α factor. An isogenic control strain deleted for the *STE4* gene (Δ*ste4*) does not generate a signal to antibody either before or after pheromone treatment. Lane B depicts the relative mobility of bacterially synthesized Ste4 protein. Arrow indicates the position of unmodified Ste4 protein; asterisks indicate modified Ste4 protein.

(B) Immunoprecipitation of ^{32}P-labeled extracts of pheromone-treated cells. Wild-type (wt) or isogenic Δ*ste4* cells were grown in [^{32}P]orthophosphate and treated with 2 μg/ml α factor. Extracts of cells at several time points after pheromone treatment were immunoprecipitated with the same anti-peptide antiserum as in (A). Antibody used in the block lane was preincubated with 100 ng of peptide for 30 min at 0°C prior to immunoprecipitation of wild-type extract. Asterisks indicate the positions of phosphorylated Ste4 protein.

STE4 gene, we prepared polyclonal antisera either to specific peptides or to recombinant Ste4 protein synthesized in Escherichia coli. An immunoblot using an affinity-purified anti-peptide antiserum is shown in Figure 1A. This antiserum recognizes a protein in whole-cell lysates from haploid cells with an apparent M_r of approximately 47 kd that comigrates with bacterially produced Ste4 protein. However, treatment of these *MAT***a** haploid cells with the mating pheromone α factor leads to a shift of the immunoreactive species to at least two forms of lower mobility. These modifications occured rapidly upon exposure to pheromone: at least half of the existing Ste4 was modified within 15 min, while essentially all the Ste4 protein was shifted to more slowly migrating species within 1 hr. Significantly, even in cells not exposed to pheromone, a small fraction of the Ste4 protein exists in the lower mobility forms that predominate in pheromone-treated cells. Lysates from an isogenic strain carrying a chromosomal deletion of the *STE4* gene did not exhibit immunoreactive material either before or after pheromone treatment.

One obvious possible explanation for the pheromone-induced mobility shift of the Ste4 protein is phosphorylation. To test this possibility, we labeled growing *MAT***a** haploid cells with inorganic [^{32}P]orthophosphate and used the same antiserum as in Figure 1A to immunoprecipitate Ste4 protein from either untreated or pheromone-treated cells (Figure 1B). The antibody recognizes two weakly labeled species in the untreated cells, migrating with apparent M_rs of 49 kd and 56 kd. These two bands were much more heavily labeled in pheromone-treated cells, reaching a maximum within 15 min of pheromone exposure. Neither species was present in extracts immunoprecipitated from an isogenic strain deleted for the *STE4* gene. Additionally, preincubation of the antiserum with antigenic peptide prior to immunoprecipitation of a wild-type extract completely blocked the ability of the antiserum to recognize the two phosphoprotein species (Figure 1B, block). Therefore, the two ^{32}P-labeled species result from a rapid phosphorylation of the G_β moiety of the mating pathway transducer in response to mating pheromone. Moreover, there appears to be a small amount of the phosphorylated species present in untreated cells: approximately 10% of the induced amount, as measured by densitometry of the autoradiograph.

*MAT*α haploid cells treated with **a** factor mating pheromone (obtained from filtered medium in which *MAT***a** cells had been cultured) exhibit a pattern of mobility-shifted bands on immunoblots that are indistinguishable from those seen in α factor–treated *MAT***a** cells. Likewise, a fraction of the total Ste4 protein in untreated *MAT*α cells is constitutively modified (data not shown).

Preliminary Characterization of the G_β Phosphoprotein

To more thoroughly elucidate the mechanism and function of G_β phosphorylation, we initiated an analysis of the Ste4 phosphoprotein. To determine the identity of phosphorylated residues in ^{32}P-labeled Ste4 immunoprecipitated from pheromone-treated cells, we performed a phosphoamino acid analysis (Figure 2A). Two-dimensional thin-layer electrophoresis of acid-hydrolyzed Ste4 protein revealed that the majority of the ^{32}P label is found as phosphoserine, although a small fraction is recovered as phosphothreonine. We did not detect phosphorylated tyrosine residues in Ste4.

Is the mobility shift observed in Ste4 in pheromone-treated cells due exclusively to phosphorylation, or is the protein modified in unrelated ways as well? To address this question, we treated immunoprecipitates from pheromone-exposed cells with potato acid phosphatase (Figure 2B). The first lane shows untreated ^{35}S-immunoprecipitated material running at the position of modified Ste4. Treatment of the same immunoprecipitate with potato acid phosphatase caused a quantitative shift in the mobility of

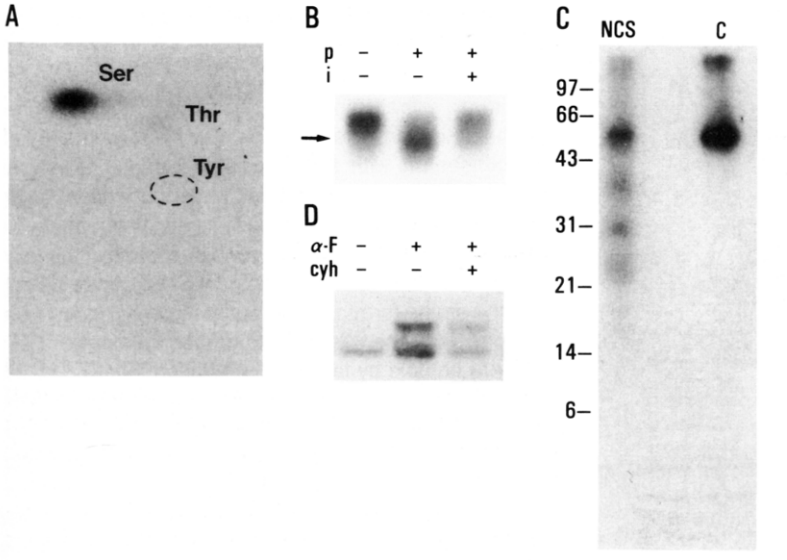

Figure 2. Characterization of the Ste4 Phosphoprotein

^{32}P-labeled extracts of pheromone-treated cells were immunoprecipitated, run on SDS–PAGE, and the band corresponding to the lowest mobility Ste4 phosphoprotein (ppSte4) was excised and subjected to proteolysis with various agents.

(A) Phosphoamino acid analysis. Ste4 is phosphorylated primarily on serine and, to a lesser extent, threonine. Ste4 protein was hydrolyzed with 6 N HCl at 110°C and electrophoresed in two dimensions on a thin-layer cellulose plate. Positions of phosphoamino acid standards are indicated.

(B) Mobility shift of Ste4 in response to pheromone is due to phosphorylation. ^{35}S-labeled immunoprecipitates from pheromone-treated cells were incubated with either potato acid phosphatase (p) or phosphatase plus inhibitors (i), analyzed by SDS–PAGE, and autoradiographed as explained in Experimental Procedures. The arrow indicates the position of unphosphorylated Ste4 from untreated cells.

(C) NCS digest of ppSte4. The tryptophan-specific cleavage agent NCS was used to generate a partial digest of ^{32}P-labeled Ste4 peptides. The digest was electrophoresed on a 20% SDS–polyacrylamide gel, dried, and visualized by autoradiography. Lane C, untreated control.

(D) Pheromone-induced phosphorylation of Ste4 can occur in the absence of de novo protein synthesis. Extracts from cultures treated first with 10 μg/ml cycloheximide (cyh) for 10 min or left untreated, then exposed to 2 μg/ml α factor (α-F) for 1 hr, were analyzed by SDS–PAGE and subsequent immunoblotting using anti-Ste4 polyclonal antisera as described in Experimental Procedures.

the Ste4 protein, in the second lane, to a position equal to Ste4 protein from untreated cells (indicated by arrow). Treatment with phosphatase in the presence of the phosphatase inhibitors sodium pyrophosphate, vanadate, EGTA, and EDTA (Figure 2B, third lane) does not lead to a mobility shift. Therefore the mobility shift observed in Ste4 in response to pheromone is due to phosphorylation. A small fraction of the Ste4 protein remains in the unshifted position in the phosphatase-treated sample. This is probably due to incomplete digestion by phosphatase, although we cannot rule out the possibility that a small fraction of the total Ste4 protein in the cell is modified in ways other than phosphorylation that lead to a shift in mobility.

Preliminary mapping data suggest that the Ste4 phosphoprotein is multiply phosphorylated in response to pheromone (data not shown). As part of an initial characterization of the phosphoprotein, we analyzed the cleavage of ^{32}P-labeled Ste4 protein with the agent N-chlorosuccinimide (NCS). Treatment of proteins with NCS leads to partial proteolysis of the polypeptide chain at tryptophan residues, resulting in a characteristic pattern of fragments for a given protein (Lischwe and Ochs, 1982). The Ste4 protein contains eight tryptophan residues, which would be expected to yield a complex partial digestion pattern of labeled bands if the protein is homogeneously labeled throughout its length. Instead, when ^{32}P-labeled Ste4 was treated with NCS and the digest analyzed by SDS–PAGE, a simple pattern of labeled fragments results (Figure 2C). Treatment with NCS generates a partial digest of four labeled bands, the smallest with an apparent M_r of 23 kd. This result suggests that the primary phosphorylation sites of the protein are probably localized to a small number of trp–trp fragments, since if many such fragments were phosphorylated, the resulting partial digest pattern would be much more complex. Furthermore, the fact that the smallest labeled fragment runs with an apparent M_r of 23 kd suggests that the primary phosphorylation target may be a large trp–trp fragment. The fact that NCS treatment results in partial proteolysis and that phosphorylation of Ste4 causes a mobility shift in the native protein that cannot be extrapolated quantitatively for proteolytic products limits drawing further conclusions.

One important question relating to pheromone-induced phosphorylation of Ste4 is whether it requires de novo protein synthesis or whether it can be carried out as a primary response by preexisting components. Many induced activities of the pheromone response pathway result from increased protein synthesis, since a primary target of the signal transduction pathway is the activation of transcription of a number of gene products involved in the response (Stetler and Thorner, 1984; Nasmyth and Shore, 1987). As Figure 2D shows, protein synthesis is not required for Ste4 phosphorylation. The first two lanes show extracts from wild-type cells before and 2 hr after treatment with pheromone. The third lane in Figure 2D is an identical extract from the same culture preincubated with 10 μg/ml cycloheximide for 15 min prior to pheromone addition. This level of cycloheximide was sufficient to arrest cell growth completely upon addition to the culture. Although the absolute level of Ste4 protein is somewhat reduced in this sample, it is evident that phosphorylation of the molecule proceeds even in the absence of de novo protein synthesis.

A Mutation of *STE4* That Eliminates Basal and Signal-Induced Phosphorylation

G protein β subunits from metazoan cells possess an interesting structural feature: they are composed of eight

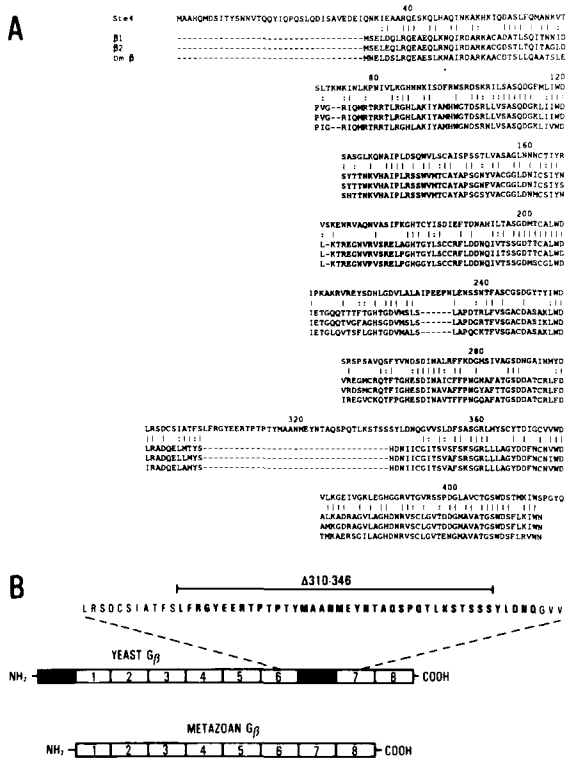

Figure 3. G_β Repeat Structure in Ste4

(A) Comparison of the Ste4 protein sequence to metazoan G_β sequences. G protein β subunits are members of a family of proteins characterized by a repeating amino acid motif of approximately 40 residues. Other members of this family include the Drosophila neurogenic locus *Enhancer of split*, the yeast cell division cycle gene *CDC4*, and *PRP4*, which encodes a component of the U4/U6 snRNP in yeast. Ste4 is compared here with two mammalian G_β subunits (β1 and β2) and with a Drosophila melanogaster homolog (Dmβ). In addition to the eight repeated motifs found in metazoan G_β subunits, Ste4 contains two nonhomologous inserted sequences, one at the amino terminus and one near the carboxyl terminus between repeats 6 and 7. Each inserted sequence is approximately one repeat unit in length.

(B) The structure of yeast G_β compared with metazoan proteins. The nonhomologous inserts in the yeast protein are shown as black boxes. The sequence deleted in the Δ310–346 mutant is indicated by the bar above the sequence from the inserted segment.

homologous domains arranged in a sequential repeating structure (Fong et al., 1986). The repeating units are approximately 40 amino acids in length and frequently terminate with the sequence trp-asp. This repeating motif is present in several proteins involved in diverse cellular functions, for example, the yeast *CDC4* gene (Yochem and Byers, 1987) and a product of the Drosophila *Enhancer of split* locus (Hartley et al., 1988), and is thought to mediate specific protein–protein interactions. The yeast *STE4* gene product is also organized in a G_β repeat structure (Figure 3). However, in addition to the β repeats, the Ste4 protein contains two nonhomologous inserts approximately one repeat unit in length, one at the amino terminus of the protein and one between the sixth and seventh homologous domains. Interestingly, these insertions occur as part of the only two large (>10 kd) trp–trp fragments of the protein. Digestion of the Ste4 phosphoprotein with NCS suggested that phosphorylation of the molecule may be localized to a large trp–trp fragment.

Since in the yeast mating pheromone response $G_{\beta\gamma}$ is the primary signal transducer, we speculated that either or both of these inserted regions may exert regulatory effects consonant with this specialized role, possibly as the site(s) of pheromone-induced phosphorylation. Using primers that precisely delete these regions separately, we amplified portions of the *STE4* gene by means of the polymerase chain reaction. Recombinant *STE4* genes lacking either the amino-terminal insert or the internal insert were used to replace the wild-type chromosomal copy of *STE4* in a *MATa* haploid by targeted integration followed by selection for intragenic recombination (Scherer and Davis, 1979). Precise replacement of the chromosomal copy with the deleted mutant was determined by Southern blot analysis.

We then assayed cells containing these constructs for the ability to respond to mating pheromone. Both strains mate at wild-type or near wild-type frequencies, with the Δ1–35 mutant mating at 100% of the wild-type level and the Δ310–346 mutant mating at 60% of wild-type level. The mating proficiency of these strains indicates that neither mutant protein is significantly impaired in signal transduction. Figure 4A is an immunoblot of whole-cell lysates from these strains. While the amino-terminal deletion of Ste4 protein (Δ1–35) undergoes a pheromone-induced phosphorylation resulting in the characteristic pattern of altered mobility, neither of two separate amplified clones containing the internal deletion (Δ310–346) is modified in response to pheromone.

To verify that Ste4Δ310–346 is in fact defective in signal-induced phosphorylation, we immunoprecipitated the mutant protein from lysates of cells labeled in vivo with [^{32}P]orthophosphate. While wild-type cells demonstrate the pheromone-induced phosphorylation of Ste4, no corresponding label could be detected in Ste4Δ310–346 protein (Figure 4B). This lack of a ^{32}P-labeled species is not due to the inability of the antibody to immunoprecipitate the truncated Ste4 protein, as we verified by immunoprecipitating ^{35}S-labeled Ste4Δ310–346, which was run in parallel to the samples in Figure 4B (data not shown). Therefore, although deletion of the internal Ste4 domain does not affect the ability of the molecule to transmit a signal, it does eliminate the signal-induced phosphorylation of Ste4.

The *ste4*Δ310–346 Mutant Is Defective in Adaptation to Pheromone

One attractive hypothesis for a functional role for G_β phosphorylation is that it leads to inactivation of the $G_{\beta\gamma}$ transducer, thus generating an adaptive response to pheromone stimulation at the level of G protein. If this hypothesis is correct, the *ste4*Δ310–346 mutant would be expected to demonstrate enhanced sensitivity to pheromone in growth-inhibition zone or halo assays (Julius et al., 1983). In this assay, a lawn of cells is spread on the surface of an agar plate. Discrete amounts of α factor are then spotted directly onto the surface of the plate. The soluble α factor diffuses outward through the agar from the application

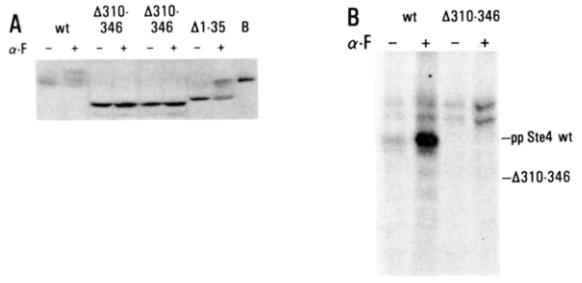

Figure 4. A Deletion of the Carboxy-Terminal Insert of Ste4 (Δ310–346) but Not the Amino-Terminal Insert (Δ1–35) Leads to a Loss of Pheromone-Induced Phosphorylation

(A) Effects of pheromone on the phosphorylation of *ste4* mutants deleted for either the amino-terminal (Δ1–35) or internal (Δ310–346) yeast segment not homologous to metazoan G_β sequences. An immunoblot of total yeast protein from isogenic strains expressing only wild-type (wt) or deleted Ste4 protein was prepared. Mutant cells were grown to midlog phase and analyzed before and after treatment with 2 μg/ml mating pheromone (α-F). Two separate clones deleted for residues 310–346 were analyzed. Lane B, bacterially produced Ste4 protein.

(B) In vivo labeling of Ste4 wild-type (wt) or Δ310–346 mutant protein with inorganic phosphate in response to mating pheromone (α-F). Immunoprecipitates from either untreated or α factor–treated (2 μg/ml) mutant and wild-type cells grown in the presence of [^{32}P]orthophosphate were analyzed by SDS–PAGE and autoradiography as described in Experimental Procedures. The tick marks at the right indicate positions of [^{35}S]methionine-labeled protein samples corresponding to the ^{32}P-labeled samples (not shown).

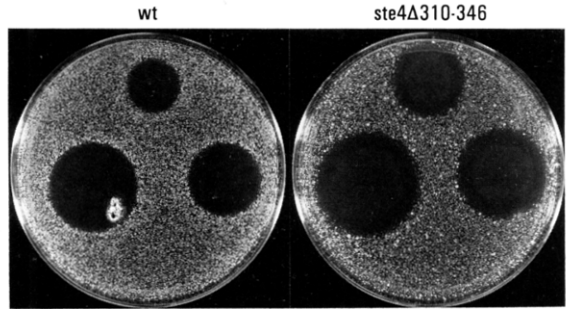

Figure 5. The *ste4*Δ310–346 Mutant Is Supersensitive to Exogenous α Factor

Approximately 10^5 cells of both wild-type (wt) and mutant strains were plated in 2% top agar on rich medium as described in Experimental Procedures, and aliquots of α factor were spotted directly on the surface of the plate. Sensitivity to pheromone can be determined by the size of the growth-inhibition halo in response to a given dose of pheromone, with more sensitive strains generating larger halos. The *ste4*Δ310–346 mutant is about 6 times more sensitive to α factor than the isogenic wild-type strain, since about 6 times less pheromone is required to generate a halo of the same size. The amounts used in these assays were, clockwise from the top spot, 0.16 μg, 0.8 μg, and 4 μg.

point to form a gradient of pheromone concentration. Cells responding to high pheromone concentrations are unable to grow, producing the clear zones of growth inhibition near the application point. At some distance from this, a threshhold level of pheromone concentration is reached below which cells can adapt and grow to form a turbid lawn. Mutants defective in adapting to pheromone stimulation exhibit a much lower threshhold level of pheromone concentration to fully inhibit growth, thus producing larger "halos" in such assays.

If phosphorylation of G_β represents an adaptive response to pheromone, the *ste4*Δ310–346 mutant, which cannot be phosphorylated, should exhibit enhanced sensitivity to pheromone. In fact, mutant cells are about 6-fold more sensitive to pheromone than wild-type cells, since 6-fold less pheromone is required to generate the same diameter halo from mutant as from wild-type cells (Figure 5). The conclusion that the *ste4*Δ310–346 mutant is defective in adaptation to pheromone is corroborated by return-to-growth experiments of pheromone-treated cells. In this assay, cells are exposed to saturating amounts of pheromone to activate the mating response, which results in the arrest of cells in G1 of the cell cycle. Pheromone is then washed out, and the cells are returned to fresh growth medium. The time required for cells to initiate budding and return to growth provides an index of their ability to adapt to pheromone: adaptation-defective cells take longer to return to growth than wild-type cells. As shown below in Figures 8B and 8C, the *ste4*Δ310–346 mutant is significantly delayed in its ability to resume growth relative to an isogenic wild-type strain. Whereas wild-type cells begin to reinitiate budding within 1 hr of pheromone removal and have tripled in cell density by 4 hr, the *ste4*Δ310–346 mutant does not initiate budding until 4 hr after pheromone removal and does not increase in cell density until approximately 6 hr. Thus, the *ste4*Δ310–346 mutant shows a significant defect in adapting to mating pheromone. Nonetheless, these mutant cells retain a significant adaptive capacity. The likely reason for this will be discussed below.

The *ste4*Δ310–346 Mutant Has a Phenotype of Partial Constitutive Pathway Activation

Wild-type haploid cells exposed to mating pheromone exhibit a number of characteristic responses: they arrest growth in the G1 phase of the cell cycle, they induce transcription of mating-specific genes, and they undergo morphological changes preparatory to conjugation that result in the formation of projections of the cell surface. *ste4*Δ310–346 mutant cells exhibit partial activation of these responses even in the absence of pheromone.

One transcript that shows a pronounced response to pheromone is encoded by the *FUS1* gene. *FUS1*, which specifies a membrane protein that is localized to the projection tip and which is required for proper cell fusion, is induced up to 100-fold by pheromone (Trueheart et al., 1987; McCaffrey et al., 1987). As shown in Table 1, *ste4*Δ310–346 mutant cells induce transcription of a *FUS1–lacZ* gene fusion during vegetative growth about 14-fold above basal levels in the wild-type strain. Isogenic wild-type cells have low basal levels of *FUS1* in the absence of pathway stimulation. However, treatment of these cells with α factor mating pheromone leads to an approximately 50-fold increase in *FUS1* transcription, as measured by β-galactosidase activity of the fusion protein. Treatment of mutant cells with pheromone leads to even higher levels of β-galactosidase activity, nearly 10-fold higher than in unstimulated mutant cells. Thus, the constitutive pathway

Table 1. *FUS1-lacZ* Transcription in *ste4*Δ310–346 vs. Wild-Type *STE4* Cells

	Units β-Galactosidase	
Deletion	Basal	α Factor Induced
wild-type	1.0	54
Δ310-346	14.6	134

β-Galactosidase activity was calculated in Miller units (see Experimental Procedures). For α factor induction, LacZ activity was determined 2 hr after addition of 2 μg/ml α factor to midlog phase cultures.

activity seen in these cells is only partial and still capable of further induction.

Wild-type cells treated with low concentrations of pheromone undergo a transient G1 arrest in the cell cycle (Bucking-Throm et al., 1973; Wilkinson and Pringle, 1974). The *ste4*Δ310–346 mutant cells appear to constitutively mimic this response to some extent. While mutant Δ310–346 cells do not arrest in G1, they grow more slowly than wild-type cells, as shown in Figure 6A. Wild-type cells of strain 15Dau in logarithmic phase exhibit a doubling time of about 1.4 hr, whereas the mutant strain grows about twice as slowly, with a doubling time of around 3 hr. Microscopic examination shows that a significant fraction of these cells, about 55%, are unbudded G1 cells. This contrasts with the 30%–40% G1 cells seen in wild-type cultures. Additionally, many of the cells in the mutant population exhibit projection formation diagnostic of cells responding to pheromone pathway stimulation (Figure 6B). The cells are large and distorted in shape, as compared with the smaller, ovoid shapes of the wild-type cells. Treatment of mutant cells with exogenous pheromone leads to gross morphological alterations not seen in wild-type cells even when treated with saturating amounts of pheromone, which again suggests that mutant cells are defective in adapting to pathway stimulation.

*MAT*α cells harboring the *ste4*Δ310–346 mutation also exhibit partial constitutive activation of the pheromone response pathway, although the growth defect is not as pronounced as in isogenic *ste4*Δ310–346 *MAT***a** cells (data not shown).

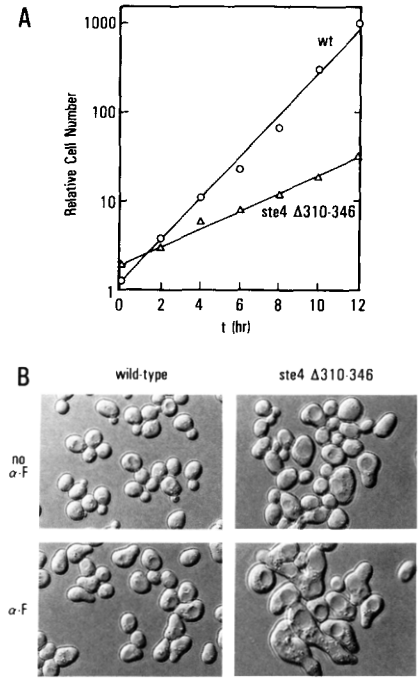

Figure 6. Cells Expressing a Nonphosphorylatable Mutant Ste4 Protein Are Partially Activated for the Pheromone Response

(A) The growth rate of mutant *ste4*Δ310–346 is reduced relative to wild-type (wt) cells. Cells were grown in rich YPD medium at 30°C to early midlog phase, and samples were taken and counted over a period of 12 hr. Whereas wild-type cells had a doubling time of about 1.4 hr, mutant cells took 3 hr to double.

(B) Morphology of the *ste4*Δ310–346 mutant compared with wild-type cells. Cells were photographed using differential interference contrast (Nomarski) optics either in midlog growth phase (no α-F) or 4 hr after addition of mating pheromone (α-F) at a concentration of 4 μg/ml. Wild-type cells treated with pheromone transiently arrest in G1 of the cell cycle and initiate projection formation. The morphology of the mutant cells resembles wild-type cells treated with pheromone, with many cells in G1 and exhibiting some degree of projection formation. Treatment of the mutant with α factor leads to an extreme activated phenotype, indicating an adaptive defect.

Dominance Relationship of *ste4*Δ310–346 to Wild-Type *STE4*

If phosphorylation of Ste4 represents a regulatory mechanism to inactivate the protein, a nonphosphorylatable G_β molecule might be expected to be at least partially dominant to wild type, since it would be unable to respond to proper regulation by the G_β kinase and would continue to transmit a mating signal when stimulated by mating pheromone. To assay for the dominance of *ste4*Δ310–346 to wild type, we first attempted to analyze wild-type haploid cells carrying either the mutant or wild-type *STE4* allele on a centromere plasmid. It has been shown that overproduction of Ste4 protein leads to constitutive induction of the pheromone response (Whiteway et al., 1990; Cole et al., 1990; Nomoto et al., 1990). In fact, we observed that the regulation of the pathway is so sensitive to Ste4 protein stoichiometry in vivo that the presence of an additional wild-type copy of *STE4* on a centromere plasmid causes cells to manifest a phenotype of constitutive pathway activation, with significantly reduced growth and strong projection formation. Therefore, owing to the impact of gene dosage on G protein subunit stoichiometry, it was not possible to assess dominance accurately in this manner.

A test for dominance that avoids the gene dosage effects that complicate the above analysis can be performed in mating-type homozygous diploids. Such cells contain a 2N complement of genetic material but behave physiologically as haploids, owing to the homozygous configuration at the mating type locus. *MAT*-homozygous diploids respond to mating pheromone identically to their haploid counterparts. When a *MAT***a**/*MAT***a** strain heterozygous for the wild-type *STE4* allele and *ste4*Δ310–346 is analyzed for pheromone sensitivity, the mutant phenotype is partially dominant (Table 2). Mutant heterozygous diploids

are still about 4-fold more sensitive to mating pheromone than the control wild-type strain. However, the phenotype of partial constitutive pathway activation in the mutant is completely recessive. The *STE4* heterozygotes no longer exhibit a growth defect relative to wild-type homozygotes, nor are they constitutively activated for *FUS1–lacZ* transcription.

This result shows that the partial-activation phenotype can be uncoupled from the adaptation defect in the mutant. This is significant, since it argues that the increased pheromone sensitivity seen in the haploid mutant cannot be merely a secondary consequence of partial pathway activation. It is likely that a small fraction of the Ste4 protein in the cell is actively transmitting signal even in unstimulated cells. In heterozygous cells, where only half the Ste4 protein is mutant and consequently unregulated by phosphorylation, the level of unregulated signaling in cells not exposed to pheromone may be insufficient to initiate induction of pheromone-responsive transcription. In haploid *ste4*Δ310–346 mutants, where all the Ste4 protein is mutant, the unregulated basal activity of the pathway may be sufficient to initiate a partial response. Complete activation of the pathway by pheromone, in which a significant fraction of the Ste4 protein is expected to be activated for pathway signaling, would generate a significantly larger signal that cannot be deactivated, leading to an adaptation defect in both haploids and heterozygous diploids. Given the extreme sensitivity of the system to small changes in the amount of Ste4 available for signaling, the slightly weaker adaptation defect in diploid heterozygotes as compared with haploids may be due to the fact that half the Ste4 protein in the diploid cell is wild type and therefore capable of being phosphorylated in response to pheromone, thus providing a degree of adaptation to signal.

Phosphorylation of G_β in Signaling Pathway Mutants

The carboxyl terminus of the α factor receptor mediates an adaptive response (Konopka et al., 1988; Reneke et al., 1988), as does the product of the *SST2* gene (Chan and Otte, 1982; Dietzel and Kurjan, 1987b). Recent evidence indicates that the activated G_α subunit encoded by *GPA1* itself generates an adaptive signal (Miyajima et al., 1989; Cole et al, 1990; Stone and Reed, 1990), although the relationship of this pathway to the previously described mechanisms is not yet clear. If phosphorylation of Ste4 is an adaptive response to pheromone sitmulation, it may occur as part of one of these desensitization pathways.

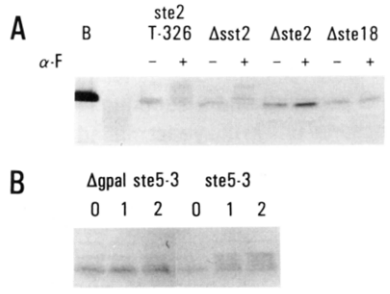

Figure 7. Effects of Mutations in Pheromone Adaptation Genes on Ste4 Modification

Total protein extracts from *MAT***a** strains carrying relevant mutations were immunoblotted with antibody prepared against Ste4. Mutants were assayed both in the presence and absence of α factor (α-F).

(A) Effects of loss of the carboxyl terminus of the pheromone receptor (*ste2*T-326), Δ*sst2*, the entire receptor (Δ*ste2*), and the G protein γ subunit (Δ*ste18*) on Ste4 phosphorylation. Lane B, bacterially produced Ste4 protein.

(B) The G protein α subunit promotes Ste4 modification. Deletion of the *GPA1* gene, which encodes a G_α subunit, leads to constitutive activation of the pheromone response pathway. This activation is suppressed by a mutation in a downstream element encoded by *STE5*. *ste5-3* is a temperature-sensitive mutation that inactivates the pathway at the restrictive temperature. *ste5-3* cells deleted for *gpa1* can be assayed for the ability of the G_α subunit to potentiate G_β modification. Cells grown at 36°C, the restrictive temperature, were shifted to 23°C and α factor was simultaneously added to 2 μg/ml. As a control, a *ste5-3* single mutant was treated identically.

To address this question, we tested the ability of strains carrying various adaptation mutants to phosphorylate Ste4 in response to pheromone (Figures 7A and 7B). *ste2*T-326 is a mutation that deletes the carboxyl terminus of the Ste2 α factor receptor (Konopka et al., 1988). As is true of mammalian receptors of the rhodopsin/β-adrenergic family, to which Ste2 is structurally homologous, this region of the Ste2 protein is phosphorylated (Reneke et al., 1988) and plays a role in adaptation to signaling (Reneke et al., 1988; Konopka et al., 1988). However, the defective receptor does not appear to affect Ste4 phosphorylation markedly in response to pheromone. *SST2* encodes a protein of unknown function (Dietzel and Kurjan, 1987b). Mutations in this gene render cells unable to adapt to even low concentrations of pheromone (Chan and Otte, 1982). Again, however, loss of this gene does not significantly affect signal-induced modification of Ste4. Not surprisingly, cells deleted for the entire α factor receptor (Δ*ste2*) do not respond to pheromone. Also, cells lack-

Table 2. Dominance Relationship of *ste4*Δ310–346 to Wild Type in *MAT* Homozygotes

Genotype	Doubling Time (hr)	Pheromone Sensitivity	*FUS1–lacZ* Basal	*FUS1–lacZ* α Factor Induced
*MAT***a**/*MAT***a** *STE4/STE4*	1.7	1.0	0.8	46
*MAT***a**/*MAT***a** *STE4/ste4*Δ310–346	1.7	3.8	0.8	42

Pheromone sensitivity was measured by halo assays as described in Experimental Procedures. *FUS1–lacZ* activity was calculated in Miller units (see Experimental Procedures); in the case of α factor induction, LacZ activity was determined 2 hr after addition of 2 μg/ml α factor to midlog phase cultures.

ing the G protein γ subunit encoded by *STE18* do not exhibit a Ste4 mobility shift upon pheromone exposure. This last result reinforces previous genetic data that suggest that both G protein β and γ subunits must be present to form a functional unit in the yeast mating response (Whiteway et al., 1989, 1990; Cole et al., 1990; Nomoto et al., 1990).

The G_α subunit encoded by *GPA1* is not essential for transmission of the mating signal. In fact, deletion of the *GPA1* gene leads to a phenotype of constitutive pathway activation (Dietzel and Kurjan, 1987a; Miyajima et al., 1987; Jahng et al., 1988). However, some hyperactivating alleles of *GPA1*, including those containing mutations in the putative GTP-binding/hydrolysis domain that are homologous to oncogenic *ras* mutations at the cognate residue, $GPA1^{val\text{-}50}$ and $GPA1^{asp\text{-}50}$, also have a dominant hyperadaptive phenotype (Miyajima et al., 1989; Stone and Reed, 1990). Overproduction of wild-type Gpa1 protein has a similar phenotype and is capable of suppressing the adaptation defect of an *sst2* mutant (Dietzel and Kurjan, 1987a; Cole et al., 1990). Thus, *GPA1* appears to play a positive role in adaptation.

We therefore assayed *gpa1*-deleted cells for the ability to phosphorylate Ste4. To maintain viablity of this strain (a *gpa1* deletion is lethal owing to constitutive pathway activation leading to growth arrest), the temperature-sensitive *ste5-3* mutation was included. *ste5-3* is a nonspecific mating defect that blocks the pheromone response downstream of *GPA1* when cells are grown at the restrictive temperature (Nakayama et al., 1988; Blinder et al., 1989). Δ*gpa1 ste5-3* cells and control *ste5-3* cells were grown at 36°C, then switched to 23°C to eliminate the signaling block at *STE5* and allow pathway activation. Additionally, a saturating amount of α factor (4 μg/ml) was added to cultures 10 min following the switch to the permissive temperature. As can be seen in the immunoblot in Figure 7B, *ste5-3* cells were able to carry out Ste4 phosphorylation within 1 hr of the switch to the permissive temperature. However, the strain lacking Gpa1 protein exhibited a very low level of Ste4 phosphorylation, even after 2 hr at the permissive temperature. This result suggests that Gpa1 facilitates, either directly or indirectly, the pheromone-induced phosphorylation of the G_β subunit encoded by *STE4*.

Epistatic Interactions of *ste4*Δ310–346 with Pheromone Pathway Adaptation Mutants

The fact that Ste4 is phosphorylated in response to pheromone in both *sst2* and *ste2*T-326 mutants suggests that adaptive responses mediated through these pathways act separately from the adaptation mediated by G protein phosphorylation. Conversely, the reason that *ste4*Δ310–346 mutants show only a limited adaptation defect may be that other mechanisms of adaptation remain available to them. To test this conclusion more directly, we assayed the pheromone sensitivity of strains doubly mutant for *ste4*Δ310–346 and either *sst2* or *ste2*T-326. When *MAT***a**/*MAT*α diploids hererozygous for *ste4*Δ310–346 and *sst2* are sporulated and dissected, all *sst2 ste4*Δ310–346 spores fail to divide and instead assume the distorted morphology of cells exposed to excess pheromone (not shown). In the absence of *SST2* and the capacity to phosphorylate Ste4 protein, cells are completely unable to adapt to even the basal level of pathway activity in the *ste4* mutant.

The interaction of *ste4*Δ310–346 with the α factor receptor mutant *ste2*T-326 is also dramatic, as shown in Figure 8A. In our wild-type strain 15Dau, the *ste2* receptor mutant is about 5 times more sensitive to pheromone than wild type. *ste4*Δ310–346 is also about 5-fold more sensitive to pheromone. Remarkably, the double mutant exhibits a strong synergism in its adaptive defects: it is about 100 times more sensitive than wild type (the halos in the *ste4*Δ310–346 *ste2*T-326 plate result from the application of 25-fold less pheromone for each spot). This synergism of defects in adaptation provides compelling evidence that Ste4 phosphorylation and the cytoplasmic domain of the α factor receptor mediate distinct desensitization responses. Cells deprived of both these adaptive mechanisms are acutely sensitive to pheromone.

This conclusion is corroborated by α factor recovery experiments (Figures 8B and 8C). In these experiments, liquid cultures of cells were arrested with 1 μg/ml α factor. After the entire population of cells was arrested, α factor was washed out and cells were incubated in fresh culture medium. Under these conditions, wild-type cells reinitiate budding within 1 hr of pheromone washout and exhibit log-phase growth within 4 hr. Both *ste4*Δ310–346 and *ste2*T-326 mutant strains do not demonstrate significant budding until 4 hr after removal and do not show significant growth until 8 hr. The double mutant *ste4*Δ310–346 *ste2*T-326 is even slower and less synchronous in overcoming pheromone-induced arrest, with a budding level comparable to single-mutant or wild-type strains only after 8 hr in the absence of pheromone. These results demonstrate a profound defect in the ability of the double mutant to recover from pheromone.

Discussion

G_β Phosphorylation Is Associated with an Adaptive Response

Haploid yeast cells maintained in the presence of mating pheromone will eventually desensitize and resume growth. Genetic dissection has revealed the existence of a number of independent mechanisms for the adaptation of yeast cells to mating pheromone. The number and complexity of these mechanisms are probably characteristic of most eukaryotic signal transduction systems, involving feedback regulation not only at the level of receptor, but also modulation of the signal itself, both intracellularly and extracellularly (reviewed by Sibley and Lefkowitz, 1985). In G protein–mediated signaling, the G protein itself is an obvious control point. Indeed, the activation of the G_α subunit by stimulated receptor, which results in the exhange of GTP for GDP, contains an intrinsic adaptive device in the hydrolysis of GTP and the return to an inactive, GDP-bound state (Stryer and Bourne, 1986; Gilman, 1987). In yeast, the $G_{\beta\gamma}$ subunit appears to be

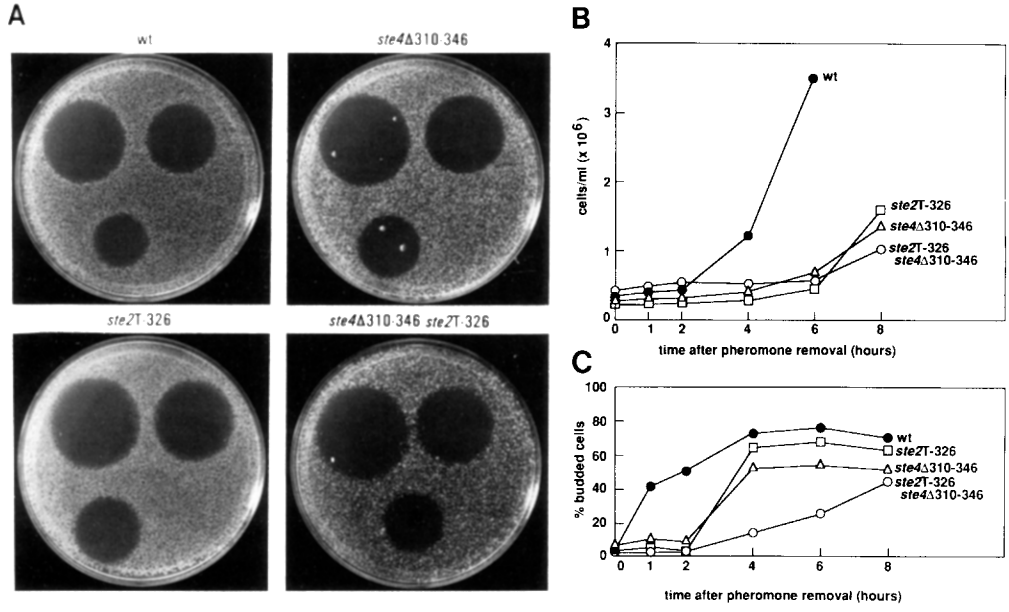

Figure 8. Epistatic Interaction of *ste4*Δ310–346 and *ste2*T-326 Mutations in Response to Mating Pheromone

(A) Growth-inhibition halo assays were performed as described in Experimental Procedures on wild-type (wt), *ste2*T-326, *ste4*Δ310–346, and *ste4*Δ310–346 *ste2*T-326 double mutants. For the double mutant, the amount of pheromone added to each spot was 1/25th that added to the corresponding spots for the wild-type and single mutants. For these, the amount of pheromone added was, clockwise from the leftmost spot, 4 μg, 0.8 μg, and 0.16 μg.

(B) Return to growth of wild-type (wt) and mutant cells after pheromone arrest followed by removal of pheromone. Midlog phase cells in YPD liquid medium were treated with saturating amounts of α factor (1 μg/ml) for 3 hr, then washed and resuspended in fresh YPD liquid. Return to growth was monitored by assaying cell density in a hemacytometer as described in Experimental Procedures.

(C) Budding indices of wild-type (wt) and mutant cells after pheromone arrest followed by removal of pheromone. Samples from (B) were scored for frequency of budded cells as a fraction of the total cell population.

the primary transducer of the mating signal. In such systems, it may be that GTP hydrolysis on G_α is not sufficient to promote the rapid reassociation of GDP–G_α and $G_{\beta\gamma}$ and the subsequent attenuation of the signal. Phosphorylation of $G_{\beta\gamma}$ may represent a control mechanism to assure the prompt inactivation of the $G_{\beta\gamma}$ signal.

We propose that the signal-induced phosphorylation of the G_β encoded by *STE4* acts as such an adaptive device to control the mating pheromone response. Signal-induced phosphorylation of Ste4 is rapid and complete, with approximately half of the Ste4 protein in the cell phosphorylated within 15 min of pheromone application. This rapid signal-induced Ste4 phosphorylation does not require de novo protein synthesis. Phosphorylation is not essential for signaling, since a mutation of *STE4* that eliminates phosphorylation (*ste4*Δ310–346) still allows essentially normal pathway activation and mating. This mutant, however, exhibits a significant defect in adaptation to mating pheromone, being up to 6-fold more sensitive to pheromone than isogenic wild-type cells as well as being strongly impaired in its ability to recover from pheromone-induced arrest. The *ste4*Δ310–346 defect becomes much more acute when cells are deprived of other adaptive pathways such as the receptor- or *SST2*-mediated responses. These phenotypes strongly suggest that Ste4 phosphorylation represents an adaptive response to pathway stimulation at the level of the G protein.

Constitutive Partial Activity of the Pheromone Response in the Mutant

Why does the *ste4*Δ310–346 exhibit a phenotype of partial activation of the mating pheromone response? Two mechanisms can be imagined. First, and most simply, it is possible that deleting residues 310–346 of Ste4, in addition to eliminating the pheromone-inducible phosphorylation of the molecule, also reduces the affinity of $G_{\beta\gamma}$ for G_α. The consequent increase in the amount of free βγ would act to stimulate a partial pheromone response. This partial response would then explain the increase in sensitivity to pheromone as an overall increase in pathway signaling at any given dose of α factor.

A more likely explanation for the partial pathway activation seen in the *ste4*Δ310–346 mutant is that these cells are unable to adapt, at the level of the G protein, to a constitutive signal in all vegetatively growing haploid cells. Several independent lines of evidence have shown that the yeast mating pheromone response pathway is active at a significant level in wild-type cells not exposed to pheromone. Many pheromone-inducible genes, for example, are expressed at significant basal levels even in the absence of pheromone (reviewed in Herskowitz, 1989). Elimination of individual elements of the signal transduction pathway (*STE4*, *STE5*, *STE7*, *STE11*, *STE12*) dramatically reduces the basal level of transcription of these genes (Hartig et al., 1986; Fields et al., 1988). Consistent

with these results, we have observed that mutations in pathway elements genetically downstream of *STE4* reduce the level of Ste4 protein about 10-fold (G. M. C. and S. I. R., unpublished data). These results may indicate that the pheromone response pathway makes a significant contribution to basal expression of responsive genes even in unstimulated cells. Further support for the idea that the pathway is constitutively active comes from the level of Ste4 phosphorylation we observe here in vegetatively growing cells. Since phosphorylation of Ste4 is a response to pathway activation by pheromone, it is significant that up to 10% of the protein is phosphorylated in unstimulated cells.

The behavior of the *ste4*Δ310–346 mutant in *MAT***a**-homozygous diploids suggests that the defect of the mutant lies not in a failure of Ste4 to interact with Gpa1, but rather in a failure to adapt to pheromone. *ste4*Δ310–346/*STE4 MAT***a**-homozygous diploids are not constitutively activated for the pheromone response yet continue to exhibit supersensitivity to pheromone. Downstream adaptive functions, such as the transcriptional induction of the *SST2* gene (which is induced ~50-fold in response to pheromone; Dietzel and Kurjan, 1987b), are not blocked by the failure to phosphorylate Ste4 and could therefore provide an adaptive response to allow the mutant to grow. Loss of other adaptive mechanisms might explain why the *ste4*Δ-310–346 *sst2* double mutation is lethal and why the *ste4*Δ-310–346 *ste2*T-326 double mutant is profoundly impaired in adaptation to pheromone.

The 6-fold defect in adaptation seen in the *ste4*Δ310–346 mutant is comparable in magnitude to that demonstrated by deletion mutations in the carboxyl terminus of the Ste2 receptor. The adaptation pathway mediated by the Ste2 receptor carboxyl terminus shares several interesting features with that of the Ste4-mediated process. Ste2 is also known to be phosphorylated by a serine/threonine-specific protein kinase in response to pheromone (Reneke et al., 1988; Konopka et al., 1988). The Ste2 receptor, like Ste4, is phosphorylated even in the absence of pheromone (Reneke et al., 1988). Interestingly, mutations in the carboxyl terminus of Ste2 that result in adaptive defects also lead to constitutive activity of the pheromone response comparable to those observed in *ste4*Δ310–346 mutants (Konopka et al., 1988; G. M. C. and S. I. R., unpublished data). Ste4 phosphorylation does not require the Ste2 carboxyl terminus, however, and epistatic interactions imply that the two genes lie on different adaptive pathways. Indeed, the pronounced synergism in pheromone supersensitivity of a *ste4*Δ310–346 *ste2*T-326 double mutant provides strong evidence that G_β phosphorylation mediates an adaptive response that becomes critical when cells are deprived of receptor-mediated adaptation.

The Role of G_α in G_β Phosphorylation

Cells lacking *GPA1* show a significantly reduced ability to phosphorylate Ste4. This phenomenon could be due to either direct or indirect effects of Gpa1 on Ste4. For example, it has recently been shown that receptor coupling to G_α requires the products of both the *STE4* and *STE18* genes (Blumer and Thorner, 1990). It is possible that coupling of Ste4 to a signal-induced kinase may likewise require Gpa1 or Gpa1–receptor coupling. Alternatively, there is increasing evidence that activated Gpa1 may stimulate an adaptive response in pheromone-treated cells independent of its presumed role of sequestering $G_{\beta\gamma}$ in an inactive heterotrimer. Some mutations in *GPA1*, including those at a residue homologous to the oncogenic *ras* mutation *ras*$^{val\text{-}12}$ (Gpa1 residue 50) have a phenotype of initial supersensitivity followed by enhanced adaptation (Miyajima et al., 1989; Stone and Reed, 1990). It is thought that these phenotypes result from the same biochemical lesion manifested by the oncogenic *ras* allele: dramatically reduced GTPase activity. Interestingly, the supersensitivity phenotype is recessive while the enhanced adaptive response is dominant, which suggests that GTP-bound Gpa1 protein may stimulate an adaptive response to pheromone. One target of this adaptive response could be Ste4, possibly as a result of stimulating a G_β–specific kinase. Consistent with this notion, a strain containing such a *GPA1*-activating allele is unable to stimulate the dominant adaptive response in cells containing the *ste4*Δ-310–346 mutation (D. Stone and S. I. R., unpublished data). It is therefore possible that the phosphorylation of Ste4 represents one aspect of this Gpa1-mediated response.

The G_β-Specific Kinase

The kinase or kinases responsible for the phosphorylation of Ste4 remain unknown. However, four genes whose products show homology to protein kinases, *STE7*, *STE11*, *KSS1*, and *FUS3*, have been implicated genetically as components of the mating pheromone response pathway (Teague et al., 1986; B. Errede, personal communication; Courchesne et al., 1989; Elion et al., 1990). It is of obvious interest to identify the Ste4 kinase, since this mode of adapting to signal transduction may be a general feature of systems mediated by $G_{\beta\gamma}$ signal transduction. While activated GTP-bound G_α carries with it the means of its own inactivation in the hydrolysis of GTP, the presumed reassociation of GDP–G_α and $G_{\beta\gamma}$ in $G_{\beta\gamma}$ signaling pathways may not be sufficient, for whatever reason, to attenuate the $G_{\beta\gamma}$ signal and allow cells to adapt to an ongoing signal.

Experimental Procedures

Yeast Strains, Media, and Microbiological Techniques

All yeast strains used in this study are isogenic derivatives of strain 15Dau (*MAT***a** *ade1 his2 leu2-3,-112 ura3* Δ*trp1*; Cole et al., 1990). Replacement of chromosomal loci with mutant derivatives was accomplished by targeted integration followed by homologous recombination (Scherer and Davis, 1979). Replacement was verified in each case by Southern blot analysis. Both rich medium (YPD) and synthetic minimal medium (SD) supplemented with various nutrients to allow growth and the maintenance of plasmids were prepared as described by Sherman et al. (1986). Genetic manipulation of yeast cells was as described by Mortimer and Hawthorne (1969). Transformation of yeast cells was by the alkali cation method of Ito et al. (1983). Formation of mating-type homozygous diploids was by spherolast fusion as described by Katz et al. (1987). The growth rate of yeast cultures was assayed by determining cell density in a hemacytometer using a Zeiss phase-contrast microscope. E. coli strains used to propagate plasmids were JA226

and DH5α. Recombinant DNA techniques were essentially as described by Sambrook et al. (1989).

Preparation of Polyclonal Antisera against Ste4 Peptides and Recombinant Ste4 Protein

The peptides CHNNKISDFRWSRDSKRIL-amide and CSAVEDEIQNKIEAARQESK-amide were a generous gift of Dr. Richard Houghton. These peptides were separately coupled to the carrier protein keyhole limpet hemocyanin (Sigma) using the heterobifunctional cross-linking agent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as described in Harlow and Lane (1988), with the exception that free amino groups on the peptides were blocked by treatment with 0.1 M sodium borohydride in ice-cold 0.1 M sodium borate buffer. Two New Zealand White rabbits were then subcutaneously injected with peptide-coupled carrier. The first injection was with 200 μg of antigen in PBS (phosphate-buffered saline: 150 mM NaCl, 20 mM sodium phosphate [pH 7.6]) mixed with an equal volume of Freund's complete adjuvant. Subsequent boosts were with 100 μg of antigen in PBS mixed with an equal volume of Freund's incomplete adjuvant. Boosts were at 2 week intervals, with bleedings at 10–14 days after boosting.

Anti-peptide antisera were affinity purified using the cognate peptide coupled to aminoalkyl agarose (Bio-Rad 2). The coupling reaction was essentially the same as that used to couple peptide to carrier protein, again using the cross-linking reagent MBS. Two to three milliliters of crude serum was cycled through a column containing 2–3 ml of peptide–Sepharose for 1 hr. The column was then washed with 10 vols of PBS (pH 7.6), 10 vols of 0.1 M sodium acetate, 1 M NaCl (pH 4.8), 10 vols of 0.1 M $NaHCO_3$, 1 M NaCl (pH 7.6), and finally 10 vols of PBS (pH 7.6). Elution of antibody was with 0.1 M glycine (pH 2.0) into a volume of 1 M Tris–HCl (pH 8.0) sufficient to neutralize the glycine.

Antisera against recombinant Ste4 protein were prepared using the method of Rosenberg et al. (1987). A NdeI site was precisely engineered at the initiation codon of the *STE4* coding sequence and a BamHI site placed just 3′ of the gene using the polymerase chain reaction. The resulting NdeI–BamHI fragment containing the *STE4* coding sequence was placed under the control of the T7 polymerase promoter in the vector pRK171 (Rosenberg et al., 1987). This construct was transformed into E. coli strain BL21 (DE3). Induction of this strain with 0.4 mM IPTG leads to rapid, high-level accumulation of Ste4 protein. This bacterially produced protein was enriched from the insoluble fraction of bacterial extracts as described by Kleid et al. (1981). Purified recombinant Ste4 was obtained by preparative SDS–PAGE followed by electroelution. Injection of rabbits and preparation of antisera were as described for peptide antisera. The crude serum against recombinant Ste4 protein was affininty purified by chromatography on bacterially produced Ste4 protein coupled to CNBr–Sepharose CL-4B, essentially as described by Wittenberg et al. (1987). Column preparation, washing, and elution were identical to that for peptide affinity columns.

Immunoblots

Cell lysates were prepared by harvesting 5 × 10^6 yeast cells in a 1.5 ml Eppendorf tube and resuspending in 100 μl of sample buffer (0.1 M Tris–HCl [pH 6.8], 2% SDS, 2% β-mercaptoethanol, 20% glycerol). Cells were then boiled for 3 min. Acid-washed glass beads (0.45 mm) were added to the suspension, and the mixture was vortexed at high speed for 2 min. Glass beads were separated from the lysate. Cell debris was pelleted by centrifugation in a microfuge for 5 min. An amount of crude lysate equal to an A_{280} of 0.3 to 0.5 was electrophoresed on a discontinuous SDS–polyacrylamide gel and dry blotted to nitrocellulose (Schleicher and Schuell) as described by Kyhse-Andersen (1984). Blots were then blocked with 2.5% nonfat dry milk, 2.5% BSA in Tris-buffered saline (TBS: 20 mM Tris–HCl [pH 7.5], 500 mM NaCl) overnight. Blots were subsequently incubated with dilute affinity-purified antibody in 0.2× blocking solution in TBS for 4 hr and washed several times with TBS. Alkaline phosphatase–conjugated anti-rabbit IgG (Pharmacia) was then used to identify antigen-bound antibody on the blots according to the manufacturer's instructions.

Immunoprecipitations of Labeled Proteins

Cells to be labeled with ^{32}P were grown overnight in low-phosphate buffer (Reed et al., 1985). The following day cultures were diltued to a density of 2.5 × 10^6/ml and allowed to double once. [^{32}P]orthophosphate (carrier free, Amersham) was added at 1 mCi/ml. Cultures were grown for 2 hr to equilibrate internal phosphate pools. α Factor pheromone (2 μg/ml) was added at this time, and aliquots were withdrawn and frozen in a dry ice–ethanol bath at various time points. Frozen samples were thawed and resuspended in lysis buffer (50 mM Tris–HCl [pH 7.5], 1% SDS, 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 0.1 mM sodium vanadate, 0.025% PMSF, 5% [vol/vol] aprotinin [Sigma], and 10 μg/ml leupeptin and pepstatin). Acid-washed glass beads were added, and the suspension was vortexed at high speed for 2 min. ^{32}P-labeled crude lysates were then boiled for 4 min. After glass beads were removed, the lysates were spun in a microfuge at 12,000 × g for 5 min to remove cell debris. BSA was added to a concentration of 5 mg/ml, and a 20-fold excess of unlabeled extract prepared from an isogenic strain deleted for the *STE4* gene was added. An equal volume of 2× TNT (2% Triton X-100, 100 mM NaCl, 40 mM Tris–HCl [pH 7.5]) was added, followed by affinity-purified antibody.

Immunoprecipitates were allowed to form at 4°C for a period of several hours. Antibody–antigen complexes were bound with fixed Staphylococcus aureus cells (Pansorbin, Calbiochem) and washed several times with TNTBPIS 0.1% (1× TNT, 2 mg/ml BSA, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 0.1% SDS). Samples were eluted from S. aureus cells by boiling in sample buffer for 3 min and then analyzed by SDS–PAGE. ^{32}P-labeled proteins were visualized by autoradiography at −70°C with one intensifying screen on XAR-5 X-ray film (Kodak).

Phosphoamino Acid Analysis and NCS Analysis of ^{32}P-Labeled Ste4 Protein

Following identification of ^{32}P-labeled Ste4 in immunoprecipitates, the relevant bands were excised from the dried polyacrylamide gel and subjected to several analytical procedures. Phosphoamino acid analysis followed the procedure described in Boyle and Hunter (1991). Briefly, the gel slice was ground to a slurry in 1 ml of 50 mM ammonium bicarbonate; 50 μl of β-mercaptoethanol and 10 μl of 10% SDS were added and the sample was boiled for 3 min. The sample was then incubated at 37°C overnight with shaking. Gel bits were removed by centrifugation for 2 min in a microfuge, then reextracted with 200 μl of 50 mM ammonium bicarbonate. The two supernatants were combined and then precipitated with 20 μg of RNAase A in 20% trichloroacetic acid (TCA). The TCA precipitate was dried and oxidized with performic acid (900 μl of 98% formic acid was added to 100 μl of 33% hydrogen peroxide; performic acid was formed after 60 min at room temperature). Seventy-five microliters of ice-cold performic acid was added to the TCA precipitate for 1 hr at 0°C; 400 μl of deionized water was added to dilute the performic acid, and the sample was then frozen and lyophilized. Fifty microliters of constant boiling HCl (5.7 M) was added to the precipitate, and the sample was placed at 110°C for 1 hr. Following hydrolysis, the sample was lyophilized and resuspended in 5 μl of pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid). It was then spotted onto thin-layer cellulose plates (MCB Reagents) with 70 μg/ml unlabeled phosphoamino acid standards. Electrophoresis in pH 1.9 buffer in the first dimension was for 20 min at 1500 V. The plate was then dried and subjected to electrophoresis in the second dimension in pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for 16 min at 1300 V. Phosphoamino acids standards were visualized with 0.25% ninhydrin in acetone. Autoradiography was as described above.

Cleavage at tryptophan residues was carried out with NCS as described by Lischwe and Ochs (1982). In short, labeled gel slices were incubated in 15 mM NCS in a urea–water–acetic acid mixture (1 g:1 ml:1 ml) for 30 min. NCS was omitted from control samples. The slices were washed for 30 min in water followed by equilibration in sample buffer. Whole gel slices were then loaded directly onto discontinuous SDS–polyacrylamide gels and electrophoresed. The partial tryptophan digest of ^{32}P-labeled fragments was then visualized by autoradiography as above.

Phosphatase Treatment of Ste4 Phosphoprotein

Cells were grown in SD minus methionine to midlog phase (5 × 10^6 cells per ml). At this point 250 μCi of ^{35}S in the form of Tran^{35}S (ICN) was added to each milliliter of culture, which was then incubated at 30°C for 2 hr. α Factor (2 μg/ml) was added, and cells were shaken at 30°C for another 1 hr. Three milliliters of culture was centrifuged for 2 min in a microfuge and washed once in ice-cold water. Cells were then lysed and immunoprecipitated in the same way as ^{32}P-labeled cells,

with the exception that protein A–Sepharose CL-4B beads (Sigma) were used to precipitate antigen–antibody complexes. Immunoprecipitates were washed two times with RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM Tris–OH [pH 7.5], 150 mM NaCl, 1 mM sodium pyrophosphate) followed by three washes with phosphatase buffer (20 mM MES [pH 5.5], 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 2% [vol/vol] aprotinin [Sigma], 4 μg/ml soybean trypsin inhibitor). Beads were then resuspended in 150 μl of phosphatase buffer and divided into three 50 μl aliquots. One was treated with 0.2 U of potato acid phosphatase (Boehringer), one with 0.2 U of phosphatase plus phosphatase inhibitors (final concentrations: 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 0.1 mM sodium orthovanadate), and one control aliquot remained untreated. Incubation was at room temperature for 30 min. Beads were washed once in RIPA followed by elution in boiling sample buffer (2 min). Samples were then analyzed by SDS–PAGE. Prior to drying, gels were immersed in the fluor Autofluor (National Diagnostics) for 2 hr and then rinsed for 15 min in distilled water. Intensifying screens were not used for ^{35}S autoradiography.

Deletions of the *STE4* Gene

Precise deletions of *STE4* were generated by the polymerase chain rection employing primers complementary to specific regions of the gene (Saiki et al., 1988). Naturally occurring restriction sites (PstI near the 5′ end and XbaI at nucleotide position 1038) were used to subclone deleted fragments back into vectors expressing *STE4*. All mutants generated by the polymerase chain reaction were represented by at least two separate amplification reactions to control for possible errors in amplification. These constructs were transformed into strains deleted for the wild-type *STE4* gene. For several experiments, replacement of the chromosomal locus with mutant alleles was effected by targeted integration followed by selection for intragenic recombination (Scherer and Davis, 1979). In this way, proteins containing precise deletions could be expressed at the proper chromosmal location and in the proper stoichiometric amount.

Pheromone Sensitivity Assays

Growth-inhibition zone or halo assays were carried out as described by Julius et al. (1983). Approximately 10^5 cells of a given strain were plated in 8 ml of molten nutrient agar on a plate of identical composition. Once the agar solidified, 2 μl of α factor at various concentrations was spotted onto the surface of the plate. This assay measures the ability of cells to grow in the presence of varying concentrations of pheromone following the initial G1 arrest induced by pathway signaling. All assays were carried out with isogenic strains deleted for the gene encoding the Bar1 protease.

For return-to-growth experiments following exposure to pheromone, log-phase cultures of cells in YPD liquid were arrested with 1 μg/ml α factor for 3 hr. Cells were then washed twice with YPD and resuspended in fresh YPD at 30°C. At the indicated time points, aliquots of each culture were placed in 1/10th vol of 37% formaldehyde (Sigma), sonicated briefly to separate clumps of cells, and then scored for budding and counted for culture density in a hemacytometer using a Zeiss phase-contrast microscope. All return-to-growth experiments were carried out with isogenic strains deleted for the gene encoding the Bar1 protease.

Mating Assays

Quantitiative mating assays were done according to the procedure of Reid and Hartwell (1977). Approximately 3.5×10^6 cells of the strain to be assayed and of a tester strain were mixed and filtered onto sterile nitrocellulose disks. These disks were placed onto YPD plates for 6 hr at 30°C. Cells were then resuspended in sterile water and serially diluted onto both rich medium and medium selective for diploids formed by mating of the two strains (containing complementing auxotrophic markers). The frequency of mating was then calculated as the frequency of diploid colonies formed relative to total colonies formed on rich medium.

FUS1 Pheromone Induction Assays

Assays for induction of the pheromone-inducible *FUS1* transcript utilized a *FUS1–lacZ* gene fusion (Trueheart et al., 1987) that had been integrated in single copy in the genome at the *FUS1* locus. The level of transcript induction was measured by β-galactosidase activity in permeabilized yeast cells, essentially using the technique of Guarente (1983). LacZ activity was calculated using the colorimetric substrate o-nitrophenyl β-D-galactopyranoside (ONPG) and was determined in Miller units: $A_{420} \times 1000/v$ (ml) $\times$ t (min) $\times A_{600}$.

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