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Receptor-Mediated Down Regulation of the Mating Signaling Cascade in *Saccharomyces cerevisiae*.

By
Andrés Couve

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in
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Receptor-Mediated Down Regulation of the Mating Signaling Cascade in *Saccharomyces cerevisiae*.

Abstract

Adviser: Professor Jeanne Hirsch

The yeast pheromone response pathway is mediated by two G protein-coupled receptors and a MAP kinase cascade. Three responses constitute the final outcome of the mating pathway. These include arrest at the G1 phase of the cell cycle, induction of transcription of mating specific genes and morphological changes that increase mating efficiency. The $STE3^{DAF}$ mutation results in inappropriate expression of the **a**-factor receptor in *MAT**a*** cells. Expression of this receptor in the inappropriate cell type confers resistance to pheromone-induced G1 arrest, a phenomenon termed receptor mediated inactivation. The ability of $STE3^{DAF}$ cells to escape cell cycle arrest in the presence of pheromone was found to correlate with reduced phosphorylation of the cyclin-dependent kinase inhibitor Far1p. Measurement of Fus3p MAP kinase activity in wild type and $STE3^{DAF}$ cells showed that induction of Fus3p activity was the same in both strains at times of up to one hour after pheromone treatment. However, after two or more hours, Fus3p activity declined in $STE3^{DAF}$ cells but remained high in wild type cells. mRNA levels of the mating specific inducible gene *FUS1* correlated with changes in Fus3p kinase activity. *STE3*^{DAF} was found to inhibit the pheromone response pathway at a step above the scaffolding protein Ste5p and above the Ste20p protein kinase. In addition, epistasis experiments demonstrated that *STE3*^{DAF} inhibited the mating pathway at the level or downstream of the Gβ subunit of the G protein. Mutational analysis of the Gβ subunit of the heterotrimeric G protein revealed that signaling through the mating pathway and receptor mediated inactivation could be uncoupled. *STE3*^{DAF} insensitive Gβ mutations mapped to essential domains for Gβ/Gα and Gβ/effector interactions. These findings indicate that receptor mediated inhibition is achieved by modification of Gβ function. Furthermore, they provide new evidence for the possible participation of Gβ and pheromone receptor in an adaptive response.

The observations significantly contribute to the understanding of the *STE3*^{DAF} mutation. They also provide new insights into the kinetics of the mating response and the relationship between the G protein coupled receptor and its effector molecules.

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Finally, I would like to thank Coty for illuminating the depths that science does not reach.

Foreword

The format of this thesis has been chosen based on the general outline for scientific publications. The separated sections of "Background", "Materials and Methods", "Results" and "Discussion" provide an easier way to understand and analyze the experiments without interruption. During the "Background" section, the mating signaling cascade is followed inward from the cell surface to the nucleus. The outward direction is regained during the "Results" section, where the experiments are described starting from the very downstream components of cell cycle control to the plasma membrane associated proteins; following the sequential order in which they were developed.

Importantly, since the $STE3^{DAF}$ phenotype constitutes an inhibitory effect, possibly associated with adaptation, I have emphasized the participation of different components in adaptation at the end of the individual topics during the "Background" section.

Finally, I would like to mention that part of this work has been published as: Loss of Sustained Fus3p Kinase Activity and the G_1 Arrest Response in Cells Expressing an Inappropriate Pheromone Receptor. 1996. Andrés Couve and Jeanne P. Hirsch. Molecular and Cellular Biology, 16: 4478-4485.

Table of Contents

List of Tables

List of Figures

Background

1. Brief introduction to the yeast *Saccharomyces cerevisiae.*

The yeast *Saccharomyces cerevisiae* belongs to the class *Ascomycetes,* one of the three classes comprised in the phylum of true fungi (162). It is a unicellular organism, also known as the budding yeast, 3 µm in diameter, round to oval in shape and surrounded by a rigid cell wall (216). The genetic material of the budding yeast consists of approximately $12X10^6$ base pairs organized in 16 chromosomes, all of which have been sequenced to date (88).

The yeast life cycle is characterized by diploid and haploid phases. In the diploid state, cells undergo vegetative mitotic division and increase in number while nutrients are available. Mitosis takes place as in higher eukaryotes although some important differences exist. Instead of giving birth to two identical daughter cells, division occurs through budding (216). Selection of a budding site, rearrangement of the cytoskeleton and polarization of secretion produce a growing bud at the tip of the mother cell. The nucleus migrates to the forming bud and the process is completed in the presence of an intact nuclear membrane.

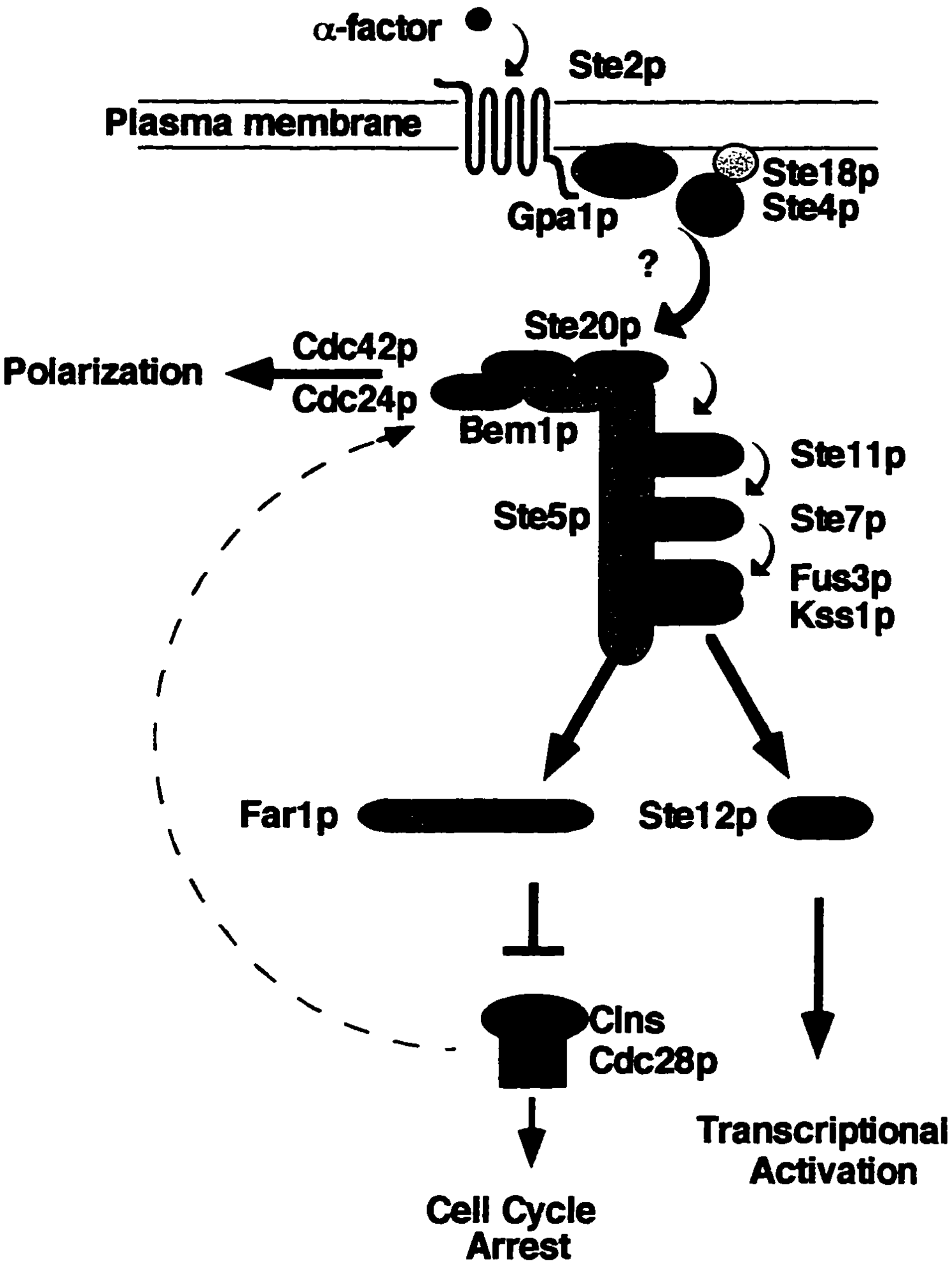
Under severe growth conditions, such as nitrogen starvation in the presence of a nonfermentable carbon source, diploid cells enter meiotic division. As a consequence, four spores form intracellularly in a spore sac. The spores form independently of each other and free from attachment to the sac wall. The result, after germination, is the formation of four haploid cells that proceed to divide mitotically through a very similar budding mechanism. Finally, diploidy is restored by the formation of a zygote between two haploid cells of opposite mating types (162).

2. Sexual reproduction in *Saccharomyces cerevisiae.*

a) Pheromones mediate sexual communication between haploid cells.

Haploid cells resulting from yeast meiotic division may belong to one of two kinds of mating types, and the mating type is controlled by the presence of specific sequences at the *MAT* locus. Thus, cells expressing ***MATa*** are designated mating type **a** whereas cells expressing *MAT*α are designated mating type α (192). As mentioned above, haploid cells divide by budding. In addition, when **a** and α cells are mixed they undergo sexual fusion, a process termed mating. To achieve mating, haploid cells produce and respond to peptide pheromones which are secreted into the medium. Binding of pheromone triggers the activation of the mating signaling pathway and prepares the cell for conjugation (Figure 1). Each mating type produces a specific kind of pheromone: **a** cells produce **a**-factor while α cells produce α-factor (for reviews see 132, 87). Synthesis and secretion of the two mating pheromones occur through different pathways in each cell type. **a**-factor is encoded by two genes, *MFa1* and *MFa2*. It is a twelve amino acid peptide,

Figure 1. Schematic representation of the mating pathway activated by α-factor. See "Background" for details.


α-factor
Ste2p
Plasma membrane
Ste18p
Ste4p
Gpa1p
?
Ste20p
Polarization
Cdc42p
Cdc24p
Bem1p
Ste11p
Ste5p
Ste7p
Fus3p
Kss1p
Far1p
Ste12p
Clns
Cdc28p
Transcriptional Activation
Cell Cycle Arrest

synthesized on free ribosomes as a propeptide and modified posttranslationaly by farnesylation and carboxy-methylation (4). **a**-factor is secreted to the medium through a membrane translocator encoded by the *STE6* gene, which belongs to the ATP binding cassette (ABC) family of proteins (111). α-factor is also encoded by two genes, *MFα1* and *MFα2*. In contrast to **a** factor, however, the propeptide contains a signal sequence for transport into the endoplasmic reticulum (58). The propeptide is, thus, translocated to the ER and later secreted through the Golgi apparatus and the typical secretory pathway. Pro-α-factor is modified by three endoproteases encoded by *KEX1, KEX2* and *STE13,* and N-linked glycosylated on three residues to produce a mature thirteen amino acid peptide (103, 37, 8, 102, 104, 113). *MAT**a*** cells secrete a protease encoded by the *BAR1* gene that cleaves and inactivates α-factor (128). This mechanism allows for faster recovery from pheromone exposure and constitutes a very early adaptation event. Secreted pheromones bind to and activate receptors located at the surface of cells of the opposite mating type. Therefore, **a**-factor, secreted by **a** cells, binds to and activates the **a**-factor receptor in α cells (11), while α-factor, secreted by α cells, binds to and activates the α-factor receptor present in **a** cells (99).

b) Pheromones activate receptors on the surface of conjugating yeast.

The **a**-factor receptor is only present in α cells and it is encoded by the *STE3* gene (148, 79). Ste3p is a 54 kDa, highly hydrophobic protein that localizes to the cell membrane as shown by immunofluorescence (148). It contains seven potential membrane spanning domains connected by extracellular and intracellular hydrophilic loops, and an intracellular hydrophilic carboxyl-terminal tail (33). Similarly, the α-factor receptor is present exclusively in **a** cells and it is encoded by the *STE2* gene (19). The predicted structure of the α-factor receptor is very similar to that of the **a**-factor receptor although no significant sequence homology has been found (148). Most pheromone binding studies have been performed using the α-factor receptor in **a** cells, mainly because of the availability of active and mature α-factor. These studies have shown that α-factor binds to **a** cells with an equilibrium dissociation constant of $6X10^{-9}$ to $2X10^{-8}$ M and that approximately 10^4 binding sites exist per cell (99, 170).

Yeast pheromone receptors show significant structural homology to the large superfamily of seven transmembrane, G protein-coupled receptors that respond to a wide variety of stimuli, and include light activated receptors such as invertebrate and vertebrate rhodopsin, hormone receptors such as substance P and bradykinin receptors, and neurotransmitter receptors such as the β-adrenergic receptor (200, 6). Studies on mammalian receptors have indicated that the transmembrane segments and extracellular domains play an important role in ligand binding (50, 40, 200). The role of residues in these positions in the yeast pheromone receptors has been investigated in some detail and the studies indicate that they also provide the ligand binding specificity (131).

Desensitization and adaptation are characteristic of most, if not all, signal transduction systems, and pheromone receptors provide an important step at which inactivation may occur in the mating pathway. Evidence to support this function has come from the observations that α-factor receptors are subject to internalization (100) and that receptors that lack the C-terminal hydrophobic tail show hypersensitivity to α-factor (109). This last finding suggests that the C-terminal domain participates in a negative regulatory event, and possibly in adaptation. In a similar way, **a**-factor receptors with point mutations or small deletions in the third intracellular loop show constitutive activation and hypersensitivity to **a**-factor (17).

In mammalian cells, β-adrenergic receptors desensitize as a result of phosphorylation by the specific protein kinase βark (12) and rhodopsin down regulates in response to phosphorylation by rhodopsin kinase (221). No protein kinases with this activity have been identified in yeast. Nevertheless, the α-factor receptor is hyperphosphorylated and endocytosed in response to pheromone exposure (224, 173, 28), so the existence of receptor kinases is certainly possible.

Upon pheromone binding, the receptor triggers the activation of a heterotrimeric guanine nucleotide-binding protein (G protein) and a downstream kinase cascade that mediate the responses that prepare the cell for mating (132, 87) (Figure 1). These responses include arrest in the G_1 phase of the cell cycle, transcriptional activation of mating specific genes and morphological changes (191, 132).

3. The yeast mating pathway.

a) A heterotrimeric G protein couples the receptor to downstream components.

A yeast heterotrimeric G protein has been implicated in the propagation of the mating signal from the pheromone receptor to downstream components of the pathway (112) (Figure 1). The heterotrimer is formed by the products of three unique genes that resemble mammalian Gα, Gβ and Gγ subunits (132).

The *GPA1* gene encodes the yeast Gα subunit and its product is a 472 amino acid protein expressed exclusively in haploid cells and 45% identical to rat $G\alpha_i$ (49, 144, 146). Recently, the crystal structure of the mammalian $G_i\alpha_1\beta_1\gamma_2$ heterotrimer and of transducin (the heterotrimeric G protein complex for the visual cascade of the rod cell) have been described (214, 116, 80). Since Gpa1p in yeast presents extensive sequence similarity to mammalian Gα, the structural features that determine the mechanism of action of this subunit are likely to be conserved. Briefly, GTP binding correlates with conformational changes that occur predominantly in three regions of Gα, termed switch regions I, II and III (115, 188, 36). Several residues in these regions are involved in Gβγ binding, and rearrangements that occur in the GTP bound state expose a new surface in Gα. These conformational changes modify the ability of Gα to interact with Gβγ subunits and effector molecules (80). In *S. cerevisiae*, stimulation of the specific pheromone receptor, either by **a**-factor or α-factor, is likely to promote the exchange of GDP for GTP in Gpa1p (112). This event triggers the activation of Gpa1p and the release of the Gβγ subunits from the heterotrimer, in a similar fashion to that described for mammalian GTP binding proteins (107).

Analysis of the null allele indicates that Gpa1p is not required to activate the mating pathway. On the contrary, Gpa1p has been classified as a negative regulator since strains bearing a disruption in the *GPA1* gene show a constitutively active signaling cascade (49, 144, 97). Gpa1p is N-terminal myristoylated and absence of the lipid moiety correlates with activation of the signaling cascade, probably due to the inability of the Gpa1p subunit to bind and, therefore, inactivate the Gβγ dimer (198). In addition, C-terminal mutations in Gpa1p prevent activation of the pathway, presumably by modifying the receptor-G protein interactions (199, 91).

Desensitization mechanisms have also been associated with Gpa1p. First, a mutation that substitutes valine for glycine at position 50 of the protein (analogous to the val12 activation mutation in the Ras GTP binding protein) causes pheromone supersensitivity, partially constitutive signaling and increased cell cycle arrest recovery (143, 199, 114). However, the relationship between supersensitivity and adaptation remains poorly understood. Second, mutations in the Sst2p protein also affect sensitivity and result in delay recovery from pheromone exposure (23). Recent evidence indicates that *GPA1* is epistatic to *SST2* (143, 114). Thus, the adaptation mechanism engaging Sst2p also appears to involve Gpa1p. A direct role of Sst2p as the desensitization effector of the Gα subunit has been postulated, but the evidence supporting this conclusion is not conclusive (51, 52).

The Gβ subunit in yeast is a 423 amino acid protein encoded by the *STE4* gene (217). It shares significant similarity to bovine Gβ1 and Gβ2 transducin subunits (217), and is 40% identical to human Gβ isoforms (123). As mentioned above, the crystal structure of the heterotrimeric G protein has been solved (214, 116, 187). Interestingly, Gβ subunits of several distantly related organisms present significant homology along the entire length of the protein. In addition, it seems that all of them, including Ste4p, fit the three dimensional model proposed for the transducin Gβγ dimer (187). Briefly, the protein is arranged as an N-terminal helix linked to seven symmetric β-propeller domains, that result from antiparallel stacking of β-sheets (214, 187). Residues from the top of the β-propeller, especially from blades 1, 2 and 3, interact extensively with the switch I and II regions of Gα (214, 116) and one side of the propeller domain interacts with the N-terminal helix of Gα (116).

In addition, the N-terminal helix binds the extended N-terminal helix of Gγ (214, 187).

The Gβ and Gγ subunits in yeast are responsible for activating the mating cascade through a still unknown downstream effector. Several findings support this model. First, overexpression of *STE4* leads to the activation of the mating response as indicated by haploid-specific cell cycle arrest (218). Deletion of the Gγ subunit suppresses this phenotype and suggest that the Gβγ dimer is the active component. Second, *STE4* haploid specific mutants (*STE4*^{Hpl}) also cause terminal cell cycle arrest as a consequence of constitutive signaling (15). The simplest interpretation of these two observations is that activation of the pathway results from an excess of free Ste4p subunits. Finally, mutations in three specific domains of the protein produce Ste4p subunits that prevent signaling even in the presence of wild type Ste4p subunits; thus these alleles behave as dominant negative mutants (119, 75, 38). Importantly, some of these mutants block constitutive signaling resulting from deletion of Gpa1p (119, 75). These findings confirm the importance of Ste4p in signaling through the mating pathway. The positive role of Gβ in signaling has now been established for a number of G protein activated pathways in mammalian cells (195).

Ste4p has also been implicated in the process of adaptation. During the mating response, the protein is phosphorylated in a non-conserved domain that includes residues 310-346. Deletion of the phosphorylated region causes supersensitivity to pheromone and cells carrying the mutant Ste4p do not recover properly from pheromone-induced cell cycle arrest (34).

The Gγ subunit in yeast is a 110 residue protein, encoded by *STE18*, similar to the bovine transducin Gγ subunit (217). As in mammalian systems (154), Ste4p and Ste18p subunits are thought to form a tight complex (218). Interestingly, Gγ subunits, including Ste18p, are more divergent than Gα or Gβ subunits, and the difference may be important in determining the specificity of the Gβγ complex (154). As seen in other Gγ subunits (154), Ste18p contains a carboxy-terminal consensus sequence for isoprenylation, but the role of this modification is not well understood (219). The crystal structure of the G protein heterotrimer reveals that the Gγ subunit contains two helices and that the N-terminal helix forms an extensive coiled-coil with the N-terminal helix of Gβ (214, 115). In addition, the results show that the conformation of Gγ does not change significantly during the GTP cycle (115). Homology between mammalian and yeast G proteins suggests that the structural characteristics of the mammalian Gγ subunit are conserved in the yeast Ste18p protein during activation of the mating signaling cascade.

b) A mitogen activated protein (MAP) kinase module functions after the G protein.

In yeast, pheromone binding triggers the activation of a specific seven transmembrane receptor and the dissociation of the Gβγ subunits from the Gα subunit. The downstream effector of Gβγ has not been identified, but several proteins seem to act in close proximity to the plasma membrane. As mentioned above, the mating response produces cell cycle arrest, increase in transcription of mating specific genes and morphological changes. To initiate at least two of these responses, namely cell cycle arrest and transcriptional induction, a MAP kinase module is activated by the incoming signal (87) (Figure 1). The problem then is to link the G protein to the MAP kinase module.

Recent evidence indicates that the mating pathway might not be linear downstream of the activation of the G protein. At this point, a large protein complex may be responsible for divergence of the signal to produce polarization of the cytoskeleton (27). Alternatively, this large complex may work to transmit the signal downstream (110) and at the same time, serve as a checkpoint for the morphological changes once the signal has been transmitted.

A key component of this protein complex is Ste20p. Ste20p is a serine/threonine protein kinase necessary for mating (169, 118, 223) and important for the generation of cell polarity (118). In addition, Ste20p is essential for cell cycle arrest and transcriptional activation (118). Epistasis experiments place Ste20p near the beginning of the pathway, downstream of the Gα and Gβ subunits and upstream of the MAP kinase module (118) (Figure 1). Early evidence indicated that Ste11p, the first enzyme in the yeast MAP kinase module, was phosphorylated by Ste20p *in vitro* (223). This finding suggested that Ste20p was the direct or indirect effector of the G protein and the bridge to the kinase cascade. In spite of this, the observation has not been supported by new data and it is reasonable to believe that the exact place of action of Ste20p remains unknown. Besides, it has been recently shown that Ste20p interacts with Cdc42p (225) and this reinforces the idea that Ste20p works predominantly in the generation of cell polarity. The role of Ste20p in the morphological response will be discussed in the next section.

Although the connection between the G protein and the MAP kinase cascade remains elusive, the activation signal is received by the MAP kinase module shortly after it originates at the cell surface. The MAP kinase module concept has received considerable attention lately and examples of it have been found in a wide variety of systems (87, 215). These include several yeast signal transduction pathways such as the responses for mating, osmotic regulation, cell wall construction and pseudohyphal differentiation (3, 66). In addition, the same organization of protein kinases has been found in the distantly related yeast *Schizosaccharomyces pombe*, in nematodes and in *Drosophila* (133). Moreover, conservation of the module has been shown for several signal transduction pathways in vertebrates such as those involved in the responses to growth factors, cytokines and stress (134, 93, 89).

The MAP kinase module consists of three proteins kinases activated sequentially by phosphorylation (133, 87) (Figure 1). In the mating pathway of *S. cerevisiae*, the three enzymes that constitute the module are: 1) The MEK kinase homolog Ste11p, 2) the MEK homolog Ste7p and 3) the partially redundant MAP kinase homologs Fus3p and Kss1p (87). The MAP kinase cascade in the mating pathway functions downstream of the G protein. This has been shown convincingly by experiments in which an activating signal originating from the G protein is blocked in the absence of components of the MAP kinase module (147, 15).

Similarly, the order of action of the enzymes in the module has been determined through *in vitro* kinase assays, site directed mutagenesis and epistasis experiments. The first enzyme to be activated is the serine/threonine kinase Ste11p; this activation occurs by unknown mechanisms. Ste11p activates the MEK homolog Ste7p, presumably through phosphorylation of a conserved threonine residue at position 363 (155). Finally, following its activation, Ste7p phosphorylates the Fus3p and Kss1p protein kinases (7, 71, 65). These observations are supported by the following facts: first, Ste11p activates Fus3p only in the presence of Ste7p; second, activation of Ste7p occurs in the absence of the MAP kinase homologs (228); and third, a Ste7p mutant that lacks catalytic activity is unable to phosphorylate Fus3p *in vitro* (228).

Fus3p is phosphorylated on threonine 180 and tyrosine 182, and these modifications correlate with activation of the protein (71). The regulation of the second MAP kinase homolog, Kss1p, is not as well documented but it is likely to occur by similar mechanisms. Kss1p is phosphorylated on serine, threonine and tyrosine residues and phosphorylation requires a functional mating pathway downstream of Ste11p (126).

As mentioned earlier, several MAP kinase cascades exist in yeast (3). In the same way, different cascades function in mammalian cells to integrate a large number of signals generated as a result of different stimuli (215, 134). It is, therefore, very interesting to note that mechanisms for isolating the components of a signaling cascade must exist in order to provide i) specificity and ii) independence to a particular response. It is also important to mention that cross-talk between different pathways is, intuitively, essential to provide a coherent response to a complex environment. In view of these essential properties, several solutions may be envisioned to provide the necessary specificity, independence and plasticity to a signaling pathway. For example, the components may localize specifically to certain compartments of the cell or function in microenvironments; alternatively, they may exist in the same compartments but be expressed at different times during growth or development. The yeast mating cascade apparently achieves independence due to the existence of the MAP kinase module as a discrete multiprotein complex (60). Evidence for this idea started to accumulate with the finding that a scaffolding protein, Ste5p, binds all the components of the MAP kinase cascade, namely Ste20p, Ste11p, Ste7p, Fus3p and Kss1p (167, 30, 120). In addition, interaction between some of the kinases was found to be, at least in part, dependent on the presence of Ste5p (30). Furthermore, Ste5p contains a metal-binding motif, similar to the LIM domain that has been implicated in protein-protein interactions and a proline-rich region that could serve as a binding site for SH3 domain containing proteins (60). Thus, it seems likely that Ste5p tethers the MAP kinase complex, and does so by physically interacting with each one of the kinases through different regions (30). The presence of this multiprotein kinase complex reflects an efficient mechanism to provide specificity to signal transmission. Surprisingly however, the yeast mating cascade is the only well characterized example where such a solution has been utilized.

Ste5p may have another interesting function in the propagation of the mating signal. Recent evidence indicates that this potential scaffolding protein interacts with Ste4p, as shown by two-hybrid and co-immunoprecipitation analyses (220). These findings suggest that activation of the cascade might depend on the subcellular localization of the kinase module. A clear example of this kind of regulation has been extensively studied in mammalian signal transduction. Here, activation of the protein kinase Raf requires membrane targeting through the ras protein to promote activation of signaling events (197, 121). In the same manner, Ste5p may recruit components of the MAP kinase cascade to the projection tip, where receptors and G proteins concentrate during the pheromone response (96, 60). The well established mammalian system should provide a useful parallel model to uncover the significance of membrane targeting of the MAP kinase module during mating in yeast.

The activation of the MAP kinase module is a critical event that takes place during the pheromone response. Clearly, inactivation must also play a key role in the regulation of the cascade, and must be a highly controlled phenomenon during adaptation to pheromone exposure. There is little evidence, however, of such mechanisms of inactivation of the yeast protein kinases. Activation of the MAP kinase module is tightly regulated by phosphorylation. Thus, an obvious way to achieve inactivation is through protein phosphatases acting in concert with the known kinases. Surprisingly, only one phosphatase, Msg5p, has been identified so far in the yeast pheromone pathway (54). Overexpression of the *MSG5* gene decreases pheromone-induced transcription by approximately 50%, and accelerates the recovery phase from pheromone-induced cell cycle arrest. Msg5p is homologous to dual specificity protein phosphatases and removes phosphate groups from threonine and tyrosine residues in the MAP kinase homolog Fus3p *in vitro* (54).

A second mechanism to achieve inactivation of the MAP kinase cascade has been suggested. This one includes the existence of a negative feedback loop controlled by the MAP kinase homologs. Activation of Fus3p and Kss1p has been proposed to mediate inactivation of the upstream kinase Ste7p through phosphorylation (65). Support for this hypothesis comes from the observations that Ste7p binds to Fus3p and Kss1p (30, 167), that Ste7p is a substrate for Fus3p and Kss1p *in vitro* (65, 228) and that Ste7p activity is higher in the absence of active Fus3p and Kss1p (65). The feedback loop awaits a formal demonstration, but it seems reasonable to suspect its existence.

After activation of the MAP kinases, the signal originated at the cell surface diverges. Thus, the MAP kinase homologs function to distribute the information by acting on at least two independent downstream components. These correspond to the transcription factor Ste12p and the cell cycle inhibitor Far1p (87) .

c) Downstream components include a cell cycle inhibitor and a transcription factor.

Pheromone binding triggers an increase in transcription of several mating-specific genes. These include genes that encode components of the signaling pathway per se, such as *STE2, STE3, STE4, STE5, GPA1, FUS3, FAR1* and genes involved in cell and nuclear fusion such as *FUS1, CHS1, Agα1, AGA1* and *KAR3* (132). The transcription factor Ste12p is responsible for mating specific transcription (56) (Figure 1). This observation is supported by epistasis experiments that place Ste12p at the most downstream position of the mating pathway (55) and by DNA binding assays that indicate that Ste12p binds to the pheromone response element (PRE), an upstream regulatory DNA sequence present in many mating specific genes (56, 64). Ste12p is rapidly phosphorylated in response to pheromone (189), and the MAP kinase homolog Fus3p appears to be responsible for the observed modification (63). In addition, Kss1p (the second MAP kinase homolog) has also been implicated in Ste12p phosphorylation (63). Although a clear correlation exists between the activation of the mating pathway and Ste12p phosphorylation, no direct evidence demonstrates that phosphorylation of Ste12p is required for activity. Nonetheless, phosphorylation mediated activation is suggested by the observation that Ste12p shows no activity in the absence of Fus3p and Kss1p (61). Ste12p is not the only protein required for mating-specific transcription and some general transcription factors, such as Mcm1p, have also been identified (87).

As mentioned above, the MAP kinase homologs participate in the activation of at least two branches of the mating pathway; these include induction of mating specific transcription, through Ste12p, and inhibition of the cell cycle, through the cell cycle inhibitor Far1p. Although a more detailed description of the participation of the cell cycle in the mating response will be presented later, a brief introduction to it is essential to understand the function of Far1p.

In *S. cerevisiae* a single cyclin-dependent protein kinase (CDK) controls the progression through the different stages of the cell cycle (171). This enzyme corresponds to the p34^{cdc2} homolog Cdc28p (222). During the G_1 to S transition, accumulation of G_1 cyclins (Cln1p, Cln2p and Cln3p) and other phosphorylation events lead to the activation Cdc28p (150, 45, 78, 175, 211). During the course of the pheromone response, haploid cells that are cycling normally leave the cell cycle, arrest at the G_1 phase and differentiate into mating-competent cells. The Far1p protein is required for pheromone mediated G_1 arrest and acts as a cyclin-dependent protein kinase inhibitor (159, 160) (Figure 1). Several findings uphold this idea. First, haploid cells do not arrest in response to pheromone in the absence of the *FAR1* product (25). Second, Far1p co-immunoprecipitates with Cdc28p and Cln2p, as well as with other G_1 cyclins, in pheromone treated cells, but not in untreated controls (209, 160). Third, purified Far1p inhibits Cdc28p-Cln2p kinase activity *in vitro* (160). Finally, the *in vitro* kinase activity of Cdc28p-Cln2p decreases in a Far1p dependent manner in response to pheromone (160).

Evidence from mammalian systems indicate that several cell cycle inhibitors are necessary for normal cell cycle progression (184, 76, 106). Structural observations demonstrated that human $p27^{Kip1}$ inhibits cyclin A-Cdk2 by binding both elements in the complex, by mimicking ATP and causing a conformational change in the catalytic region of Cdk2 (177). The precise mechanism of Far1p mediated inhibition in yeast and its relation to other cell cycle inhibitors, such as $p27^{Kip1}$, $p40^{SIC1}$, p17 and p21 (180, 124, 106, 177), remains to be determined.

It is probable that several inputs converge at Far1p and regulate the activity of this large protein. Far1p is phosphorylated in the presence of pheromone just before START (26, 137) and phosphorylation requires the MAP kinase homolog Fus3p (63). In addition, Far1p is phosphorylated by Fus3p *in vitro* (63, 159) and the phosphorylation pattern partially overlaps the phosphorylation pattern of Far1p *in vivo* (159). Fus3p is not the only protein kinase that acts upon the inhibitor, since Cdc28p also phosphorylates Far1p (159). The protein levels of Far1p fluctuate throughout the cell cycle and are high exclusively during early G_1, at the time when inhibition of cell cycle progression takes place (137). Thus, evidence gathered so far suggests that accumulation and phosphorylation activate the cell cycle inhibitor. Likewise, the findings indicate that as a consequence of these modifications, Far1p binds and inactivates Cdc28p thereby preventing progression through the G_1 phase of the cell cycle in response to pheromone.

Taken together, the observations described above imply that a signal that originates at the cell surface is propagated through a MAP kinase cascade and evokes a complex response that includes several aspects of yeast physiology.

d) The morphology complex receives a divergent signal upstream of the MAP kinase module.

The mating cascade outlined above can be visualized as a linear pathway bifurcated at the end to mediate transcriptional induction and cell cycle arrest. A large amount of recent evidence indicates that the system is far more complex than this model suggests. Findings from one of the specific responses to pheromone exposure, namely polarization of the cytoskeleton, support this observation.

Morphology is altered on several occasions during the life cycle of *Saccharomyces cerevisiae*. Cell division occurs by budding in haploid and diploid stages, and this process requires the establishment of an asymmetric organization of organelles, the cytoskeleton and membrane components (27). Additionally, pheromone binding during the mating response causes dramatic changes in cell morphology and cytoskeletal polarization (181) (Figure 1). Thus, a cell that is normally round or oval in shape develops a membrane projection towards the source of pheromone and adopts a pear-like form. Most of the evidence indicates that a large multiprotein complex promotes these changes in response to the mating signal originated at the cell surface (120, 225). In addition, the data suggest that the protein complex functions upstream of the MAP kinase cascade, although the exact way in which the signal impinges on it is still a matter of debate.

At the core of this complex lies Cdc42p, a low molecular weight GTP-binding protein of the Rho family of proteins, that appears to be involved in the generation of cell polarity during normal budding and during mating projection formation (1, 101, 226). Rho type proteins have been implicated in several aspects of morphology in *S. cerevisiae* (1, 229, 57). In particular, cells become round, large, multinucleate and fail to bud in the absence of Cdc42p, indicating that the protein plays a crucial role in morphological changes during the normal cell cycle (1). Cdc42p is localized to the plasma membrane (230) and directly associates with the (GDP)-GTP exchange factor Cdc24p (226) and the GTPase-activating proteins Rga1p (196) and Bem3p (227).

In addition to the role of Cdc42p in the generation of cell polarity, recent evidence suggests that the protein is directly involved in signaling. In spite of the fact that the exact mechanism of activation of Cdc42p and the protein complex responsible for polarization are not known, some interesting observations connect morphogenesis to signaling through the mating cascade. First, the mating signaling pathway is defective in cells lacking Cdc42p or Cdc24p (186, 225). Second, overexpression of *CDC42* or expression of an activated allele of *CDC42* result in hyperinduction of mating specific transcription (225). Finally, a number of physical and genetic interactions, that link the proteins responsible for morphogenesis to the signaling cascade, have been reported. In this way, GTP-Cdc42p has been shown to interact with Ste20p and Cdc24p has been shown to associate with Ste4p, the Gβ subunit of the heterotrimeric G protein (225). Thus, emerging evidence favors a model in which activation of the pheromone receptor stimulates the heterotrimeric G protein to activate Cdc42p and the mating cascade. The central role of Cdc42p in signaling is further supported by the finding that GTP-Cdc42p is able to stimulate the kinase activity of Ste20p *in vitro* (186) and by observations which suggest that Cdc42p-related molecules activate a wide variety of protein kinases in mammalian systems (129, 135, 31, 5, 39, 142). As mentioned above, the evidence that links Ste20p to the MAP kinase module is not conclusive, therefore, these findings are not to be taken as definitive proof that Cdc42p is the effector of Gβ and the activator of the mating kinase cascade.

One more piece of evidence is essential to understand the function of morphogenesis. This evidence concerns Bem1p, a protein required for mating projection formation (9, 29). Bem1p physically associates with Cdc42p (161), Ste20p, Ste5p and actin (120, 125). In addition, recent findings suggest that Bem1p participates directly in the activation of the mating signaling cascade and place the protein upstream of the MAP kinases Fus3p and Kss1p (125). These results are in agreement with the role of other polarity gene products, such as Cdc42p, in the activation of the mating cascade.

Interestingly, Bem1p also interacts with Far1p, although the type of association (e.g. direct or indirect) has not been investigated (125). The cell cycle inhibitor Far1p has also been implicated in projection formation during the mating response (213). Thus, the interaction between these two proteins strengthens a model which places the multiprotein complex at a key intersection between direct signaling, generation of polarity and integration of cell cycle information.

Thus, in summary, with the components identified so far, a model can be proposed that physically connects and functionally associates G proteins, the actin cytoskeleton, the MAP kinase cascade and the cell cycle. Whether the interactions between morphological components and proteins involved in the kinase cascade reflect direct activation steps remains to be determined.

e) Cell cycle components also participate in the regulation of the pathway.

A large multiprotein complex transmits the pheromone signal to the proteins responsible for morphological changes and possibly to the kinase cascade (110). The view presented so far allows for a semi-linear pathway in which the signal diverges twice; once just downstream of activation of the G protein and a second time downstream of the MAP kinase module (Figure 1). There is still, however, another event that works to decrease the linearity of the mating signaling pathway and involves cell cycle progression. The basic observation is that the mating pathway is optimally activated during the G_1 phase of the cell cycle (172).

The connection between cell cycle control and the mating pathway appears to engage Far1p, the G_1 cyclins and possibly Ste20p. Far1p accumulates before G1 and is rapidly degraded after cells pass through START (138). Thus, Far1p may regulate the G_1 specificity of the mating response by only inhibiting cell cycle progression at G_1. On the other hand, G_1 cyclin protein levels, in particular Cln1p and Cln2p, fluctuate during the cell cycle and are high in the G_1 phase, just before START (222). Accumulating evidence indicates that high levels of Cln2p repress pheromone signaling and that Cln2p-mediated inhibition requires Cdc28p activity (157). These findings suggest that a substrate of the Cdc28p-Cln2p complex acts as an inhibitor of the mating pathway. The Ste20p protein kinase has been proposed as a candidate for such role. The Cdc28p-Cln2p complex could phosphorylate and maintain Ste20p in an inactive state during the cell cycle (158). When cells pass START,

G_1 cyclins are degraded and the activity of Cdc28p-Cln2p declines (222). This decrease in Cdc28p kinase activity could relieve Ste20p inhibition and the protein could work as a checkpoint component for the activation of the mating pathway. In other words, Ste20p could have a permissive function over a critical activator and could allow communication of the cell cycle machinery and the signaling cascade.

Thus, it seems likely that cell cycle progression critically influences the mating pathway. There is, however, one inconsistency in the two suggestions presented above. Far1p and G_1 cyclin accumulation occur roughly at the same time. Therefore, according to this model, Far1p causes cell cycle arrest exactly at the point in the cell cycle where the G_1 cyclins, in particular Cln2p, are most potent in inhibiting the mating signal. The inconsistency has been partially resolved by experiments that show that Far1p is required for a rapid decrease of Cln2p protein levels (212). This implies that Far1p is at the same time responsible for releasing the inhibitory effect of Cln2p and arresting the cells at the G_1 phase of the cell cycle. The optimal time for signaling through the mating pathway is thus restricted to a short interval just before START. Conditions during the rest of the cycle probably force the cells to attain this stage before responding properly.

An implication of these observations is that stabilization of the G_1 cyclin activities constitutes a mechanism of pheromone resistance; thus, the cell cycle has some relationship with different recovery processes of the mating cascade. Interestingly, Cln2p-mediated signal inhibition is dependent on Cdc28p kinase activity and independent of the adaptation mechanisms mediated by Sst2p and Ste2p carboxyl-terminus (157).

4. The *DAF* phenotype.

a) A screen for pheromone resistant cells yielded several mutants defective in cell cycle arrest.

The review presented above stipulates that components of the mating signal transduction pathway are involved in the generation of cell cycle arrest, mating specific transcription and morphological changes in response to pheromone. Of these, cell cycle arrest represents a reliable reporter for signaling through the mating pathway. Previously, a genetic screen based on these observations was devised to search for mutations that resulted in dominant resistance to α-factor (*DAF* mutations) in *MAT**a*** cells (45). The screen selected cells that escaped cell cycle arrest in the presence of pheromone. Several mutants were obtained and three have been characterized so far.

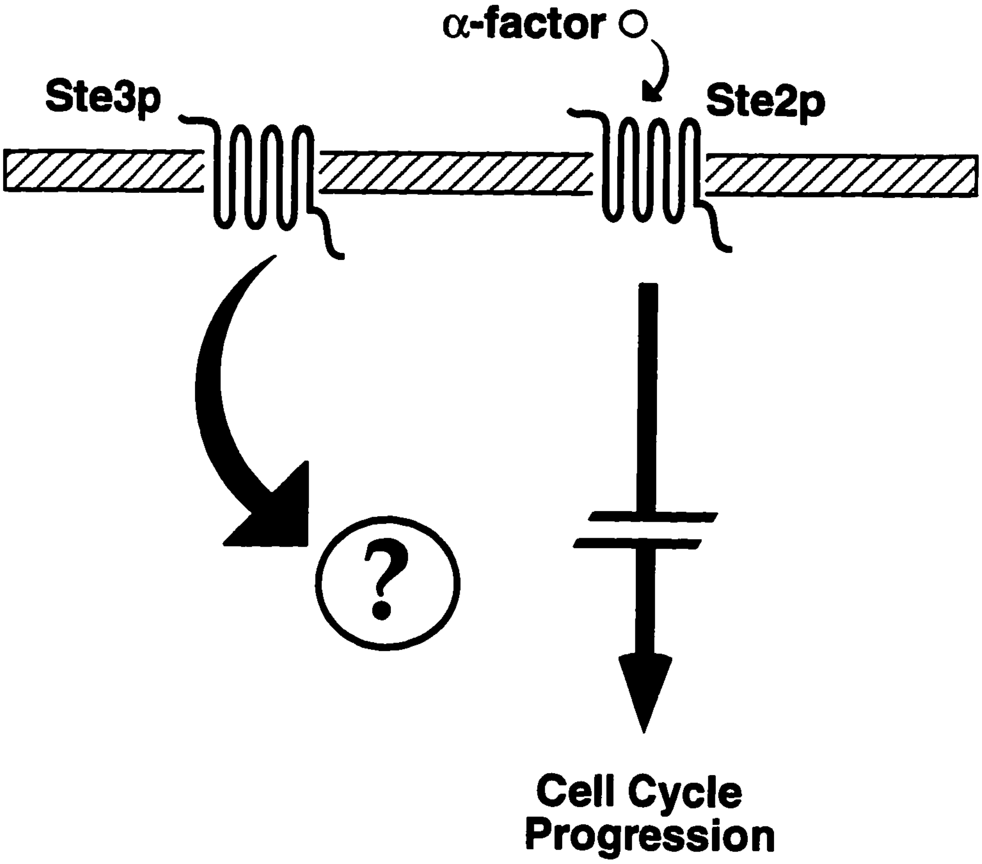
DAF1-1 was shown to be a mutant allele of the *CLN3* gene (45, 175), and similarly, *DAF3-2* was shown to correspond to an allele of *CLN2* (47). Cln2p and Cln3p are G_1 cyclins, and isolation of *CLN2* and *CLN3* in the *DAF* screen indicated that cell cycle components are highly regulated during the pheromone response.

Another mutation that was isolated was *DAF2-2*. Cells bearing this mutation arrested transiently in response to α-factor, but resumed growth at a time when control cells remained fully arrested (46). In addition, cells retained the *DAF2-2* phenotype in the presence of two mutations that activated the signaling pathway independent of pheromone, namely deletion of *GPA1* and expression of $STE4^{Hpl}$. These results indicated that *DAF2-2* inhibited cell cycle arrest and that the site of action of the mutation was at the level of, or downstream of the G protein (46).

b) *DAF2-2 is an allele of STE3.*

Using a genomic DNA library from *DAF2-2* cells, it was further demonstrated that *DAF2-2* is an allele of *STE3*, the gene that encodes the **a**-factor receptor and is normally expressed exclusively in *MATα* cells (90) (Figure 2). *DAF2-2* differed, however, from *STE3* in that the former contained a repetitive element upstream of the initiation codon. Surprisingly, the insertion of this element caused inappropriate expression of *STE3* ($STE3^{DAF}$) in *MAT**a*** cells (90). It is extremely important to clearly establish the nature of the $STE3^{DAF}$ phenotype for the complete understanding of the mutation. *MAT**a*** cells normally express the *STE2* gene. This means that they express α-factor receptor and respond to α factor. In contrast, *MAT**a*** $STE3^{DAF}$ mutants express an intact *STE3* gene in addition to *STE2*. Expression of wild-type *STE3* in *MAT**a*** cells mimicked the resistance to cell cycle arrest originally described

Figure 2. The *STE3*^{DAF} phenotype. Expression of the *STE3*^{DAF} receptor in *MAT**a*** cells interferes with the pheromone response and causes resistance to cell cycle arrest.


α-factor
Ste3p
Ste2p
?
Cell Cycle Progression

for the mutation, indicating that the insertion of the repetitive element upstream of the *STE3* gene interfered with the normal regulation of gene expression and not with *STE3* function (90).

The phenomenon of receptor-mediated inhibition of cell cycle arrest had been explored before using a different system (11, 10). *MATα* cells carrying a mutant *MATα2* gene express both α-specific and **a**-specific genes, thus, haploid *matα2* cells secrete both pheromones and express both pheromone receptors at the surface. Using this mutation, strains that secreted only **a**-factor but that expressed **a**-factor and α-factor receptors were constructed. These cells were unable to arrest in response to pheromone and they exhibited a phenotype extremely similar to *STE3*^{DAF} cells. However, the experiments did not explain the cause of the receptor-mediated interference (10).

A characteristic of *MAT**a*** cells carrying the *STE3*^{DAF} mutation is their inability to arrest in response to α-factor. *MAT**a*** cells normally produce and secrete **a**-factor and *MAT**a*** *STE3*^{DAF} cells, in turn, secrete **a**-factor and inappropriately express the **a**-factor receptor. Thus, the system immediately raises the complication of an autocrine feedback loop. This circuit originates when mutant cells secrete **a**-factor that binds to the inappropriately expressed receptor at the surface of the same cell. The mutation may, therefore, generate the observed phenotype by partially activating the mating response and triggering a desensitization mechanism. This cycle produced much of the confusion in the early studies of the *STE3*^{DAF} mutation and complicated the findings of mutations in the *MATα2* gene (46, 90, 10).

To clarify this issue, the *STE3*^{DAF} mutation was studied in cells that no longer produced **a**-factor (90). This allowed a more detailed understanding of the direct effect of *STE3*^{DAF} on the mating pathway. Surprisingly, *STE3*^{DAF} cells that lacked the genes for **a**-factor still failed to arrest in response to pheromone (90).

c) *STE3*^{DAF} is a novel inactivating component of the mating pathway.

Many studies in signal transduction have been performed with the help of hyperactive or constitutively active alleles used to order other components in a particular signaling pathway or to test the participation of more than one protein in the same cascade of events. Deletion of *GPA1*, the gene encoding the yeast Gα subunit of the heterotrimeric G protein, causes permanent activation of the mating pathway probably due to an excess of free Ste4p subunits (49, 144, 97). Suppression of the *gpa1* phenotype in *STE3*^{DAF} strains indicated the existence of an inactivation step either at the level of, or downstream of the G protein (46). In addition, the cell cycle arrest caused by deletion of *GPA1* was used successfully in the study of *STE3*^{DAF} and its relationship with different aspects of the mating response (90). For example, *STE3*^{DAF} suppressed the *gpa1* phenotype in the absence of **a**-factor synthesis. Furthermore, expression of *STE2*, the α-factor receptor gene, was not required to suppress the *gpa1* phenotype (90). These observations situated the point of action of the mutation downstream of the pheromone receptor (90), in agreement with previous findings that localized it downstream of the G protein (46). They also demonstrated that interaction between the two pheromone receptors was not relevant to the mutant phenotype. Finally, the *gpa1* phenotype was also suppressed by *STE3*^{DAF} in the absence of Sst2p (90). Sst2p is a protein involved in adaptation (24, 49) and this finding suggested that insensitivity to pheromone in *STE3*^{DAF} cells did not involve the participation of this known adaptation mechanism.

The absence of **a**-factor synthesis also provided a better model to investigate mating-specific transcription in *STE3*^{DAF} cells. Previous studies using cells that synthesized **a**-factor and inappropriately expressed the **a**-factor receptor had reported intermediate transcriptional induction in response to α-factor (10, 46). Surprisingly, induction of mating-specific mRNA levels in *STE3*^{DAF} cells occurred almost normally when the autocrine feedback loop was disrupted (90). This suggested that the intermediate transcriptional response observed previously was a consequence of desensitization due to permanent stimulation of the *STE3*^{DAF} receptor by **a**-factor. The observations also indicated that a nearly full transcriptional response was restored when cells were not autostimulated.

d) Cell type specificity of receptor inhibition.

The *STE3*^{DAF} phenotype is characterized by expression of the *STE3* gene in a *MAT***a** cell type. Interestingly, a similar albeit weaker phenomenon is observed if the *STE2* gene (encoding the α-factor receptor) is expressed in *MATα* cells which normally express *STE3,* the **a**-factor receptor. An important difference between the two equivalent systems is demonstrated by the finding that pheromone-independent signaling in the absence of *GPA1* is suppressed by *STE3*^{DAF} in *MAT***a** cells, whereas the *gpa1* growth defect is not inhibited by the inappropriate expression of *STE2* in *MATα* cells (90). However, the reciprocal phenomenon is not altogether different since pheromone-independent signaling as a consequence of a specific activating mutation of *GPA1* is in fact suppressed by expression of *STE2* in *MATα* cells (90). Thus, the observations suggest that the mechanism of receptor-mediated inactivation exists to some degree in both mating types. However, the partial asymmetry of the phenotype associated with expression of opposite mating type receptors suggests that the mechanism of interference may differ in *MAT***a** and *MATα* cells.

e) Aims and perspectives.

Thus, the picture that emerged from these studies was a clean but a complex one. The inappropriate expression of the **a**-factor receptor in *MAT***a** cells caused resistance to pheromone-induced cell cycle arrest, but left transcription of mating-specific genes largely untouched. In addition, the observations suggested that the *STE3*^{DAF} mutation acted at the level of, or downstream of the G protein. Taken together, these preliminary experiments provided an interesting framework to develop studies oriented at uncovering the site of action and the mechanism of *STE3*^{DAF} receptor-mediated inhibition. Furthermore, the implication of the pheromone receptor in a negative regulatory function suggested that the *STE3*^{DAF} mutation could add important clues to the understanding of new functions of the G protein-coupled receptor, the pheromone signaling cascade and adaptation during mating.

Materials and Methods

Plasmid construction:

Null alleles of the *MFA1* and *MFA2* genes disrupted with *HIS3* were constructed from existing *LEU2* and *URA3* disruption alleles (141) as follows: for the *MFA1* disruption, the *HIS3* gene on a 1.7-kb SalI-SmaI fragment from pUC18::HIS3 was cloned into the XhoI-HpaI sites of pSM86 to create pmfa1-Δ3::HIS3. For the *MFA2* disruption, pSM35 was digested with SmaI and EcoRV and religated to remove the *URA3* gene, and then the *HIS3* gene on a 1.7-kb BamHI fragment from pUC18::HIS3 was cloned into the BamHI site of pSM35 to create pmfa2-Δ2::HIS3.

To construct a *URA3* disruption of *FAR1*, a 1.2-kb HindIII-BamHI fragment from pAC100-2 containing the *URA3* gene was cloned into the HindIII-BamHI sites of *FAR1* in pJM306 (138) to produce pFar1-U1.

To construct a *LEU2* disruption of *CLN1*, a 2.9-kb BglII fragment from YEp13 was cloned into the BglII sites in the *HIS3* portion of the *cln1::HIS3* gene in pMT256 (207) to produce pCln1-L2.

To construct a *URA3* version of pGA1903, which contains a Myc-tagged *FUS3* gene under control of the *TPI1* promoter (228), a 1.2-kb XbaI fragment containing the *URA3* gene from pAC100-2 was cloned into the XbaI site in the *TRP1* gene of pGA1903 to produce pGA1903-U.

To construct a *URA3* disruption of *MSG5*, a 1.1-kb SphI-EcoRI fragment containing the *URA3* gene was cloned into pMSG5-1.1 to produce pmsg5::URA3.

To construct a plasmid containing the *STE5*^{Hyp} allele (82) under the control of the *GAL* promoter, a 3.2-kb PstI-DraI fragment from pDJ174 (kindly provided by D. Jenness) containing the *STE5*^{Hyp} gene was first cloned into the PstI-SmaI sites of Bluescript, and then the 3.2-kb BamHI fragment from this construct was cloned into the BamHI site of pMB272 to create pGAL-STEH.

To construct a *LEU2* plasmid containing the *GAL-STE20* gene, a fragment creating a myc-tagged *STE20* was generated by PCR (48) using oligonucleotide primers oSTE20-1, 5′-CCTCTAGAAATGG AGCAAAAGCTCATTTCTGAAGAGGACTTGAATAGCAATGATCCATCT-3′ and oSTE20-2, 5′-ACTGCTTCTAGAGATTTG-3′. The template used for PCR was plasmid pDH166, which contains the entire coding region of *STE20* under the control of the *GAL* promoter (kindly provided by M. Whiteway). The SpeI-XbaI N-terminal fragment of pDH166 was then replaced with the myc-tagged *STE20* PCR fragment digested with XbaI to produce pMSTE20. The XhoI-SacII fragment from pMSTE20 was subcloned into the XhoI-SacII sites of pRS315 (185) to create pMSTE20-L2. To construct a *LEU2* plasmid for overexpression of the untagged version of *STE20*, a 3.5-kb XhoI-SacII fragment from pDH166 was cloned into pRS315 to produce pDL166.

To produce a centromeric, *URA3* plasmid containing *STE4*, a 5-kb SphI-BamHI fragment was obtained from M81p12 and cloned into YCplac33 to produce YCpSTE4. YCpSTE4-Δ36 was constructed by cloning a XhoI-HpaI fragment from pL19-D62N-Δ310-346 (75) containing the 36 residue deletion into the XhoI-HpaI sites (partial digestion for HpaI) of YCpSTE4. The pL19 plasmid has been described (75).

To produce a pUC19 based *STE4* plasmid, a 5-kb SphI-BamHI fragment was obtained from M81p12 and cloned into pUC19 to produce pUC-STE4.1.

To produce centromeric, *LEU2* plasmids containing *STE4* or *STE4-SSD1*, a 5-kb SphI-BamHI fragment was obtained from M81p12 or YCpSSD1 and cloned into YCplac111 to produce YCpl-STE4 and YCpl-SSD1.

Finally, the reporter plasmid used for β-Galactosidase expression was made by inserting the PstI fragment from pSB234, containing the *FUS1*-β-galactosidase fusion construct, into the PstI site of Ycplac111 to produce YCplacZ.

The YEp351-CDC42 and YEp183-CDC24 were kindly provided by J. Pringle.

Strains and media: Strains used in this study are listed in Table 1.

$STE3^{DAF}$ alleles were made from the integrating plasmid piDAF2m-5, which contains the $STE3^{DAF2\text{-}2}$ gene (90). The *TRP1*:: $STE3^{DAF2.5}$ allele was made by linearizing piDAF2m-5 with XbaI to direct integration into *TRP1*; $STE3^{DAF2.5}$ was made by linearizing piDAF2m-5 with SalI to direct integration into *STE3*.

Disruptions of the **a**-factor genes were made by transformation with a 3.8-kb EcoRI-XbaI fragment from pmfa1-Δ3::HIS3 to create mfa1-Δ3::HIS3, and by transformation with a 3.2-kb EcoRI fragment from pmfa2-Δ2::HIS3 to create mfa2-Δ2::HIS3.

The *sst1::his* allele was constructed by transformation with a 5.7-kb EcoRI-SalI fragment from pJGSST1 to create *sst1::URA3* followed by deletion of *URA3* from the chromosome through recombination of the adjacent *hisG* sequences as described (63).

The *LEU2::GAL1::FAR1/+N* allele was made by linearizing pJM306 with ClaI to direct integration into *LEU2* (138). The *FAR1* gene was disrupted by transformation with an approximately 4 kb XhoI-SacI fragment from pfar1-U1 to create *far1::URA3*.

The *CLN::LEU2* disruptions were made using the following fragments: an approximately 5-kb PvuII fragment from pcln1-L2 for *cln1::LEU2*; a 3.5-kb PvuII fragment from pMT160 (209) for *cln2::LEU2*; a 3-kb SacI fragment from pBFcln3Δ::leu for *cln3::LEU2*.

The *MSG5*, disruption was made by transformation with the HindIII fragment of pmsg5::URA3, kindly provided by B. Errede. The *ZDS1* disruption was made by transformation with the EcoRI fragment of pNC419, kindly provided by B. Errede. The *STE6* disruption was made by transformation with the SphI-FspI fragment of pSM280 (13). All strain constructions involving transformations were confirmed by Southern blots (190).

Strains were grown on YEPD (2% glucose) or YEP-Gal (3% galactose), and strains under selection were grown on synthetic complete (SC) dropout media, as described (183).

Yeast methods:

Yeast transformations were performed by the lithium acetate method (95) modified as described previously (90). Yeast RNA was extracted from cells as described previously (47) and isolation of genomic DNA and plasmids from yeast was performed as reported (77).

Preparation of cell extracts for immunoprecipitation was done by modification of a method described previously (65). 20 ml of log phase cells were pelleted, resuspended in 1.25 ml stop buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 15 mM p-nitrophenyl phosphate (NPP), 15 mM $Na_2H_2P_2O_7$, 10 mM NaF, 0.1 mM sodium orthovanadate (NaV), 5 mM phenylmethylsulfonyl fluoride (PMSF)), and pelleted again. The cells were resuspended in lysis buffer (stop buffer plus 25 mM HEPES pH 7.2 with 40 µg/ml aprotinin and 20 µg/ml leupeptin) and approximately 0.25 ml of glass beads were added to the tube. The suspension was vortexed 8-10 min and centrifuged for 5 min at 4°C. After estimating the volume of the lysates, 2/3 volume of a saturated ammonium sulfate solution was added and the samples were incubated on a rocker for 30 min at 4°C. The precipitates were pelleted in a microfuge for 10 min and resuspended in storage buffer (25 mM

HEPES pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.1% NP40, 20% glycerol, 15 mM NPP, 0.1 mM NaV, 1 mM PMSF, 40 µg/ml aprotinin, 20 µg/ml leupeptin). The protein concentration of the samples was determined using a bicinchoninic acid protein assay kit (Pierce), and they were aliquoted, flash frozen in liquid nitrogen, and stored at -80°C.

Cell extracts used for Far1p immunoblots were prepared by growing cells in YEP-Gal overnight to induce expression of Far1p. The washed log phase cells were resuspended in 200 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 1% SDS, 1 mM PMSF, and 1 µg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin) and frozen in a dry ice/ethanol bath. After thawing, the cell suspension was lysed with approximately 0.25 ml of glass beads by vortexing for 10 min at 4°C and centrifuging for 5 min at 4°C to clear the lysate. The protein concentration was determined, and equal amounts of protein (40 µg) were loaded in each lane for SDS-PAGE.

Cell extracts used for Ste20p immunoblots were prepared as for Far1p, except that the cells were grown on synthetic selective media for retention of the *STE20* plasmid.

For experiments performed with the anti-myc antibody, in which both immunoprecipitation and immunoblotting was done on the same samples, the extracts were prepared by the immunoprecipitation method.

Halo assays were performed by plating a lawn of cells to be tested and placing a filter paper disk containing 5 µl of 1 mM α-factor onto the plate or by placing 5 µl of 1 mM α-factor directly onto the plate, and incubating it at 30° C for 1-2 days. Halo assays for **a**-factor were performed by depositing 5 µl of 100 µg/ml **a**-factor directly onto the plate and incubating as above.

Diploid sporulation and tetrad dissection were performed as described (77).

Production of blue color as a result of β-galactosidase activity in response to α-factor was achieved by spreading 15 µl of 1 mM α-factor and 80 µl of 40 mg/ml X-GAL on top of modified synthetic plates (pH 7.0). Plates were allowed to dry for approximately 20 minutes before replica plating. They were incubated at 30°C for 24 hours and stored at 4°C for 48 hours to enhance the result of the color assay.

Northern blots:

RNA was transferred to a nitrocellulose membrane after formaldehyde-agarose gel electrophoresis as described (122). The membranes were UV cross-linked using a Stratalinker UV box. Prehybridization and hybridization were done at 65°C in a buffer containing 1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 5% SDS. The probes used were gel-purified DNA restriction fragments ^{32}P-labeled by random primer labeling using a Prime-It kit (Stratagene). The fragments used were: *FUS1*, a 1.4-kb EcoRI-HindIII fragment from plasmid pSL589 (136); *TCM1* (179), a 0.8-kb HpaI-SalI fragment from plasmid pAB309D; *PGK1* (kindly provided by Igor Karpichev), a 0.5-kb BamHI-NdeI from plasmid pT7-PGK1.

Immunoblots:

For immunoblots to detect Far1p, SDS-PAGE was performed using a 6% polyacrylamide gel, separated proteins were transferred to nitrocellulose and the blot was probed with an anti-Far1p (51-830) rabbit polyclonal antibody (138) at a 1/5000 dilution. Donkey anti-rabbit Ig horseradish peroxidase (Amersham) was used at a dilution of 1/7500, and immune complexes were detected with an enhanced chemiluminescence (ECL) kit (Amersham).

For immunoblots to detect Ste20p, SDS-PAGE was performed using a 7% polyacrylamide gel, separated proteins were transferred to nitrocellulose, and the blot was probed with an anti-Ste20 rabbit polyclonal antibody (Upstate Biotechnology Incorporated) at a 1/2000 dilution. Donkey anti-rabbit Ig horseradish peroxidase (Amersham) was used as a secondary antibody at a dilution of 1/7500, and immune complexes were detected using ECL.

For immunoblots to detect myc-tagged proteins, immunoblots were performed as above and blots were probed with anti-myc monoclonal antibodies (Myc1-9E10, Oncogene Science) at a 1/1000 dilution. Rabbit anti-mouse Ig horseradish peroxidase (Amersham) was used at a dilution of 1/7500 and immune complexes were detected using ECL.

Immune-complex kinase assays:

Proteins were immunoprecipitated from cell extracts by incubating 100 μg of protein with 1.7 μg of Myc1-9E10 antibody (Oncogene Science) (68) on a rocker for 60 min at 4°C. Protein A Sepharose beads were swelled in 0.1 M Tris-HCl pH 7.5 for 15 min at room temperature, washed two times in 0.1 M Tris-HCl pH 7.5, washed three times in kinase extract buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP40), and resuspended in an equal volume of kinase extract buffer plus 5 mg/ml bovine serum albumin, 15 mM NPP, 0.1 mM NaV, 1 mM PMSF, 40 µg/ml aprotinin, and 20 µg/ml leupeptin. The beads were incubated on a rocker for 60 min at 4°C, and 25 µl of equilibrated beads in 0.5 ml of kinase extract buffer plus 5 mg/ml bovine serum albumin, 15 mM NPP, 0.1 mM NaV, 1 mM PMSF, 40 µg/ml aprotinin, and 20 µg/ml leupeptin were added to the antigen-antibody complexes. These mixtures were incubated on a rocker for 90 min at 4°C. The immune complexes were washed five times with buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 15 mM NPP, 0.1 mM NaV), three times with buffer B (25 mM HEPES, 15 mM NPP, 0.1 mM NaV) and one time with buffer C (wash B with 15 mM $MgCl_2$). Kinase assays were performed with by adding 10 µl of 2x kinase assay buffer (50 mM HEPES pH 7.5, 30 mM $MgCl_2$, 10 mM EGTA, 2 mM DTT, 30 mM NPP, 0.2 mM NaV, 80 µg/ml aprotinin, 40 µg/ml leupeptin), 7.5 µl myelin basic protein (1 µg/µl), and 2.2 µl ATP mix (2 µl 1 mM ATP and 0.2 µl [γ-^{32}P]ATP [10 µCi/µl]) to the adsorbed immune complexes. The reactions were incubated for 20 min at 30°C, and stopped by the addition of 15 µl of 2x SDS-PAGE loading buffer. Before loading on a gel, the samples were incubated for 5 min at 100°C and 3 µl of 1 mM ATP was added to them. The beads were then pelleted and 30 µl of supernatant was loaded. Before drying, the gels were fixed in 10% methanol, 10% acetic acid, 0.5% phosphoric acid, and 10 mM KH_2PO_4 for 1 hr, and then in 10% methanol and 10% acetic acid for 30 min.

Screen for *STE4* suppressors of *STE3^{DAF}*:

An approach described by Ma et al (127) was used to randomly mutagenize the coding region of *STE4*, and express the mutant proteins in yeast. *STE4* was amplified from YCpSTE4 by 30 cycles of Polymerase Chain Reaction (PCR) using the oligonucleotide primers oMSTE4.1, 5′-AAGAGTACACTAGATCCATTC-3′, and oMSTE4.3, 5′-AAAGGAAGCA AATGACAATGC-3′. Denaturation, primer annealing and extension were done at 95°C for 1 minute, 42°C for 2 minutes and 72°C for 3 minutes respectively. Erroneous insertion of nucleotides was achieved by performing PCR reactions under imbalance of nucleotides (80 µM A, 400 µM C, 400 µM G, 400 µM T or 400 µM A, 400 µM C, 80 µM G, 400 µM T) and modified Taq polymerase buffer containing 100 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.25 mM $MnCl_2$. The reaction products were then pooled to constitute a single library of altered *STE4* genes. Approximately 800 ng of amplified, linear *STE4* fragments and 300 ng of XhoI-AflII digested YCpSTE4 were transformed into the AC17-2B strain (*TRP:: STE3^{DAF} ste4::HIS3 mfa1::HIS3 mfa2::HIS3*) carrying the YCplacZ plasmid (*FUS1*-β-galactosidase) as a reporter for mating specific transcription. Digestion of YCplacSTE4 removed the majority of the coding region of *STE4*, but left enough flanking sequence (100-200 bp on each side) to allow homologous recombination (*in vivo*) between the gapped YCpSTE4 plasmid and the mutagenized PCR fragments. An approximate efficiency of 1.5-2.0 X 10^4 transformants/μg YCpSTE4 (+ PCR library) was obtained using this method. Transformation of the digested YCpSTE4 plasmid alone (- PCR library) was responsible for approximately 10% of the transformants. This background probably reflects incomplete gapping of the plasmid population. In addition, approximately 10% of the mutant plasmids produced non-functional Ste4p proteins as determined by the ability of the library to rescue pheromone dependent signaling in a *ste4* strain. A total of 24,000 colonies, containing approximately 80% functional and potentially mutant *STE4* alleles were screened. Transformants were selected on synthetic complete medium (SC) lacking uracil and leucine (SC-URA-LEU), incubated for two days at 30°C and then replica plated to SC-URA-LEU + α-factor + X-GAL to detect pheromone-dependent transcription of the *FUS1*-β-galactosidase fusion construct. Colonies that did not grow and turned blue in response to α-factor were chosen and patched onto fresh plates. Potentially mutagenized plasmids were then isolated from clones (77) and retransformed to test plasmid dependency of the phenotype. Alleles that conserved the phenotype after retransformation were sequenced according to the dideoxy chain-termination method (178). Site directed mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech) and the pUC-STE4.1 plasmid.

Table 1

Strain	Genotype	Source
W303	*MAT**a**/α leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
W3031A	*MAT**a** leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
W3031B	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein

All of the following strains are isogenic to W3031A

Strain	Genotype	Source
AC9	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln1::LEU2*	this study
AC8	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* *STE3*$^{DAF2.5}$ *sst1::hisG cln1::LEU2*	this study
AC3	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln2::LEU2*	this study
AC2	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* *STE3*$^{DAF2.5}$ *sst1::hisG cln2::LEU2*	this study
AC5	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln3::LEU2*	this study
AC4	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* *STE3*$^{DAF2.5}$ *sst1::hisG cln3::LEU2*	this study
AC9-F1	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln1::LEU2 far1::URA3*	this study
AC8-F1	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* *STE3*$^{DAF2.5}$ *sst1::hisG cln1::LEU2 far1::URA3*	this study
AC3-F1	*MAT**a** mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln2::LEU2 far1::URA3*	this study

Table 1 (continued)

AC2-F1	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* $STE3^{DAF2.5}$ *sst1::hisG cln2::LEU2 far1::URA3*	this study
AC5-F1	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln3::LEU2 far1::URA3*	this study
AC4-F1	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* $STE3^{DAF2.5}$ *sst1::hisG cln3::LEU2 far1::URA3*	this study
AC13-3B1	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG cln1::LEU2 cln2::LEU2*	this study
AC14-15B1	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* $STE3^{DAF2.5}$ *sst1::hisG cln1::LEU2 cln2::LEU2*	this study
H67-9D.FBa	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 LEU2::GAL1::FAR1/+N sst1::hisG*	this study
H67-6C.FBa	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* $STE3^{DAF2.5}$ *LEU2::GAL1::FAR1/+N sst1::hisG*	this study
H67-9D.Ba	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG*	this study
H67-6C.Ba	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* $STE3^{DAF2.5}$ *sst1::hisG*	this study
AC12	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG far1::URA3*	this study
H72-5C.Ba	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 fus3::LEU2 sst1::hisG*	this study
AC1	*MAT***a***/α mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 STE3/*$STE3^{DAF2.5}$ *MSG5/msg5::URA3 GPA1/gpa1::LEU2 sst1::hisG*	this study

Table 1 (continued)

AC10	*MATa/α mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 STE3/STE3*$^{DAF2.5}$ *QPA1/qpa1::URA3 GPA1/gpa1::LEU2 sst1::hisG*	this study
D109-1B	*MATa ste12::LEU2*	this study
AC21	*MATa mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG ste6::URA3*	this study
AC20	*MATa mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 STE3*$^{DAF2.5}$ *ste6::URA3 sst1::hisG*	this study
AC17-7B	*MATa mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG ste4::HIS3*	this study
AC17-2B	*MATa mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 STE3*$^{DAF2.5}$ *ste4::HIS3 sst1::hisG*	this study
H100-10C	*MATa mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG ste4::HIS3 gpa1*^{LYS388}	this study
H100-10D	*MATa mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 STE3*$^{DAF2.5}$ *ste4::HIS3 gpa1*^{LYS388} *sst1::hisG*	this study

Results: Part I

1. The *STE3*^{DAF} phenotype and the cell cycle.

a) G_1 cyclins are not directly responsible for the generation of the phenotype.

*MAT**a*** cells that carry the *STE3*^{DAF} allele are resistant to cell cycle arrest but show nearly wild type induction of mating specific genes (90). This suggested the possibility that the inappropriately expressed *STE3*^{DAF} receptor affected only one branch of the mating pathway, namely cell cycle arrest, and left the second one, that is transcriptional induction, intact. If cell cycle progression alone was altered, a malfunction of the Cdc28p/Clnp complex could be envisioned. G_1 to S transition in *S. cerevisiae* is controlled by several G_1 cyclins. Deletion of *CLN1, CLN2* and *CLN3* simultaneously causes lethality, and at least one G_1 cyclin is required to progress through the cell cycle (45, 150, 175, 78, 222, 44, 210). Thus, the *STE3*^{DAF} phenotype may result from sustained activation of one or more G_1 cyclins. For example, the inappropriately expressed receptor may stabilize one particular cyclin and promote permanent activation of Cdc28p, thus causing resistance to pheromone induced cell cycle arrest.

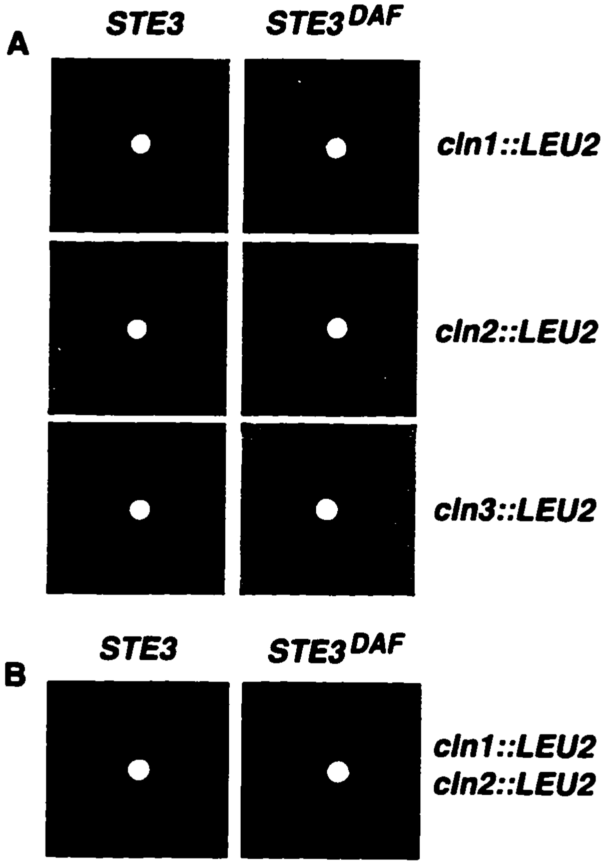
To address this issue, strains that carried null alleles of each individual G_1 cyclin were constructed. In addition, a *cln1::LEU2 cln2::LEU2* double deleted strain was made. The reasoning was that if *STE3*^{DAF} acted by stabilizing the activity of one or more cyclins, strains lacking these particular proteins would arrest in response to pheromone due to the presence of remaining cyclins that were now under proper control.

As expected, wild type strains containing null alleles of individual G_1 cyclins or a combination of *cln1* and *cln2* null alleles arrested in response to α-factor, as indicated by the formation of a clear zone of no growth, or halo, around the source of pheromone (Figures 3A and 3B, left). In contrast, isogenic *STE3^{DAF}* strains did not arrest under the same conditions (Figures 3A and 3B, right). These results suggest that *STE3^{DAF}* does not act by stabilizing the activity of an individual G_1 cyclin. Moreover, they rule out the possibility that Cln1p and Cln2p activities are stabilized together by the inappropriately expressed receptor to promote escape from cell cycle arrest. Thus, the *STE3^{DAF}* phenotype is likely to be caused by inhibition of all three G_1 cyclins simultaneously, probably through inactivation of components acting upstream of the G_1 cyclins.

b) The Far1p-independent mechanism of cell cycle arrest is also affected by *STE3^{DAF}*.

Arrest in the G_1 phase of the cell cycle is achieved by binding of the cell cycle inhibitor Far1p to Cdc28p-Cln1p and Cdc28p-Cln2p complexes (160). This mechanism of arrest, although very important under normal conditions, is not the only one that exists in yeast. A Far1p independent mechanism is evidenced by the finding that a strain lacking *FAR1* and *CLN2*

Figure 3. Inhibition of G_1 arrest by *STE3*^{DAF} occurs in the absence of individual G_1 cyclins and in the absence of Cln1p and Cln2p. (A) Cell cycle arrest was tested using halo assays with 5 µl of 1 mM α-factor. The strains used were: *MAT**a** cln1::LEU2* (AC9), *MAT**a** cln1::LEU2 STE3*^{DAF} (AC8) (top); *MAT**a** cln2::LEU2* (AC3), *MAT**a** cln2::LEU2 STE3*^{DAF} (AC2) (middle); *MAT**a** cln3::LEU2* (AC5), *MAT**a** cln3::LEU2 STE3*^{DAF} (AC4) (bottom). (B) Halo assays were performed as for panel (A). The strains used were: *MAT**a** cln1::LEU2 cln2::LEU2* (AC13-3B1) (left), and *MAT**a** cln1::LEU2 cln2::LEU2 STE3*^{DAF} (AC14-15B1) (right).


A
STE3
STE3 DAF
cln1::LEU2
cln2::LEU2
cln3::LEU2
B
STE3
STE3 DAF
cln1::LEU2
cln2::LEU2

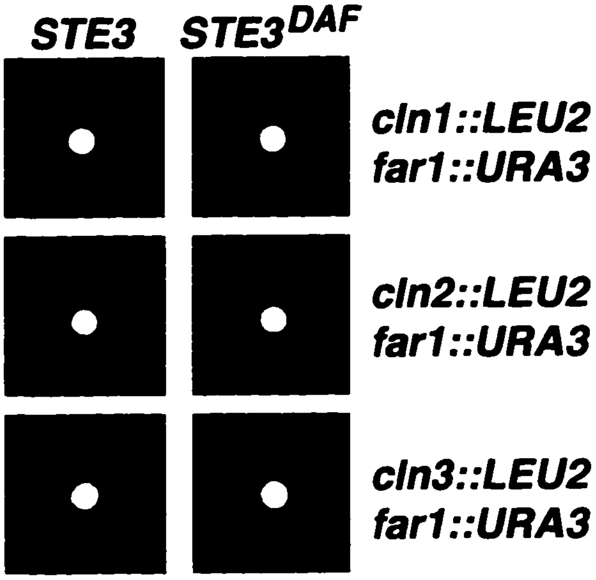
is able to arrest normally in response to pheromone (25). Since deletion of the G_1 cyclins suggested that escape from cell cycle arrest involves components further upstream of the G_1 cyclins, it was of interest to analyze the response of *STE3*^{DAF} cells under conditions where the Far1p-independent mechanism of arrest was apparent.

Wild type and *STE3*^{DAF} strains were constructed that carried deletions in each individual G_1 cyclin in addition to a disruption in the *FAR1* gene. As expected, the *Δcln2 Δfar1* strain arrested in response to pheromone (Figure 4 middle, left). In contrast, a strain carrying the double deletion in a *STE3*^{DAF} background was still resistant to cell cycle arrest (Figure 4 middle, right). Furthermore, the small amount of Far1p-independent arrest observed in the *Δcln1 Δfar1* and *Δcln3 Δfar1* strains was suppressed by *STE3*^{DAF} (Figure 4 top and bottom). These results indicate that the inappropriate expression of the pheromone receptor affects both Far1p-dependent and Far1p-independent mechanisms of arrest. The findings are also in agreement with the notion that *STE3*^{DAF} acts upstream of the complexes directly responsible for mediating cell cycle arrest.

c) Far1p phosphorylation is incomplete in *STE3*^{DAF} cells.

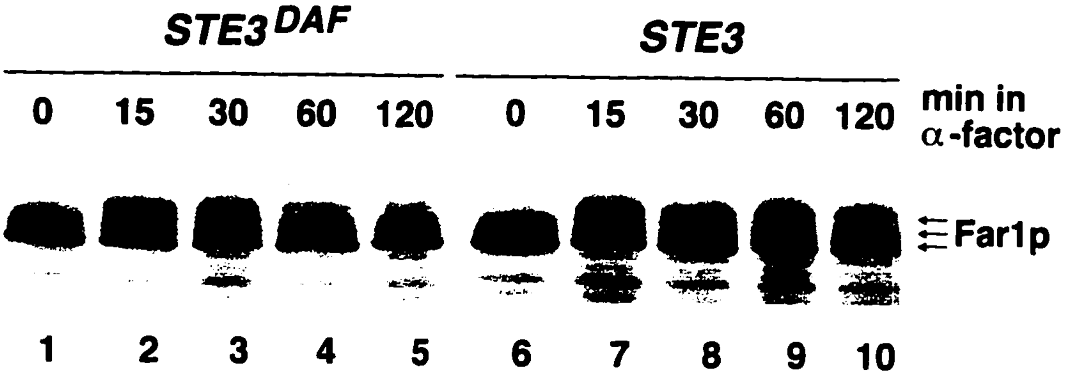
The signal originated at the cell surface results in changes in the phosphorylation state of Far1p. Far1p is phosphorylated on multiple residues by Cdc28p and by the MAP kinase Fus3p (159, 209). These changes can be

Figure 4. Inhibition of G_1 arrest by *STE3*^{DAF} in *MAT**a*** cells also affects the Far1p independent mechanism of cell cycle arrest. Cell cycle arrest was tested using halo assays with 5 µl of 1 mM α-factor. The strains used were: *MAT**a*** *cln1::LEU2 far1::URA3* (AC9-F1), *MAT**a*** *cln1::LEU2 far1::URA3 STE3*^{DAF} (AC8-F1) (top); *MAT**a*** *cln2::LEU2 far1::URA3* (AC3-F1), *MAT**a*** *cln2::LEU2 far1::URA3 STE3*^{DAF} (AC2-F1) (middle); *MAT**a*** *cln3::LEU2 far1::URA3* (AC5-F1), *MAT**a*** *cln3::LEU2 far1::URA3 STE3*^{DAF} (AC4-F1) (bottom).


STE3
STE3^DAF
cln1::LEU2
far1::URA3
cln2::LEU2
far1::URA3
cln3::LEU2
far1::URA3

observed on SDS-PAGE, since the modifications alter the migration of the protein significantly (26, 138). To investigate the activation kinetics of Far1p in the presence of *STE3*^{DAF}, Far1p phosphorylation was followed in wild type and *STE3*^{DAF} cells for 120 minutes after exposure to α-factor by probing a Western blot containing cell extracts with an anti-Far1p antibody (137). In wild type cells, Far1p migrated as a doublet, with the fastest mobility form of the protein being the most abundant (Figure 5, lane 6). As early as 15 minutes after treatment with pheromone, slower mobility forms of Far1p appeared and were stable for at least 120 minutes in wild type cells (Figure 5, lanes 7-10). Far1p migration was very similar in *STE3*^{DAF} and wild type cells under noninduced conditions (Figure 5, lane 1). In addition, a slower migrating form of Far1p also appeared after 15 minutes of pheromone induction in *STE3*^{DAF} cells (Figure 5, lane 2). However, a significant difference was observed after 30-60 minutes of treatment with α-factor. Slower migrating forms of Far1p did not persist after the initial increase in phosphorylation in *STE3*^{DAF} cells (Figure 5, lanes 3-5). These results demonstrate that the inability of *STE3*^{DAF} cells to arrest upon pheromone binding correlates with the loss of phosphorylation of the cell cycle inhibitor Far1p. Since activity of Far1p depends on phosphorylation (26, 137), escape from cell cycle arrest is likely to result from failure to maintain Far1p in an activated state. In addition, the observations point to a step further upstream as the inhibitory site of action of the receptor.

Figure 5. Far1p is underphosphorylated in *STE3*^{DAF} cells. Log phase cultures of *MAT**a*** *STE3*^{DAF} (H67-6C.FBa, lanes 1-5) and *MAT**a*** *STE3* (H67-9D.FBa, lanes 6-10) strains were treated with α-factor at 0.1 μM, and samples were removed at the times (in min) indicated. Cell extracts were prepared and resolved on a 7% SDS-PAGE gel. A Western blot was performed with an anti-Far1p antibody.

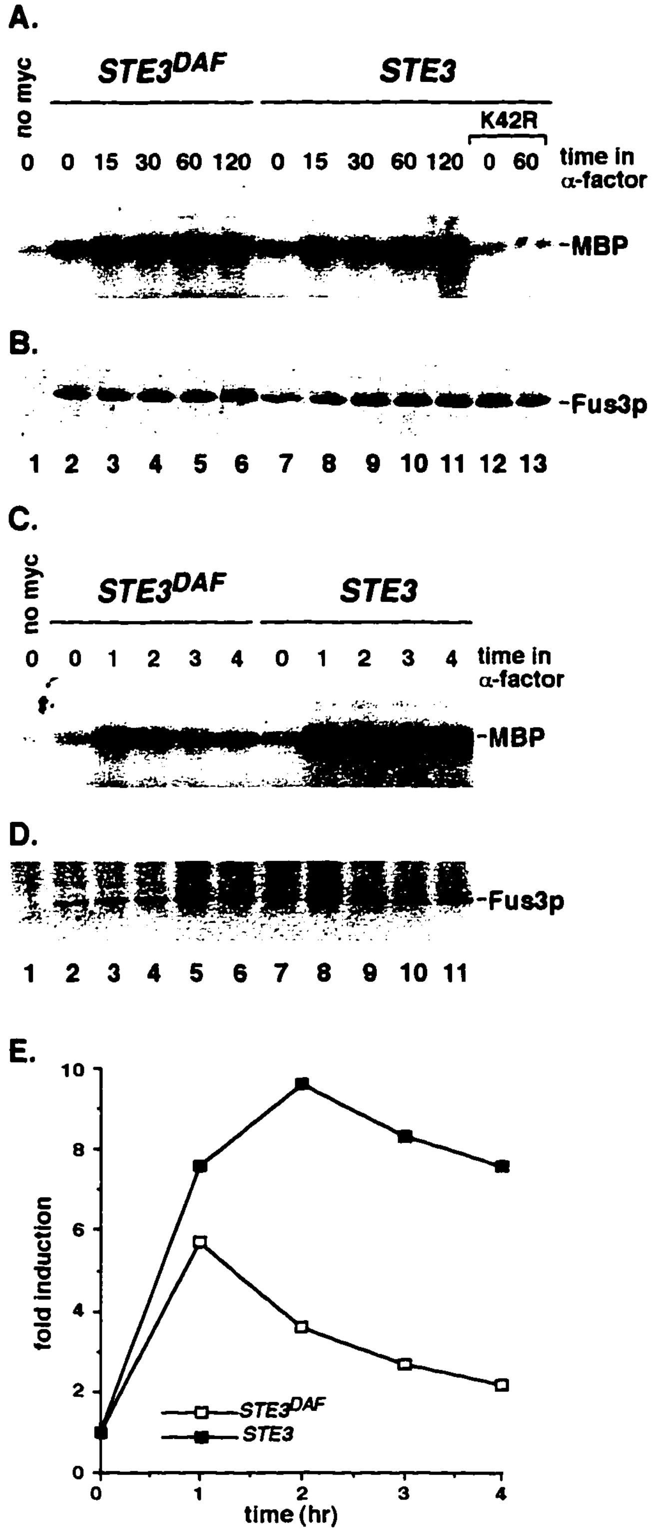

STE3^{DAF}
STE3
0
15
30
60
120
0
15
30
60
120
min in α-factor
Far1p
1
2
3
4
5
6
7
8
9
10

2. *STE3*^{DAF} and downstream components of the mating pathway.

a) The Fus3p MAP kinase is inactivated prematurely in *STE3*^{DAF} cells.

In order to investigate a possible correlation between inactivation of Far1p and a defect in upstream signaling, the activity of the Fus3p kinase was followed in an *in vitro* kinase assay (65). To allow isolation of Fus3p, wild type and *STE3*^{DAF} cells were transformed with a myc-tagged version of Fus3p. This protein was immunoprecipitated efficiently with anti-myc monoclonal antibodies from extracts of cells incubated in the absence and the presence of pheromone as shown by immunoblots against the Fus3p-myc protein (Figures 6B and 6D). To measure Fus3p kinase activity, cells were treated for different amounts of time with α-factor and cell extracts were immunoprecipitated with anti-myc antibodies. The *in vitro* kinase reaction was performed by adding the artificial substrate myelin basic protein (MBP) to the immune complexes in the presence of [γ-^{32}P] ATP. The proteins in this reaction were finally separated on SDS-PAGE and exposed to X-ray film to detect the presence of phosphorylated MBP. Immunoprecipitates from wild type cells lacking Fus3p-myc showed low, basal phosphorylation of MBP (Figure 6A, lane 1). Immunoprecipitates from transformed *STE3*^{DAF} and wild type cells, on the other hand, displayed a higher basal kinase activity towards MBP (Figure 6A, lanes 2 and 7) and, significantly, showed an increase in kinase activity when prepared from cells exposed to α-factor (Figure 6A, lanes

Figure 6. Fus3p kinase activity is decreased at late times after pheromone treatment of *STE3*^{DAF} cells. (A) A *MATa* *STE3*^{DAF} strain (H67-6C.Ba) transformed with plasmid pGA1903-U (lanes 2-6) and a *MATa* *STE3* strain (H67-9D.Ba) either untransformed (lane 1) or transformed with plasmid pGA1903-U (lanes 7-11) or pGA1905 (lanes 12 and 13) were treated with α-factor at 0.1 µM, and samples were removed at the times indicated. Cell extracts were prepared, Fus3p-myc was immunoprecipitated with 1.7 µg of Myc1-9E10 antibody, and immune-complex kinase assays were performed using 7.5 µg of myelin basic protein. (B) Parallel immunoprecipitations were performed with the extracts described in (A), and immunoblotted with anti-myc antibody. (C) α-factor treatment was as described in (A) except that the time of sample removal is indicated in hr. (D) Parallel immunoprecipitations were performed with the extracts described in (C), and immunoblotted with anti-myc antibody. (E) The fold induction of Fus3p kinase activity from the experiment shown in (C) was calculated as follows. Relative amounts of phosphate incorporated into MBP were determined by PhosphoImager analysis. After subtracting the amount incorporated by a sample that did not contain Fus3p-myc from all the other amounts, the fold induction was calculated as the amount incorporated at a given time point divided by the amount at the zero time point. Values obtained from *STE3*^{DAF} strains are shown in open squares and values from *STE3* strains are shown in closed squares.


A.
no myc
STE3^DAF
STE3
K42R
0 0 15 30 60 120 0 15 30 60 120 0 60 time in α-factor
-MBP
B.
-Fus3p
1 2 3 4 5 6 7 8 9 10 11 12 13
C.
no myc
STE3^DAF
STE3
0 0 1 2 3 4 0 1 2 3 4 time in α-factor
-MBP
D.
-Fus3p
1 2 3 4 5 6 7 8 9 10 11
E.
fold induction
0
2
4
6
8
10
0
1
2
3
4
time (hr)
STE3^DAF
STE3

3 and 8). Fus3p kinase activity increased as early as 15 minutes after treatment with pheromone and persisted for at least 120 minutes in both *STE3*^{DAF} and wild type cells (Figure 6A, lanes 3-6 and 8-11). To corroborate the specificity of the observed kinase activity, catalytically inactive myc-tagged Fus3p-K42R (71) was immunoprecipitated from extracts of wild type cells incubated in the presence and absence of α-factor. As expected, the mutant form of Fus3p showed no increase in kinase activity (Figure 6A, lanes 12 and 13), thus confirming the Fus3p specificity of the *in vitro* kinase assay.

STE3^{DAF} and wild type cells showed a similar kinetics of increase in Fus3p activity early in response to pheromone. Nevertheless, a slight difference was observed at 120 minutes of α-factor treatment (Figure 6A, lanes 6 and 11). To further investigate this difference, a longer time course was performed under the same conditions. Fus3p kinase activity was followed for four hours after pheromone exposure. In wild type cells, the kinase activity rose continuously for approximately 2 hours and remained at elevated levels for at least 2 more hours (Figures 6C, lanes 7-11 and 6E). Fus3 activity in *STE3*^{DAF} cells, in contrast, increased for 1-2 hours and slowly declined thereafter (Figures 6C, lanes 2-6 and 6E). Fus3p protein levels showed no significant variation during the course of the experiments, as indicated by immunoprecipitation and immunoblotting of parallel samples (Figures 6B and 6D). These findings indicate that cell cycle arrest in response to pheromone requires sustained MAP kinase activation. In addition, the results suggest that $STE3^{DAF}$ cells respond normally early during pheromone exposure, but fail to maintain the appropriate signal at elevated levels. Moreover, kinase levels correlate with the kinetics of phosphorylation of the cell cycle inhibitor Far1p, and are consistent with a model in which escape from cell cycle arrest is the result of a late inactivation of the mating response. Finally, inactivation of Fus3p indicates that the inappropriately expressed receptor affects the mating pathway at the level of or upstream of the Fus3p MAP kinase.

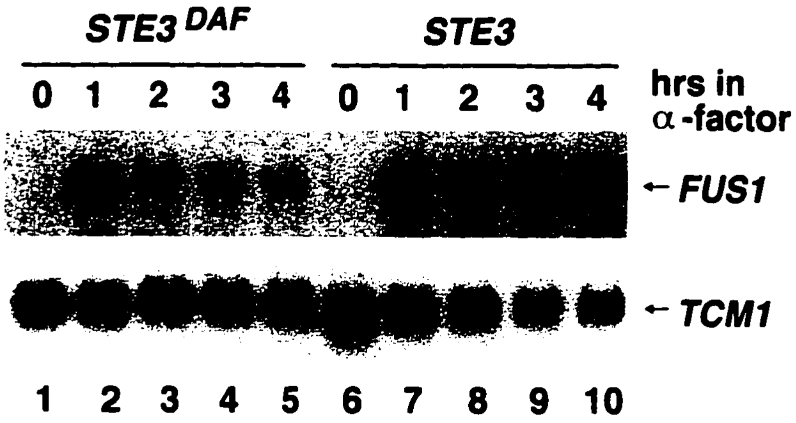
b) Mating specific transcription occurs transiently in $STE3^{DAF}$ cells.

An important contradiction seemed to be present in the original description of the $STE3^{DAF}$ phenotype. Cells escaped cell cycle arrest, as measured by halo assays, but showed transcriptional induction of mating specific genes, as indicated by Northern blot analysis (90). These two assays, however, measured the state of the pathway at very different times. RNA extracts for Northern blots were made shortly after pheromone exposure while halo assays required at least 24 hours of treatment.

The results from the Fus3p kinase assays suggested that the mating pathway was subject to an early, transient activation, followed by a slow inactivation. Fus3p is responsible for activating the cell cycle inhibitor Far1p and the transcription factor *STE12* (63). It follows that if the inappropriately expressed receptor inactivates the pathway upstream of Fus3p, the level of Fus3p kinase activity should also correlate with transcriptional levels of mating specific genes. To test this possibility, transcription of *FUS1*, a mating specific inducible gene (208, 136), was followed for four hours. α-factor was added to wild type and *STE3*^{DAF} cultures, aliquots were removed at the indicated times and RNA was extracted from the cells. Northern blots were performed using a ^{32}P labeled *FUS1* probe. *FUS1* mRNA levels in wild type cells were very low under noninduced conditions (Figure 7, lane 6). However, soon after pheromone exposure, significant *FUS1* mRNA levels accumulated and remained high for at least four hours (Figure 7, lanes 7-10). *FUS1* transcript levels in *STE3*^{DAF} cells, on the other hand, were also very low in untreated cells and accumulated significantly in response to α-factor (Figure 7, lanes 1 and 2). Nevertheless, after an initial increase, mRNA levels decreased gradually for the remaining 2-4 hours of the time course, and did not persist like in wild type cells (Figure 7, lanes 2-5). These findings indicate that the kinetics of Fus3p activity correlate closely with transcriptional levels of *FUS1*, a mating specific gene, during the pheromone response in *STE3*^{DAF} cells. The observations also suggest that the decrease in mRNA accumulation results from the inactivation of the MAP kinases, and corroborate the idea that inactivation of the mating signal occurs either at the level or upstream of the MAP kinases in *STE3*^{DAF} cells.

Evidence obtained from deletion of the G$_1$ cyclins suggested that escape from cell cycle arrest was not caused by sustained Clnp activity in mutant cells. However, the decrease in Fus3p kinase activity and *FUS1* mRNA levels

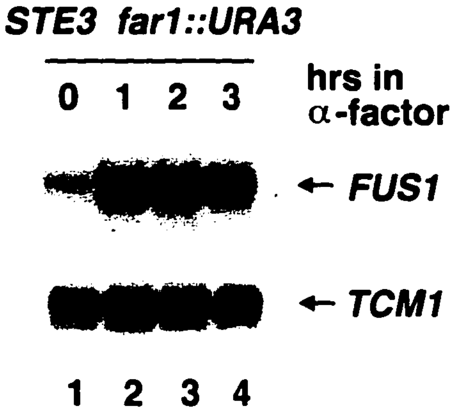
Figure 7. *FUS1* transcript levels are decreased at late times after pheromone treatment in *STE3*^{DAF} cells. *MAT**a*** *STE3*^{DAF} (H67-6C.Ba) (lanes 1-5) and *MAT**a*** *STE3* (H67-9D.Ba) (lanes 6-10) strains were treated with α-factor at 0.1 µM for the indicated periods of time. RNA was isolated, transferred to nitrocellulose, and hybridized with *FUS1* probe. The blot was rehybridized with *TCM1* probe to determine the amount of RNA per lane.


STE3 DAF
STE3
0 1 2 3 4 0 1 2 3 4
hrs in α-factor
← FUS1
← TCM1
1 2 3 4 5 6 7 8 9 10

in *STE3*^{DAF} cells could still be explained by cell cycle progression. Progression is largely controlled by Cdc28p, and elevated kinase levels could force *STE3*^{DAF} cells to continue cycling even in the presence of pheromone. Thus, Cdc28p could be directly affected by the inappropriately expressed receptor to produce the *STE3*^{DAF} phenotype, and could inhibit the mating pathway through a negative feedback mechanism. To test this possibility, a strain that failed to arrest in response to pheromone was constructed. This was achieved by deleting the gene encoding the cell cycle inhibitor Far1p from a wild type strain (25). In the *far1Δ* strain, the Cdc28p-Clnp complexes remain active in the presence of pheromone and, thus, the system should be useful to differentiate direct from indirect effects of cell cycle elements on the mating pathway.

far1Δ cells were treated with α-factor and RNA was extracted at different time points after addition of pheromone. A Northern blot was performed using a *FUS1* probe to follow induction of mating-specific genes. Significant accumulation of *FUS1* RNA was observed shortly after exposure to pheromone (Figure 8, lanes 1 and 2) and, interestingly, RNA levels remained high for at least 3 hours in the *far1Δ* strain (Figure 8, lanes 2-4). These results indicate that cell cycle progression, as a consequence of the *far1Δ* mutation, does not inhibit transcriptional induction. Thus, the observations suggest that sustained activation of Cdc28p-Clnp complexes is not responsible for direct inhibition of the mating pathway in *STE3*^{DAF} cells and favor an

Figure 8. *FUS1* transcript levels remain high in normally cycling cells. A *MATa STE3 far1::URA3* strain (AC12), was treated with α-factor at 0.1 μM for the indicated periods of time. RNA extraction and Northern blot analysis were performed as in Figure 7.


STE3 far1::URA3
0 1 2 3
hrs in α-factor
← FUS1
← TCM1
1 2 3 4

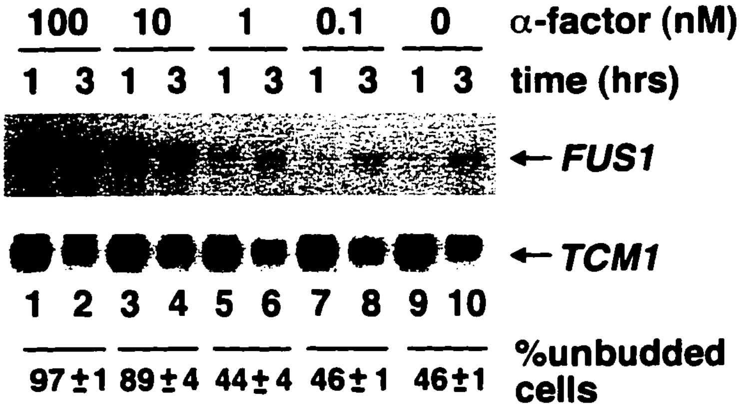
alternative model in which cell cycle progression is a consequence of the inactivated mating signaling pathway.

c) Inhibition in *STE3*^{DAF} cells does not result from partial signaling.

The observations described above suggest that down regulation of the mating pathway in *STE3*^{DAF} cells results most likely from a direct inhibition of the signaling cascade and not from altered cell cycle signals that feed into the response. Considering the existence of a negative, inhibitory function in a branched signaling pathway, several mechanisms of inactivation may be conceived. An interesting one is that in *STE3*^{DAF} cells the appropriate receptor (Ste2p) may only generate a weak signal in response to pheromone. Consequently, the ability of the signal to reach a threshold level could be limited and this partial activation could be responsible for the pattern of activity observed for Fus3p, Far1p, cell cycle arrest (46) and transcriptional induction. In other words, the binding of α-factor to Ste2p receptors, in the presence of *STE3*^{DAF}, could trigger a submaximal response under pheromone concentrations that normally elicit a complete response in the absence of *STE3*^{DAF}. This partial response could fail to generate a sustained signal, and the pathway could inactivate as a consequence of an overall low level of signal.

If the proposed model is correct, wild type cells exposed to lower concentrations of pheromone should mimic the *STE3*^{DAF} response, namely, the mating pathway should activate transiently. To test this idea, wild type cells were exposed to decreasing concentrations of α-factor for 1 and 3 hours. After pheromone treatment, cells were collected, RNA was extracted and Northern blots were performed to follow transcriptional induction of the mating specific gene *FUS1*. Cell cycle arrest of the same samples was measured by counting accumulation of unbudded cells. As expected, little or no *FUS1* mRNA was observed when cells were not treated with α-factor (Figure 9, lanes 9 and 10). In addition, unbudded cells in untreated cultures constituted 46% of the population, in agreement with values for actively growing cells (166). Upon treatment with 100 nM α-factor, *FUS1* mRNA levels increased significantly and remained high for 3 hours (Figure 9, lanes 1 and 2). In addition, cells arrested in G1, as indicated by an index of 97% unbudded cells in the population. A 10-fold dilution of pheromone elicited a similar response albeit somewhat decreased (Figure 9, lanes 3 and 4). However, concentrations of pheromone of 1 and 0.1 nM were completely unable to activate mating-specific transcription or cell cycle arrest (Figure 9, lanes 5-8). If wild type cells were able to reproduce the $STE3^{DAF}$ phenotype, the response to low concentrations of pheromone should present the following characteristics: no arrest in the G_1 phase of the cell cycle (46), high *FUS1* mRNA levels at 1 hour and very low mRNA levels at 3 hours (Figure 7, lanes 1-5). Interestingly, these conditions were not satisfied simultaneously by any of the pheromone concentrations tested. In contrast, α-factor evoked a complete response at high or moderate concentrations, and failed to activate

Figure 9. The *STE3*^{DAF} effect cannot be mimicked by low doses of pheromone. A *MAT**a*** *STE3* strain (H67-9D.Ba) was treated with α-factor at the indicated concentrations for 1 and 3 hr, and RNA was isolated. Northern blots were prepared as described in the legend to Figure 7. The percent unbudded cells was calculated by counting the number of budded cells in a total of 200 cells after 3 hours of treatment with α-factor at 0.1 µM. The counting process was repeated three times and an average of the individual counts was calculated and converted to percent unbudded.


100 10 1 0.1 0
α-factor (nM)
1 3 1 3 1 3 1 3 1 3
time (hrs)
← FUS1
← TCM1
1 2 3 4 5 6 7 8 9 10
97±1 89±4 44±4 46±1 46±1
%unbudded cells

the pathway at very low concentrations. These results suggest that inactivation of the mating pathway in *STE3*^{DAF} cells is not the consequence of an overall low level of signal. On the contrary, they suggest that the pathway is inhibited after the wild type receptor triggers a normal response to pheromone.

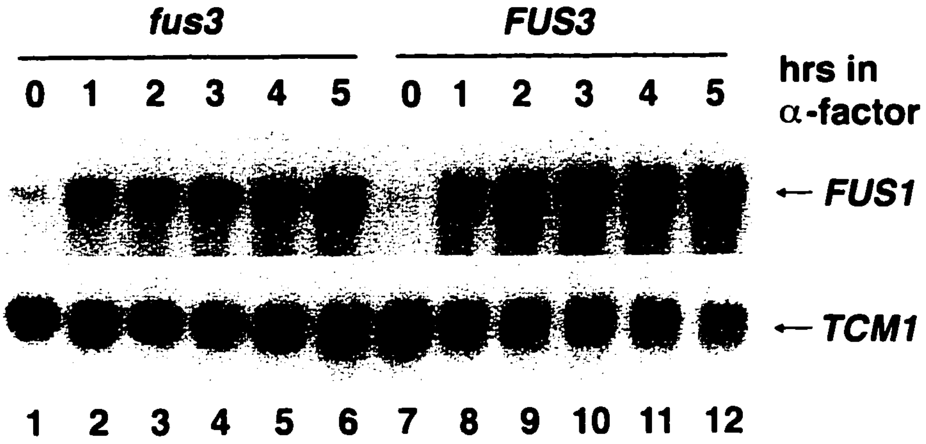
d) The Kss1p MAP kinase behaves like its homolog Fus3p.

Considerable evidence supports the idea that Fus3p and Kss1p have partially overlapping functions (61, 41, 71). However, only Fus3p phosphorylates the cell cycle inhibitor Far1p (159, 63) and *fus3* cells escape cell cycle arrest in the presence of pheromone (62). Thus, Kss1p is primarily involved in the activation of transcription and not in cell cycle arrest. A prediction of these observations is that Kss1p activity persists during the pheromone response, but that the protein has specific substrates different from Far1p. Alternatively, Kss1p activity may be naturally short lived. Since i) the cell cycle arrest response in *fus3* cells resembles the *STE3*^{DAF} phenotype and ii) Far1p phosphorylation and mating-specific transcription are down regulated in *STE3*^{DAF} cells, it was of interest to determine the levels of Kss1p activity during pheromone exposure in *STE3*^{DAF} cells. To explore this issue, *FUS1* mRNA levels were followed in a strain that carried a disruption in the *FUS3* gene. In this strain, *FUS1* mRNA should increase exclusively in response to Kss1p activity. As observed previously, *FUS1* mRNA levels in wild type cells increased significantly and remained elevated for extended periods of time (Figure 10, lanes 7-12). The results in the strain lacking Fus3p showed that *FUS1* mRNA levels were also induced and remained high for at least 4 hours (Figure 10, lanes 1-6). These findings indicate that Kss1p is not naturally short lived. Recent evidence concerning duration of Kss1p activity in response to pheromone support these observations (126). Taken together, these results strongly imply that the Kss1p protein is inactivated in *STE3*^{DAF} cells and further support the existence of an upstream inactivating event in the *STE3*^{DAF} phenotype.

e) Known negative regulators do not participate in the *STE3*^{DAF} phenotype.

Msg5p is a dual-specificity phosphatase that acts on threonine and tyrosine residues of the MAP kinase Fus3p (54). In *STE3*^{DAF} cells, Fus3p kinase activity was significantly reduced after a transient activation. Since Msg5p is a phosphatase that inactivates Fus3p, it was of interest to determine the role of this protein in the *STE3*^{DAF} phenotype. If the inappropriately expressed receptor induced inactivation of the mating pathway through Msg5p, then inhibition should be prevented in the absence of this particular phosphatase. In order to investigate this idea, the interaction between Msg5p and *STE3*^{DAF} was tested genetically. A diploid strain heterozygous for a disruption of the *MSG5* gene, heterozygous for *STE3*^{DAF} and heterozygous for a *gpa1* null allele was constructed (AC1 strain). Disruption of *GPA1* in an otherwise wild type background results in constitutive signaling through the mating pathway and non-growth due to pheromone independent cell cycle

Figure 10. *FUS1* transcript levels remain high in cells containing Kss1p as the only MAP kinase. *MATa STE3 fus3::LEU2* (H72-5C.Ba) and *MATa STE3 FUS3* (H67-9D.Ba) strains were treated with α-factor at 0.1 μM for the indicated periods of time. RNA was isolated, transferred to nitrocellulose, and hybridized with *FUS1* probe. The blot was rehybridized with *TCM1* probe to determine the amount of RNA per lane.


fus3
FUS3
0 1 2 3 4 5 0 1 2 3 4 5
hrs in α-factor
← FUS1
← TCM1
1 2 3 4 5 6 7 8 9 10 11 12

arrest (49). In addition, *STE3*^{DAF} suppresses the cell cycle arrest phenotype associated with *gpa1* (46, 90). The AC1 diploid strain was sporulated and tetrads were dissected. As expected, disruption of the *MSG5* gene in a wild type background (*STE3*) did not affect the *gpa1* phenotype. Thus, *MATa STE3 gpa1::LEU2 msg5::URA3* spores did not form large colonies. Furthermore, deletion of *MSG5* did not alter the suppression of the *gpa1* phenotype by the *STE3*^{DAF} receptor. Thus, *MATa TRP1::STE3*^{DAF} *gpa1::LEU2 msg5::URA3* spores were viable and indistinguishable from *MATa TRP1::STE3*^{DAF} *gpa1::LEU2* spores. These results indicate that Msg5p is not required to promote receptor-mediated inhibition. However, other phosphatases exist in *S. cerevisiae* and many have not been well characterized. Therefore, the involvement of protein phosphatases other than Msg5p remains to be thoroughly explored.

Recent reports suggest that another protein, Zds1p (Qpa1p), participates in polarization and inactivation of the mating signaling cascade (14). In contrast to Msg5p, however, no particular function has been associated with Zds1p (14). Nevertheless, at least some findings suggest that the protein localizes in the proximity of Cdc42p (14). To study the participation of Zds1p in the *STE3*^{DAF} receptor-mediated inactivation, an experiment very similar to the one explained above for Msg5p was performed. *ZDS1* was disrupted in a diploid strain heterozygous for *STE3*^{DAF} and carrying a disruption in one allele of the *GPA1* gene (AC10 strain). AC10 cells were sporulated and tetrads were dissected to analyze the suppression of the *gpa1* phenotype by *STE3*^{DAF}

in *zds1* spores. Again, disruption of the *ZDS1* gene in a wild type background (*STE3*) did not affect arrest due to the *gpa1* allele and the disruption did not affect the ability of *STE3*^{DAF} to suppress the *gpa1* phenotype. Thus, *MATa TRP1::STE3*^{DAF} *gpa1::LEU2 zds1::URA3* cells were recovered successfully in the analysis. These results, like those for Msg5p, indicate that receptor-mediated inactivation of the mating pathway occurs independently of the negative regulator Zds1p.

The Msg5p and Zds1p experiments, together with previous observations showing that *STE3*^{DAF} inhibition is independent of Sst2p, the carboxyl-terminus of Ste2p and *BAR1* (90), further support the notion that *STE3*^{DAF} mediated inactivation of the mating signaling cascade constitutes a novel inhibition mechanism.

f) Summary.

Taken together the results presented so far indicate that *STE3*^{DAF} causes a late inactivation of the mating pathway. They also suggest that inactivation takes place at the level of, or upstream of the MAP kinases (Fus3p and Kss1p). Furthermore, they indicate that inactivation is not a secondary effect of a cell cycle input but a direct result of a novel inhibitory mechanism, and they imply that sustained MAP kinase activity is required to promote cell cycle arrest during the mating response.

Finally, *STE3*^{DAF} cells do not undergo significant morphological changes in response to pheromone (42). The observations described so far suggest that absence of morphological changes in *STE3*^{DAF} cells reflects an inactive signaling cascade, and not a direct effect of the mutation in the generation of cell polarity during mating.

3. *STE3*^{DAF} and upstream components of the mating pathway.

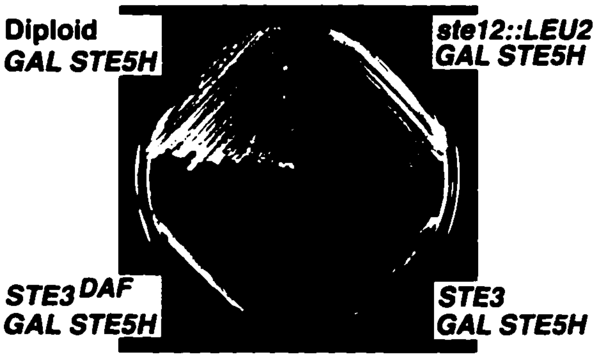
A fundamental question regarding the effect of the *STE3*^{DAF} mutation is the step at which the inappropriately expressed receptor causes the inactivation of the pheromone response pathway. In the previous section, conclusive evidence demonstrated that *STE3*^{DAF} acted upstream of, or at the level of the MAP kinases. In order to address the question further, a series of epistasis experiments were performed using a variety of alleles of genes involved in the mating response.

In *S. cerevisiae*, the mating pathway can be activated in a pheromone-independent manner either by deletion of negative regulators (49), overexpression of activators (218) or expression of hyperactive alleles (20, 82). The use of these techniques represents a powerful tool to study the order of components in signaling pathways.

a) An allele of *STE5* suppresses the *STE3*^{DAF} phenotype.

To further understand the mechanism of action of the *STE3*^{DAF}-mediated inhibition, it was of interest to study if the receptor acted upstream of the entire MAP kinase module. To test this, a hyperactive allele of the scaffolding protein Ste5p, *STE5H*, was used (82). Ste5p binds several proteins involved in the mating response, in particular, all the proteins from the MAP kinase module (30, 167). Furthermore, each one of these proteins seem to bind a specific domain of Ste5p (30). The *STE5H* allele activates the mating pathway in a pheromone-independent manner (82). The hyperactivity of *STE5H* is not the consequence of a single mutation, but probably represents the sum of several mutations that increase binding of some components and render the complex more stable. To investigate whether $STE3^{DAF}$ acts upstream or downstream of Ste5p, *STE5H* was placed under the control of the *GAL* promoter and introduced into wild type and $STE3^{DAF}$ strains. As expected, the hyperactive allele caused cell cycle arrest in wild type cells when they were grown on galactose-containing plates (Figure 11, bottom right). Two observations suggest that the cell cycle arrest caused by *STE5H* was mating pathway specific. First, when the allele was introduced into a diploid strain, cells did not undergo cell cycle arrest in the presence of galactose (Figure 11, top left). Since diploid cells do not express all the components of the mating pathway (49, 217, 132, 191), it follows that cell cycle arrest mediated by *STE5H* requires these components. Second, a disruption in the *STE12* gene also prevented G_1 arrest in cells that expressed *STE5H* under the control of the *GAL* promoter (Figure 11, top right). *STE12* is required for transcription of mating specific genes, and the absence of this particular transcription factor prevents essential components from being expressed during the mating response (56, 64). Taken together these results indicate that *STE5H* causes cell

Figure 11. *STE3*^{DAF} is unable to suppress the arrest phenotype mediated by a constitutively active allele of *STE5*. *MATa STE3*^{DAF} (H67-6C.Ba) (bottom left) and *MATa STE3* (H67-9D.Ba) (bottom right) strains were transformed with a plasmid containing *STE5*^{Hyp} under the control of the *GAL* promoter (pGAL-STE5H). Control wild-type diploid (W303) (top left) and haploid *MATa ste12::LEU2* (D109-1B) (top right) strains were also transformed with pGAL-STE5H. Cells were streaked onto galactose-containing plates and grown at 30°C for 2 to 3 days.

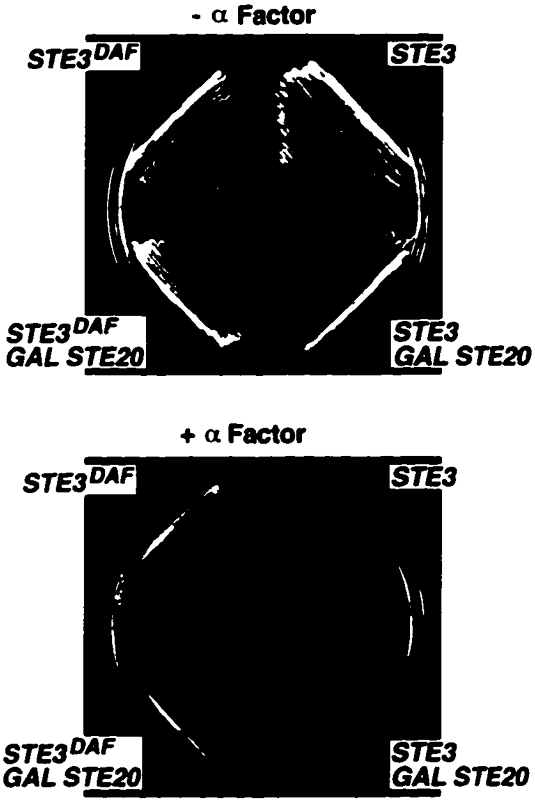

Diploid
GAL STE5H
ste12::LEU2
GAL STE5H
STE3^{DAF}
GAL STE5H
STE3
GAL STE5H

cycle arrest in wild type cells, and that it does so by specifically affecting components of the mating pathway responsible for G_1 arrest. Interestingly, when *STE5H* was introduced into a *STE3*^{DAF} strain, cells arrested in the presence of galactose (Figure 11, bottom left). These findings indicate that *STE5H* is able to suppress the inhibition caused by expression of the inappropriate pheromone receptor in a haploid strain. In addition, they suggest that *STE3*^{DAF} acts upstream of Ste5p. Moreover, since the MAP kinase module and Ste5p have been shown to act as a protein complex, the results presented above suggest that *STE3*^{DAF} acts upstream of the entire MAP kinase module.

b) Overexpression of *STE20* but not *CDC42* or *CDC24* blocks *STE3*^{DAF} inhibition.

Ste20p plays a significant role in mating, as evidenced by the fact that deletion of the *STE20* gene results in sterility and extremely weak, or absent, signaling in response to pheromone (118, 169, 2). It was of interest to determine the site of action of *STE3*^{DAF} with respect to Ste20p. To achieve this, the *STE20* gene was placed under the control of *GAL* promoter and introduced into wild type and *STE3*^{DAF} strains. Overexpression of Ste20p had no immediate effect on growth, either in the wild type or in the *STE3*^{DAF} strain. This was indicated by the normal growth of the two transformed strains on galactose-containing plates compared to untransformed control strains (Figure 12, top panel). To allow activation of the mating response, and

Figure 12. Overexpression of *STE20* blocks the *STE3*^{DAF} effect. *MAT**a*** *STE3*^{DAF} (H67-6C.Ba) and *MAT**a*** *STE3* (H67-9D.Ba) strains were transformed either with a plasmid containing *STE20* under the control of the *GAL* promoter (pMSTE20-L2) or with vector alone (pRS-315). (A) Cells were streaked onto galactose-containing plates grown at 30°C for 2 to 3 days. (B) Cells were streaked onto galactose containing plates with 20 µl of 1mM α-factor and incubated as in (A).

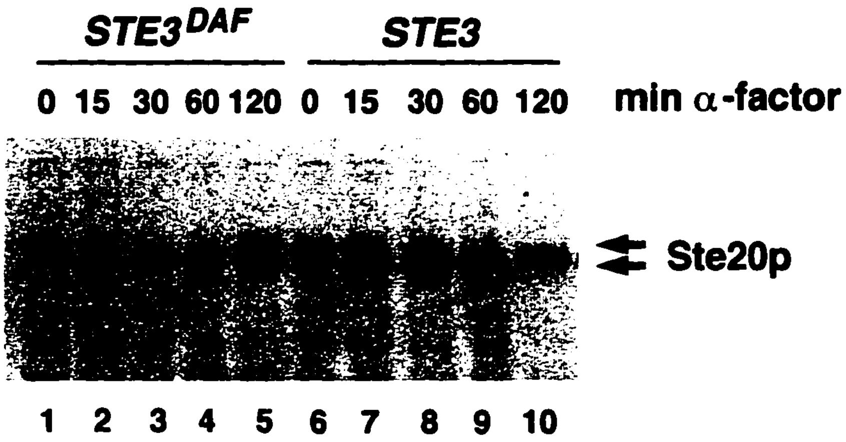

- α Factor
$STE3^{DAF}$
STE3
$STE3^{DAF}$ GAL STE20
STE3 GAL STE20
+ α Factor
$STE3^{DAF}$
STE3
$STE3^{DAF}$ GAL STE20
STE3 GAL STE20

simultaneous overexpression of *STE20*, cells were then transferred to plates containing galactose and α-factor. As expected, wild type cells arrested in the presence of pheromone (Figure 12, bottom panel, top right) and *STE3*^{DAF} cells escaped cell cycle arrest (Figure 12 bottom panel, top left). Interestingly, *STE3*^{DAF} cells overexpressing *STE20* displayed cell cycle arrest in response to α-factor (Figure 12 bottom panel, bottom left) as wild type cells did (Figure 12 bottom panel, bottom right). These results indicate that overexpression of *STE20* suppresses the *STE3*^{DAF} phenotype in the presence of pheromone. In addition, they suggest the possibility that the receptor acts at a step that is subject to direct competition by excess of Ste20p.

Ste20p is a low abundance, phosphorylated protein (223) and interestingly, the phosphorylation state of Ste20p fluctuates during the cell cycle (158). In addition, phosphorylation results in mobility changes that can be resolved by SDS-PAGE and detected by immunoblotting with anti-Ste20p antibodies. Fast and slow migrating forms of the protein are present during most of the G_2, M and G_1 phases. During G_1, however, high molecular weight species disappear and a single form of the protein becomes prominent (158). These observations suggest that Ste20p dephosphorylation and the beginning of G_1 are coordinated events. The importance of this correlation is stressed by fact that the mating pathway is activated optimally during G_1 (172), immediately after G_1 cyclins are degraded and Cdc28p-Clnp complexes are inactivated (222, 151). Interestingly, a similar decrease in mobility of Ste20p was observed when cells were exposed to pheromone (42, 158). These last observations strongly suggest that activation of the mating pathway also requires dephosphorylation of Ste20p.

To study the phosphorylation state of Ste20p in the *STE3*^{DAF} system, wild type and *STE3*^{DAF} cells were transformed with a multicopy plasmid carrying *STE20* under the control of its own promoter. This was done to allow efficient detection of Ste20p, which is normally present in low abundance, by immunoblot analysis. Cells were treated with α-factor, protein extracts were made at different time points after the addition and extracts were separated by SDS-PAGE. Ste20p was then detected by immunoblot analysis using specific antibodies. In the wild type asynchronous population of cells, different forms of the protein were observed prior to the addition of pheromone (Figure 13, lane 6). After 60 minutes of pheromone exposure, Ste20p was only present as a single species of approximately 100 kDa (Figure 13, lanes 7-10). These results confirm previous observations concerning Ste20p phosphorylation during the cell cycle and after pheromone treatment (158). As expected, Ste20p also migrated as multiple bands before α-factor exposure in the *STE3*^{DAF} cells, in agreement with the presence of a normal cell cycle machinery in the mutant strain (Figure 13, lane 1). Surprisingly, the slower migrating forms of Ste20p were still apparent when cells were treated with pheromone (Figure 13, lanes 2-5). In other words, Ste20p in *STE3*^{DAF} cells resembled wild type Ste20p for the first 30 minutes but reappeared as

Figure 13. Ste20p is dephosphorylated transiently in *STE3^{DAF}* cells. *MAT**a*** *STE3^{DAF}* (H67-6C.Ba) and *MAT**a*** *STE3* (H67-9D.Ba) strains were transformed with a plasmid containing *STE20* under the control of the *GAL* promoter (pDL166) and exposed to α-factor at 0.1 μM. Samples were removed at the times (in min) indicated. Cell extracts were prepared and separated on a 7% SDS-PAGE gel. A Western blot was performed using an anti-Ste20p antibody.


STE3DAF
STE3
0 15 30 60 120 0 15 30 60 120
min α-factor
Ste20p
1 2 3 4 5 6 7 8 9 10

multiple species thereafter. These results suggest that Ste20p dephosphorylation occurs only partially in *STE3*^{DAF} cells.

As these studies were being completed, preliminary experiments performed elsewhere demonstrated that phosphorylation levels of Ste20p were affected by Cdc28p-Clnp complexes and suggested that the different forms of the protein reflected different stages of the cell cycle (158). Phosphorylation of Ste20p was severely reduced at elevated temperatures in a *CDC28* temperature sensitive strain (158) and in strains carrying deletions in *CLN1* and *CLN2* (42). Thus, Ste20p is presumably phosphorylated as Cdc28p kinase activity increases during the cell cycle and is dephosphorylated just before START, when G_1 cyclins are degraded. Therefore, accumulation of the fast migrating form of Ste20p during pheromone exposure is likely to be the result of the G_1 arrest, and not a direct effect of pheromone treatment over Ste20p. In view of these additional observations, partial Ste20p dephosphorylation in *STE3*^{DAF} cells is probably a consequence of active cell cycle elements and not a direct effect of receptor-mediated inhibition.

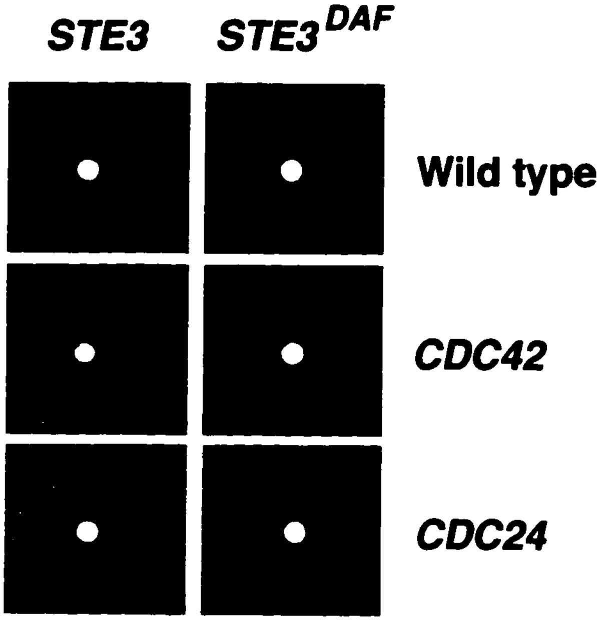
Ste20p interacts physically and functionally with a variety of proteins involved in the generation of cell polarity during the mating response (120, 186). The role of these proteins in signaling is still under intense investigation. However, recent experiments indicate that three of them, Bem1p (125), Cdc42p and Cdc24p (225) enhance signaling through the mating pathway when overexpressed. On the other hand, studies performed in mammalian systems indicate that the mammalian homolog of Cdc42p participates in the activation of the PAK kinases, the mammalian counterparts of Ste20p (129, 135). Thus, it seemed possible to have a positive or inhibitory effect in the mating cascade by altering the function of some component of this particular GTP-binding protein complex. For this reason, it was of interest to investigate the role of Cdc42p and Cdc24p in the *STE3*^{DAF} phenotype. To accomplish this, *CDC42* and *CDC24* were overexpressed (229) in wild type and *STE3*^{DAF} strains. Neither overexpression of *CDC42* nor *CDC24* had an effect on the growth rate of wild type cells (not shown), and cell cycle arrest occurred normally when the strains were exposed to pheromone and compared to untransformed control cells (Figure 14, left). Similarly, overexpression of *CDC42* or *CDC24* had no effect on the behavior of the *STE3*^{DAF} strain in the presence of α-factor, and thus, cells escaped arrest in a manner similar to the same strain without overexpression of these genes (Figure 14, right). These results indicate that Cdc42p and Cdc24p have no effect in the *STE3*^{DAF} phenotype.

4. *STE3*^{DAF} and receptor interference.

a) Inhibition in *STE3*^{DAF} is not the result of the presence of an inactive receptor.

The work presented here suggests that activation of the mating cascade does not occur normally in the presence of a second, inappropriately expressed receptor, characteristic of the opposite mating type. The *STE3*^{DAF}

Figure 14. Overexpression of *CDC42* or *CDC24* have no effect on the *STE3*^{DAF} phenotype. *MAT**a*** *STE3*^{DAF} (H67-6C.Ba) and *MAT**a*** *STE3* (H67-9D.Ba) strains were transformed with either with a plasmid for *CDC42* overexpression (YEp351-CDC42) (middle panels) or *CDC24* overexpression (YEp183-CDC24) (bottom panels). Cell cycle arrest was tested using halo assays with 5 µl of 1 mM α-factor. Untransformed *STE3*^{DAF} (H67-6C.Ba) and *STE3* (H67-9D.Ba) strains were used as controls (top panels).


STE3
STE3^{DAF}
Wild type
CDC42
CDC24

case, however, is not the only example of receptor-mediated interference. At least two systems present similar properties in *Saccharomyces cerevisiae.*

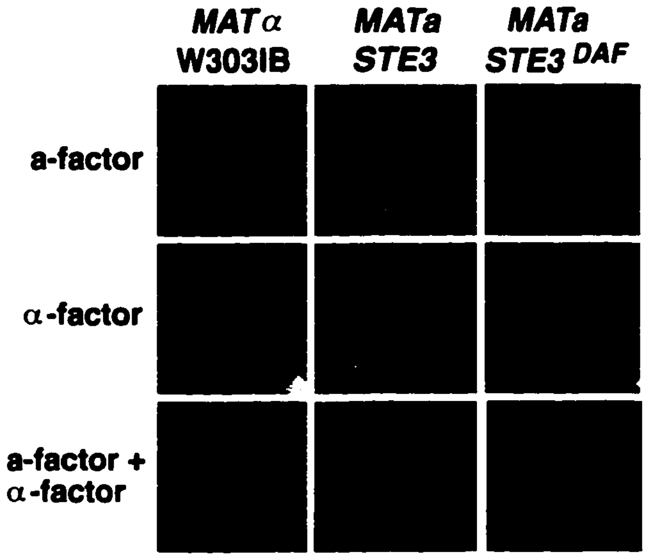
In the first one, stimulation of the mating pathway by an α-factor agonist has been studied. The peptide dTA-α-factor acts as a partial α-factor agonist on the chimeric receptor CKC4, and treatment of cells expressing exclusively CKC4 with dTA-α-factor leads to activation of the mating cascade (182). Interestingly, signaling from the chimeric receptor is significantly reduced when the normal α-factor receptor is also present at the cell surface (182). These results suggest that the presence of the inactive receptor inhibits signaling through the CKC4 receptor. In other words, the existence of a negative function of the wild type receptor is uncovered.

In the second group of studies, heterologous expression of mammalian receptors has been employed to follow G protein function and drug potency in yeast. In these systems, the mating pathway can be activated artificially by a variety of compounds as long as the appropriate receptor is expressed, and as long as the receptor is properly coupled to a functional heterotrimeric G protein. In this manner, expression of β-adrenergic receptors activate the mating cascade upon binding of adrenaline (108), and rat A2a-adenosine receptors trigger activation upon treatment with adenosine agonists (165). Surprisingly, sensitivity to adenosine agonists in *MATa* cells increased in the absence of *STE2*, the gene that encodes the α-factor receptor (165).

The two examples presented above suggest the existence of a negative function of the pheromone receptor in its inactive state. If the model is correct, activation of both receptors simultaneously should eliminate the negative function of the inactive one, and signaling should be restored. Interestingly, this is exactly what happens if cells expressing CKC4 and *STE2* are treated with α-factor, a pheromone that acts on both types of receptors (182).

To address the issue of a negative function of the inactive receptor in the *STE3*^{DAF} phenotype, wild type *MAT**a***, wild type *MATα* and *MAT**a*** *STE3*^{DAF} cells were treated either with **a**-factor, α-factor or both simultaneously. Sensitivity to pheromone was measured by the formation of a halo around the source of pheromone. It is important to mention at this point that halos produced by α-factor are generally larger than those produced by **a**-factor, partly because the *MAT**a*** strains used in these studies contain a disruption in the *BAR1* gene (the product of *BAR1* is a protease that cleaves α-factor specifically, and thus makes the *MAT**a*** strain less sensitive to pheromone). As expected, *MAT**a*** cells arrested in the presence of α-factor and did not arrest in the presence of **a**-factor (Figure 15, center column, middle and top). Conversely, *MATα* cells arrested in the presence of **a**-factor but not in the presence of α-factor (Figure 15, left column, top and middle). In addition, wild type cells from both mating types arrested in the presence of

Figure 15. The inhibitory effect of the *STE3*^{DAF} receptor is not associated with its inactive state. Cell cycle arrest in *MAT*α *STE3* (W3031B) (left column), *MAT**a*** *STE3* (H67-9D.Ba) (center column) and *MAT**a*** *STE3*^{DAF} (H67-6C.Ba) (right column) strains was tested using halo assays with 5 μl of 100 μg/ml of **a**-factor (top), 5 μl of 1 mM α-factor (middle), or 5 μl of 100 μg/ml **a**-factor + 5 μl of 1 mM α-factor (bottom).


MATα
W303IB
MATa
STE3
MATa
STE3 DAF
a-factor
α-factor
a-factor +
α-factor

both pheromones added together (Figure 15, center and left columns, bottom). These control experiments indicate that both pheromones activate the mating pathway if cells of the appropriate mating type are tested. Moreover, they show that the effect of the appropriate pheromone is not affected by the presence of a second (inappropriate) pheromone. As shown previously, *STE3*^{DAF} cells did not arrest in the presence of α-factor (46) (Figure 15, right column, middle). In addition, no cell cycle arrest was observed when the *STE3*^{DAF} strain was treated with **a**-factor (Figure 15, right column, top). Intriguingly, treatment of *STE3*^{DAF} cells with both pheromones simultaneously did not bypass the pheromone resistance of the strain, and the activated *STE3*^{DAF} receptor still inhibited the pathway (Figure 15, right column, bottom). These results indicate that negative signaling from unoccupied receptors is not the primary cause of the *STE3*^{DAF} phenotype, and suggest that the mechanism of inactivation of the mating response, in the case of *STE3*^{DAF}, differs from the other receptor-mediated inhibition phenomena described above.

It is interesting to note that activation of CKC4 by α-factor did not fully restore signaling in the presence of Ste2p (182). Contrary to dTA-α-factor, α-factor activates both CKC4 and Ste2p. Thus, some inactivation is still present when the two receptors are expressed at the surface of a single cell, and are activated by a common peptide. The similarity between this residual inactivation and the $STE3^{DAF}$ phenotype remains to be explored in more detail.

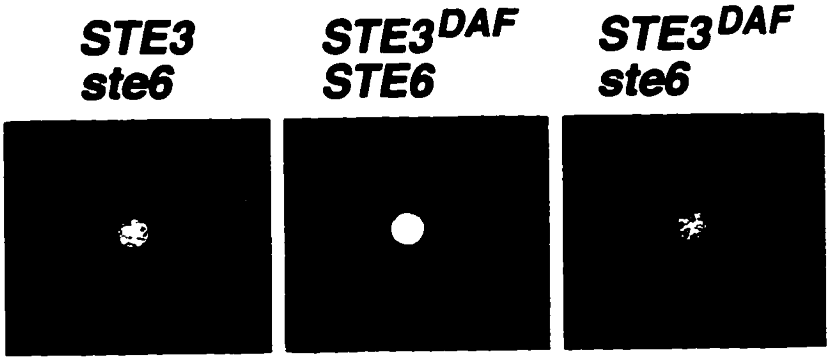
b) The mating type context of the $STE3^{DAF}$ phenotype remains unresolved.

Another interesting question regarding receptor-mediated down regulation concerns the mating type specificity of the $STE3^{DAF}$ phenotype. *STE3* causes signaling inactivation only when expressed abnormally in *MAT***a** cells and not when present in *MATα* cells (10). One interpretation of these observations is that they result from the existence of a function in the receptor that "senses" the mating type by way of specific signaling molecules. This is supported by the fact that overexpression of the proper receptor has no apparent effect on signaling through the mating pathway (42). One possibility is that proteins expressed exclusively in one of the two cell types define the mating type context and prevent signaling if the context is altered. Not many proteins are expressed in a mating type specific manner in *S. cerevisiae*. These include pheromones, pheromone receptors and a few enzymes involved in pheromone processing and secretion (10). The participation of pheromones and pheromone receptors in the $STE3^{DAF}$ phenotype has been explored previously. Absence of the **a**-factor structural genes or deletion of the wild type receptor, *STE2*, had no effect on the ability of $STE3^{DAF}$ cells to escape cell cycle arrest (90). These results indicate that the appropriate receptor and pheromone production do not participate in the generation of the $STE3^{DAF}$ phenotype. They also suggest that the mating type context for

$STE3^{DAF}$ is not defined by them. However, the observations do not rule out the possibility that other mating type specific proteins interact with the appropriate or inappropriate receptor at the surface of $STE3^{DAF}$ cells and induce a negative function.

The participation of *STE6* in the $STE3^{DAF}$ phenotype remained unexplored. Ste6p is an ideal candidate for a sensor of the mating type context in receptor-mediated inactivation. It is exclusively expressed in *MAT***a** cells, and it is presumably located at the plasma membrane, where it has a role in **a**-factor transport to the extracellular medium (140). In addition, Ste6p is required for efficient mating even in the presence of exogenously added **a**-factor (130), and recent experiments suggest a role of the protein in fusion to *MATα* cells (59). Previous experiments that analyzed the involvement of Ste6p in the inactivation of the mating pathway were not conclusive (10). To begin addressing the question of the mating type context in *MAT***a** $STE3^{DAF}$ cells, the *STE6* gene was deleted from wild type and $STE3^{DAF}$ strains. Cell cycle arrest was assayed by exposing the cells to a source of α-factor and monitoring the formation of halos. As expected, disruption of the *STE6* gene had no effect on the ability of wild type cells to arrest in the presence of pheromone (Figure 16, left). Similarly, the *ste6* disruption did not affect escape from cell cycle arrest in the $STE3^{DAF}$ strain (Figure 16, right), and the response was indistinguishable from the one produced by $STE3^{DAF}$ cells containing wild type *STE6* (Figure 16, center). These results indicate that

Figure 16. Ste6p is not involved in the definition of the mating type context in *STE3*^{DAF} cells. The *STE6* gene was disrupted in wild type and *STE3*^{DAF} strains. In these strains cell cycle arrest was tested using halo assays with 5 µl of 1 mM α-factor. The strains used were: *MAT**a** STE3 ste6::URA3* (AC21) (left), *MAT**a** STE3*^{DAF} (H67-6C.Ba) (center) and *MAT**a** STE3*^{DAF} *ste6::URA3* (AC20).


STE3
ste6
STE3^{DAF}
STE6
STE3^{DAF}
ste6

Ste6p is not involved in the production of the *STE3*^{DAF} phenotype. However, they do not exclude the possibility that mating type context effectors, or sensors, exist in yeast, and that *STE3*^{DAF} is affected by them.

Results: Part II

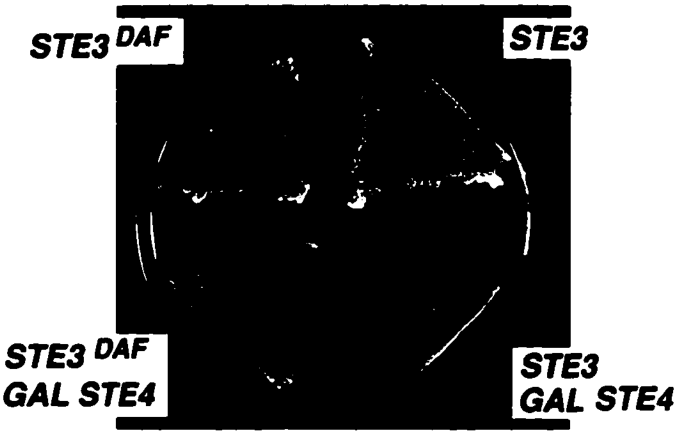
1. *STE3*^{DAF} and the Gβ subunit (*STE4*) of the heterotrimeric G protein.

A significant amount of effort has been directed towards understanding the mechanism by which the heterotrimeric G protein activates the mating signaling pathway. The exact process, however, remains elusive. In any event, similar to what has been extensively studied in mammalian systems (80), activation of the seven transmembrane, G protein-coupled receptor appears to trigger the exchange of GDP for GTP in the Gα subunit of the G protein, Gpa1p (132, 53, 85). In the GTP bound state, Gpa1p dissociates from Ste4p, and the latter subunit is believed to activate an unknown downstream effector of the mating cascade (217). These observations have been demonstrated by two sets of experiments. First, overexpression of Ste4p, the yeast Gβ subunit, is sufficient to activate the pathway in a pheromone independent manner (218). In a similar way, dominant constitutive alleles of *STE4* result in cell cycle arrest as a consequence of permanent activation of the mating pathway (15). Second, deletion of *GPA1* causes lethality due to the presence of free Ste4p subunits and constitutive signaling (49, 144, 97). Thus, both situations favor a model in which free Gβ subunits promote activation of the pheromone response.

a) *STE3*^{DAF} acts at the level of or downstream of Ste4p, the Gβ subunit.

It had been shown previously that *STE3*^{DAF} suppressed cell cycle arrest associated with the absence of Gpa1p (46, 90). These results indicated that inactivation of the mating pathway was independent of Gα. In addition, they strongly suggested that the receptor was able to down regulate the signal generated by Gβ in a pheromone independent manner. In this context, it was important to evaluate the effect of *STE3*^{DAF} during overexpression of the Gβ subunit (218). To study this, wild type and *STE3*^{DAF} cells were transformed with a plasmid containing *STE4* under the control of the *GAL* promoter. As expected, untransformed strains were able to grow in the presence of galactose and no difference in the growth rate was detected between wild type and *STE3*^{DAF} cells (Figure 17, top). In addition, overexpression of *STE4* in the wild type strain caused cell cycle arrest confirming previous results (218) (Figure 17, bottom right). Interestingly, *STE3*^{DAF} was able to suppress the growth defect caused by excess Gβ. Thus, *STE3*^{DAF} cells did not arrest even in excess of Ste4p (Figure 17, bottom left). These findings suggest that *STE3*^{DAF} acts downstream of or at the level of the Gβ subunit of the heterotrimeric G protein. Furthermore, they confirm previous results concerning *STE3*^{DAF} inactivation of the mating signal in the absence of Gpa1p.

Figure 17. *STE3*^{DAF} is able to suppress cell cycle arrest mediated by overexpression of *STE4*. *MAT**a*** *STE3*^{DAF} (H67-6C.Ba) and *MAT**a*** *STE3* (H67-9D.Ba) strains were transformed either with a plasmid containing *STE4* under the control of the GAL promoter (pL19) or with vector alone (pMB272). Cells were streaked onto galactose-containing plates and grown at 30°C for 2 to 3 days.

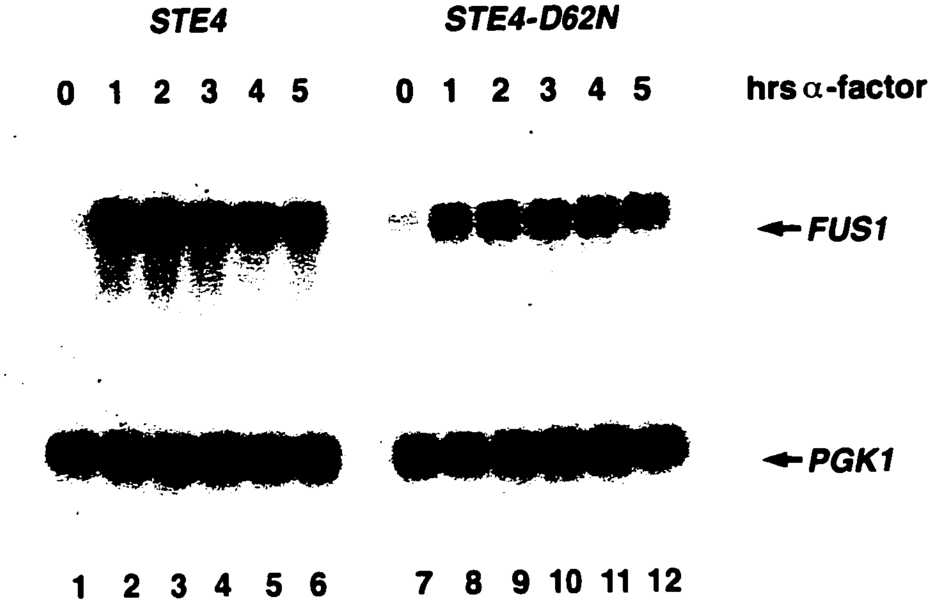

STE3 DAF
STE3
STE3 DAF
GAL STE4
STE3
GAL STE4

b) Dominant negative mutations in *STE4* do not mimic the *STE3*^{DAF} phenotype.

Several observations indicate that certain *STE4* mutants display important similarities to the *STE3*^{DAF} phenotype. Dominant negative alleles of *STE4* that interfere with the endogenous Gβ function, and prevent signal transmission, have been identified (119, 75). Sequence analysis of a group of such mutants revealed that the changes were concentrated in two regions close to the amino-terminus of *STE4*. These domains are likely to participate in Gβ/effector interactions (119, 75). Expression of these mutants in cells containing the wild type version of Ste4p resulted in resistance to cell cycle arrest (119). These findings indicate that Ste4p has an essential role during the propagation of the mating signal. In addition, they suggest that interference of Gβ function can account for inactivation of the mating pathway.

The *STE3*^{DAF} phenotype is also characterized by inactivation of the mating signal and escape from cell cycle arrest. Thus, it was of interest to determine if any relationship existed between the *STE3*^{DAF} phenotype and dominant negative *STE4* mutants. In order to test this possibility, the kinetics of inactivation of the mating pathway in the presence of a dominant negative *STE4* were followed by measuring *FUS1* mRNA levels. Wild type cells were transformed with a *STE4* dominant negative allele that contained a single amino acid substitution, which replaced an aspartic acid with asparagine at position 62 (*STE4-D62N*) (119). Transformed and untransformed control cells were then incubated with α-factor for different amounts of time, RNA was isolated and Northern blots were performed with a *FUS1* probe. As shown previously, *FUS1* mRNA levels in untransformed wild type cells increased significantly after treatment with pheromone (Figure 18, lanes 1 and 2). Moreover, levels remained elevated for at least 4-5 hours, suggesting a prolonged activation of the mating pathway in the continuous presence of α-factor (Figure 18, lanes 2-6). As shown in Figure 7, *STE3*^{DAF} cells, in contrast, had a transient activation of signaling. Interestingly, *FUS1* mRNA also increased after pheromone treatment in *STE4-D62N* transformed cells (Figure 18, lanes 7 and 8). However, mRNA showed constant and overall lower levels of accumulation in response to pheromone (Figure 18, lanes 8-12). These findings suggest that escape from cell cycle arrest results from the inability of the mating signal to reach high enough levels in *STE4-D62N* cells. More importantly, the kinetics of activation of the mating pathway in *STE4-D62N* and *STE3*^{DAF} indicate that both mutations trigger completely different responses upon pheromone exposure. Briefly, while *STE3*^{DAF} causes resistance to cell cycle arrest by preventing sustained activation of the mating pathway, *STE4-D62N* does so by decreasing the overall levels of signal. The results stress the differences between the two mutations, but they also indicate that signaling may be regulated differently to produce similar outcomes. This is important because intermediate responses, or activation of branching pathways, may depend on different levels of signals at particular

Figure 18. *FUS1* transcript levels in cells expressing the dominant negative *STE4-D62N* allele do not resemble *STE3*^{DAF}. *MAT***a** *STE3* (H67-9D.FBa) cells either untransformed (lanes 1-6) or transformed with a plasmid containing the dominant negative allele *STE4-D62N* under the control of the *GAL* promoter (pL19/D62N) (lanes 7-12) were treated with α-factor at 0.1 μM for the indicated periods of time. RNA was isolated, transferred to nitrocellulose, and hybridized with *FUS1* probe. The blot was rehybridized with *PGK1* probe to determine the amount of RNA per lane.


STE4
STE4-D62N
0 1 2 3 4 5
0 1 2 3 4 5
hrs α-factor
FUS1
PGK1
1 2 3 4 5 6
7 8 9 10 11 12

points in the pathway. Thus, intensity and duration may determine a complex set of responses to a particular stimulus.

c) Adaptation mediated by residues 310-346 of Ste4p is not related to *STE3*^{DAF}.

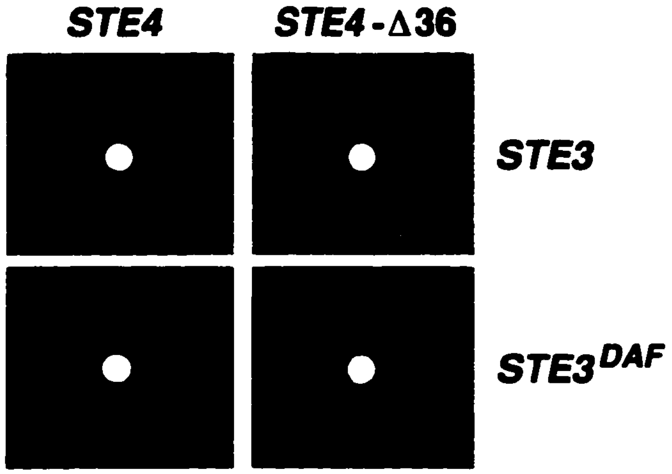
Several observations suggest that the Ste4p subunit of the heterotrimeric G protein has an adaptive role (34, 119, 75) in addition to signaling (217). Adaptation through Gβ was particularly intriguing because of its resemblance to the *STE3*^{DAF} phenotype. Ste4p is phosphorylated during the mating response and phosphorylation occurs in a non-conserved region of the protein (34). Interestingly, deletion of the 36 residues that comprise this non-conserved region abolish phosphorylation, result in delayed recovery from cell cycle arrest and cause supersensitivity to pheromone. The observations suggest that the domain participates in adaptation to pheromone exposure. One interpretation of these findings is that residues 310-346 are required for the interaction of Ste4p with an essential component of the adaptation machinery. Since *STE3*^{DAF} promotes inactivation of the mating pathway in a manner comparable to adaptation by *STE4*, it was of interest to determine the role of residues 310-346 in the *STE3*^{DAF} phenotype. The rationale was that an adaptive-deficient Ste4p could signal normally in *STE3*^{DAF} cells, but could be, in principle, unable to down regulate the pathway at a later stage.

To test this particular model, wild type and *STE3*^{DAF} strains that carried a disruption in the *STE4* gene were constructed. The strains were transformed either with a plasmid carrying wild type *STE4* or *STE4-Δ310-346*. Cells were then exposed to pheromone and cell cycle arrest was measured by halo assay 24 to 36 hours later. As expected, wild type cells expressing *STE4* formed a halo around the source of pheromone (Figure 19, top left). In addition, wild type cells expressing *STE4-Δ310-346* formed a wider halo than cells expressing *STE4* (~40 mm versus ~36 mm) (Figure 19, top right). These findings indicate that *STE4-Δ310-346* causes supersensitivity to pheromone, and are in agreement with the original observations (34). $STE3^{DAF}$ cells, on the other hand, did not alter their response to pheromone in the presence of *STE4-Δ310-346*, and escaped cell cycle arrest in the same way that $STE3^{DAF}$ cells carrying wild type *STE4* did (Figure 19, bottom). These results clearly indicate that inactivation of the mating pathway in $STE3^{DAF}$ does not operate through residues 310-346 of Ste4p.

d) Novel alleles of *STE4* suppress the $STE3^{DAF}$ phenotype.

Although residues 310-346 of Ste4p were not relevant to the $STE3^{DAF}$ phenotype, an interesting piece of evidence suggested that Ste4p might still have a key role in the production of the phenotype. A particular dominant negative allele of *STE4* that caused resistance to cell cycle arrest was recently isolated. Interestingly, the negative effect of the allele was only observable in the presence of the pheromone receptor (38). The kinetics of the inhibition have not been investigated, but the existence of a receptor-dependent

Figure 19. Residues 310-346 of Ste4p are not involved in the inhibition mediated by the *STE3^{DAF}* receptor. *MAT**a** STE3^{DAF} ste4::HIS3* (AC17-7B) (top) and *MAT**a** STE3^{DAF} ste4::HIS3* (AC17-2B) (bottom) strains were transformed either with a plasmid containing *STE4* (YCpSTE4) (left) or with a plasmid containing a truncated version of *STE4,* that lacks the nucleotide sequence corresponding to residues 310-346 (YCpΔ36) (right). Cell cycle arrest was tested using halo assays with 5 µl of 1 mM α-factor.

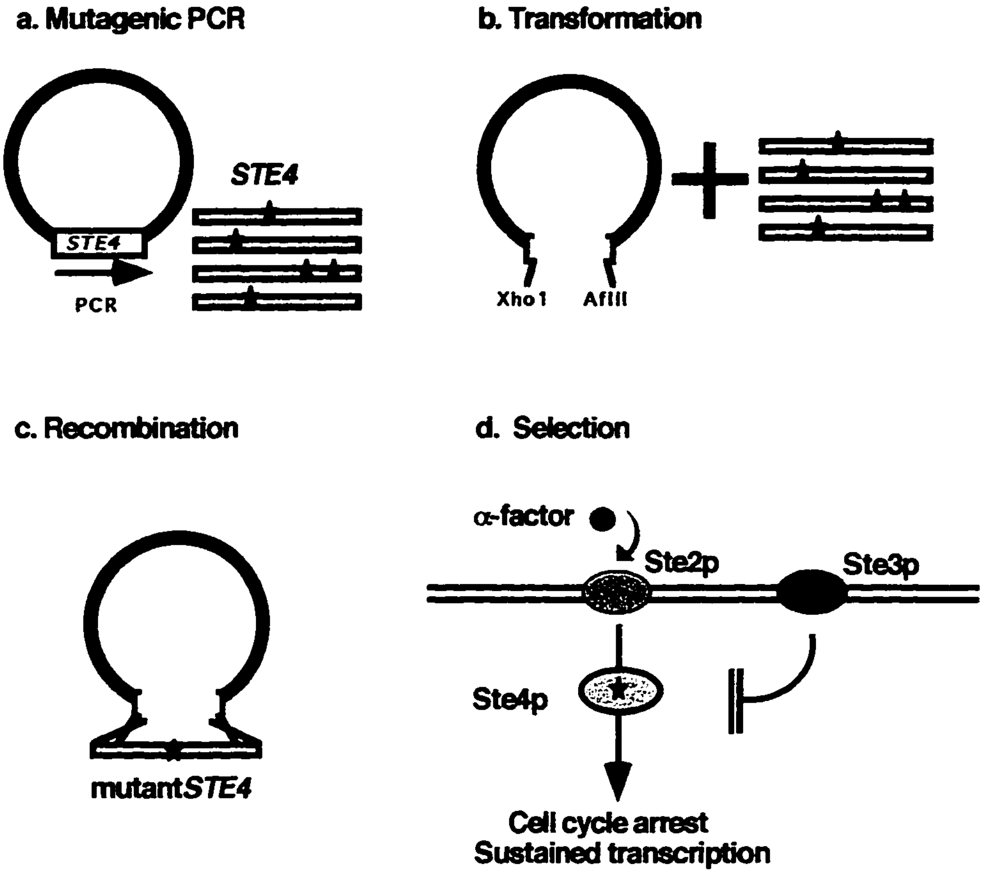

STE4
STE4-Δ36
STE3
STE3^DAF

dominant negative allele of Ste4p strongly suggests a functional interaction between the pheromone receptor and Ste4p.

The observations allow the conception of a model to account for *STE3*^{DAF} mediated inactivation. Concisely, the presence of the pheromone receptor in particular, and of seven transmembrane receptors in general, could be required to achieve adaptation through Gβ. This could be explained in terms of the formation of a complex, between free Gβγ subunits and the inactive receptor, that acted to return the system to an inactive state after a cycle of activity. This putative function of the receptor and Gβγ could be mediated by specific domains that interacted directly. Alternatively, it could require the presence of an adapter or regulatory molecule to place the receptor and the Gβγ subunits in close proximity. In the yeast mating pathway, receptor-dependent, Ste4p-mediated adaptation could operate normally in haploid cells. In addition, due to the presence of alternative or increased affinity sites for the regulatory component in the inappropriately expressed receptor (*STE3* in *MAT**a*** cells), the kinetics of desensitization could simply be enhanced in *STE3*^{DAF}.

Since the *STE3*^{DAF} phenotype involves inactivation of the mating pathway and the experiments described above suggest that inhibition takes place upstream of Ste5p and at the level of or downstream of Ste4p, it was of interest to use the system to test the predictions made by the *STE3*^{DAF} receptor-dependent, Ste4p-mediated inactivation model. On the assumption that Ste4p interacts with the *STE3*^{DAF} receptor (directly or indirectly) and that this results in inhibition of the mating pathway, it was reasonable to think that specific mutations in Ste4p could block this effect. For this purpose, the inability of *STE3*^{DAF} cells to arrest in the presence of pheromone was utilized to select mutant Ste4p molecules that restored prolonged signaling (high levels of mating specific transcription and cell cycle arrest) in the arrest resistant, *STE3*^{DAF} background. Thus, selection of i) signaling-competent and ii) *STE3*^{DAF} receptor-insensitive Ste4p subunits was pursued. A combined genetic and molecular biological screen was devised to obtain such mutations (Figure 20). Briefly, PCR based mutagenesis of *STE4* was used to introduce random changes in the Gβ subunit. The library of mutagenized *STE4* fragments was then transformed into a *MATa ste4 STE3*^{DAF} strain together with a plasmid containing a gapped *STE4* gene under its own promoter. Expression of the mutant molecules was achieved by *in vivo* gap repair recombination between the ends of the PCR fragments and the linear yeast centromeric vector containing 5′ and 3′ sequences complementary to *STE4*. Selection was based on restoration of cell cycle arrest in response to pheromone. In addition, to facilitate selection of prolonged mating specific transcription, a *FUS1*-β-galactosidase fusion reporter construct was introduced into the host strain. Thus, colonies containing *STE3*^{DAF} receptor-insensitive Ste4p would arrest and turn blue in response to pheromone. Approximately 20,000 *STE4* clones were screened (see Materials and Methods for details) and

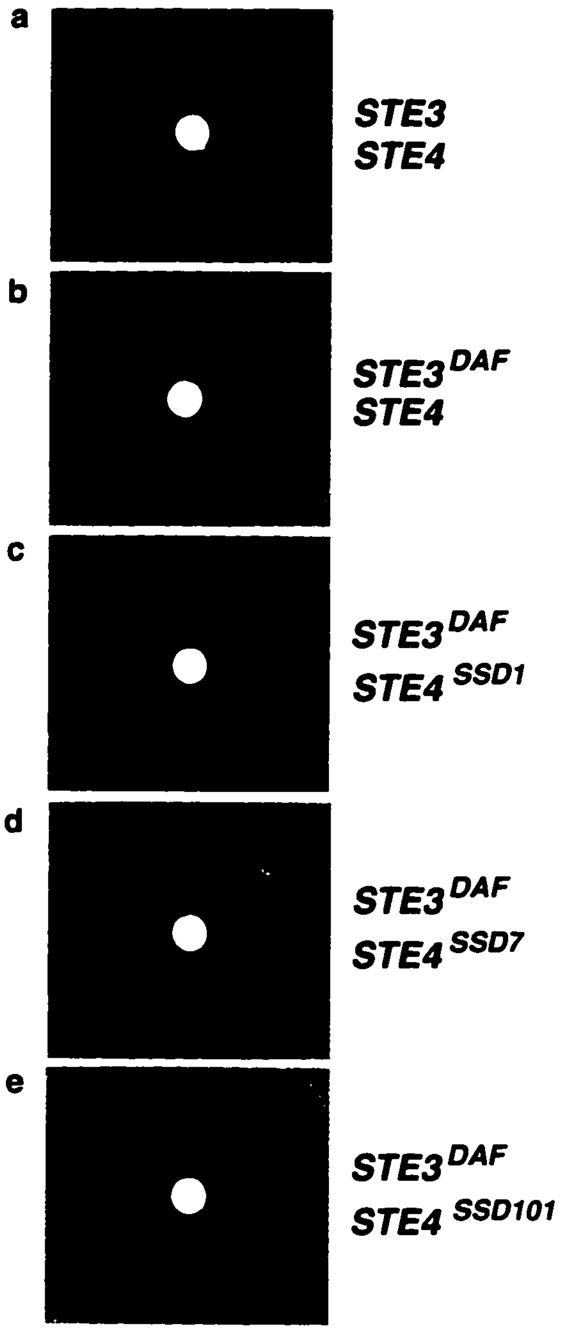
Figure 20. Schematic representation of the screen to isolate *STE4* suppressors of the *STE3*^{DAF} phenotype. For details see "Materials and Methods". Briefly, a PCR mutagenized library of *STE4* clones was generated (a), and transformed into a *MAT**a*** *STE3*^{DAF} *ste4::HIS3* strain (AC17-7B) (b). Homologous recombination *in vivo* was achieved by transforming simultaneously the PCR fragments and a gapped plasmid containing *STE4* flanking sequences (c). Transformed cells were treated with α-factor and cells in which cell cycle arrest and sustained transcription from a *FUS1*-β-galactosidase fusion reporter construct were restored, were selected (d). Plasmids containing the *STE4* alleles were isolated and sequenced.


a. Mutagenic PCR
STE4
STE4
PCR
b. Transformation
Xho1
AflII
c. Recombination
mutantSTE4
d. Selection
α-factor
Ste2p
Ste3p
Ste4p
Cell cycle arrest
Sustained transcription

several mutants with the desired phenotype were obtained. The novel *STE4* alleles were named *STE4-SSD*, for *STE4* Supressors of the *STE3*$^{\underline{D}AF}$ phenotype. Three of the *STE4-SSD* clones, *STE4-SSD1, STE4-SSD7, STE4-SSD101* showed increased mating specific transcription in response to pheromone, and all three of them restored at least partial sensitivity to pheromone induced cell cycle arrest in a *STE3*^{DAF} background.

Retransformation of *STE3*^{DAF} cells with the mutant *STE4* plasmids confirmed that the plasmids conferred increased sensitivity to α-factor. Interestingly, *STE4-SSD1, STE4-SSD7* and *STE4-SSD101* conferred different degrees of sensitivity when cell cycle arrest was tested by halo assays. As expected, *MAT***a** *ste4 STE3*^{DAF} cells expressing *STE4* did not arrest in the presence of pheromone (Figure 21, panel b). Expression of *STE4-SSD1* resulted in a clear halo (Figure 21, panel c) and the size of it was very similar, if not indistinguishable, from the one produced by wild type cells expressing *STE4* (Figure 21, panel a). Cells expressing *STE4-SSD7*, on the other hand, were less sensitive as indicated by the growth of colonies within the halo (Figure 21, panel d). Finally, cells containing *STE4-SSD101* displayed an intermediate response between *STE4-SSD1* and *STE4-SSD7* (Figure 21, panel e). These results indicate that mutations in *STE4* revert the sensitivity to pheromone in *STE3*^{DAF} cells. Furthermore, they imply that prolonged signaling through the mating pathway is restored in response to pheromone. Thus, the existence of the *STE4-SSD* alleles strongly suggests that propagation

Figure 21. Novel alleles of *STE4* suppress the *STE3*^{DAF} phenotype. A *MATa STE3*^{DAF} *ste4::HIS3* strain (AC17-2B) was transformed either with a plasmid containing wild type *STE4* (YCpSTE4) (b), or with plasmids containing the mutant alleles *STE4-SSD1* (YCp-SSD1) (c), *STE4-SSD7* (YCp-SSD7) (d) and *STE4-SSD101* (YCp-SSD101) (e). As a control for cell cycle arrest, a *MATa ste4::HIS3* strain (AC17-7B) was transformed with a plasmid containing wild type *STE4* (YCpSTE4) (a). Cell cycle arrest was tested using halo assays with 5 μl of 1 mM α-factor.


a
STE3
STE4
b
STE3^{DAF}
STE4
c
STE3^{DAF}
STE4^{SSD1}
d
STE3^{DAF}
STE4^{SSD7}
e
STE3^{DAF}
STE4^{SSD101}

of the mating signal through mutant Gβs occurs as a result of insensitivity of the mutant alleles to the inhibitory effect of the *STE3*^{DAF} receptor.

A series of experiments were performed to investigate the participation of the *STE4-SSD* alleles in the *STE3*^{DAF} phenotype and to study the possible participation of Gβ in receptor interaction and receptor-mediated adaptation.

e) Sequencing of the *STE4-SSD* alleles revealed important changes in the protein binding domain of *STE4.*

In order to fully understand the implications of the changes in *STE4,* for the *STE3*^{DAF} phenotype and for G protein signaling in general, it was of interest to map the mutations in the *STE4-SSD1, STE4-SSD7* and *STE4-SSD101* alleles. Sequence analysis revealed that the mutations were distributed within different domains of the *STE4* gene. *STE4-SSD1* and *STE4-SSD7* contained mutations in the WD repeat motifs of *STE4. STE4-SSD101,* on the other hand, contained mutations in one WD repeat and also in a non-conserved region of *STE4.*

In *STE4-SSD1* the following changes were detected in WD repeats 2 and 3: Arg162→Gly, Cys182→Arg, Ile195→Val. The mutations lie in close proximity to sequences encoding critical residues for Gα interaction (see Figure 25B for position of the mutations). Interestingly, cysteine 182 is located between two Gα interacting residues, and the change introduces a basic amino acid which could affect the binding properties of the site. However, analysis of a *STE4* allele containing the Cys182→Arg replacement exclusively showed that the allele did not suppress receptor-mediated inhibition, and *STE3*^{DAF} cells escaped pheromone-induced cell cycle arrest. The *STE4-SSD1* allele carries, in addition, two changes in residues that are farther from amino acids involved in Gα or effector interaction. Nevertheless, analysis of single mutants revealed that replacement of an arginine for a glycine in the connecting sequence between WD repeats 2 and 3, resulted in a phenotype that was similar to, but not as strong as, *STE4-SSD1*. One interpretation of this observation is that the change produces a rearrangement of the outer strands of the second blade of the propeller of the Gβ subunit. According to the structure of mammalian Gβ, this region is involved in binding Gα (187) and at least one effector extensively (72) (see Figure 25A). Finally, analysis of the single mutation corresponding to the replacement of an isoleucine for a valine residue at position 165 indicated that the change also produced a partial insensitivity to the *STE3*^{DAF} receptor. According to the alignment of several Gβ subunits (187), this residue is well conserved between distantly related organisms and maps to a region of Gβ that contacts Gα extensively. These results suggest that the ability of the *STE4-SSD1* allele to bypass receptor-mediated inhibition results from the additive effect of the Arg162→Gly and Ile195→Val replacements.

The *STE4-SSD101* allele was found to have similar, but weaker, effects on the suppression of the *STE3*^{DAF} phenotype. The changes in this allele did not affect the immediate region of any residue known to have an effect in signaling; these changes were: Gln17→Leu, Gln21→Arg and Met183→Val. The first two mutations mapped to the N-terminus of Ste4p, a region that corresponds to a unique feature of Ste4p among the Gβ subunits (see Figure 25B for position of the mutations). The changes replaced two glutamine residues for leucine and arginine respectively, thus altering the distribution of charges within this domain. Analysis of a *STE4* allele carrying exclusively these two mutations demonstrated that partial suppression of the *STE3*^{DAF} phenotype was conserved in this allele. The third modification in *STE4-SSD101* corresponded to a valine that replaced a methionine in the fifth WD repeat and had no effect in the generation of receptor insensitivity.

In *STE4-SSD7*, three residues were modified within the sequence of WD repeats 2 and 4, these included: Leu132→Ile, Asn155→Asp, Asp270→Gly. The regions involve critical residues for Gα interaction. An asparagine is replaced by an aspartic acid between two residues that contact Gα, and the mutation introduces a negative charge that, again, might have important influences on the critical site. A second change is also proximal to a Gα contacting site, and it eliminates the negative charge of an aspartic acid residue by replacing it with a glycine. The third mutation exchanges an isoleucine for a leucine residue in WD repeat number 2 and the change conserves the hydrophobicity of the site. However, because of the proximity to Gα contacting residues, the influence of this mutation could be significant. Site directed mutagenesis should help clarify the contribution of each one of these changes in suppression of the *STE3*^{DAF} phenotype.

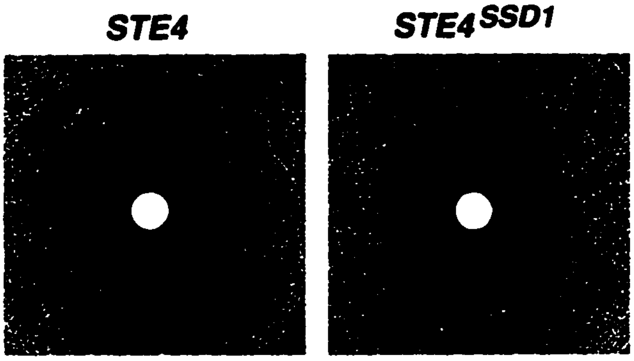
None of the mutations mapped to the same position in the *STE4-SSD* alleles. Thus, the screen did not saturate the possible sites in *STE4* for suppression of the *STE3*^{DAF} phenotype.

Since suppression by *STE4-SSD1* gave the strongest phenotype, this allele was characterized in more detail.

f) *STE4-SSD1* is dominant over *STE4*.

As part of the characterization of the *STE4-SSD1* allele, it was of interest to determine if the mutation conferred dominant or recessive functions to the Ste4p protein. To address this issue, *STE3*^{DAF} cells containing the endogenous copy of *STE4* were transformed with centromeric plasmids containing either *STE4* or *STE4-SSD1*. Cells were then plated and tested for sensitivity to α-factor by halo assays. As expected, *STE3*^{DAF} cells containing one extra copy of Ste4p did not arrest in the presence of pheromone (Figure 22, left). Interestingly, *STE4-SSD1* restored pheromone sensitivity to *STE4* *STE3*^{DAF} cells as indicated by the formation of a clear halo (Figure 22, right), indistinguishable from the one generated in the absence of the genomic *STE4*

Figure 22. The *STE4-SSD1* allele is dominant over *STE4*. A *MAT**a*** *STE4* *STE3*^{DAF} strain (H67-6C.Ba) was transformed either with a plasmid containing wild type *STE4* (YCpSTE4) (left) or with a plasmid containing the mutant *STE4-SSD1* allele (YCp-SSD1) (right). Cell cycle arrest was tested using halo assays with 5 µl of 1 mM α-factor.


STE4
STE4 SSD1

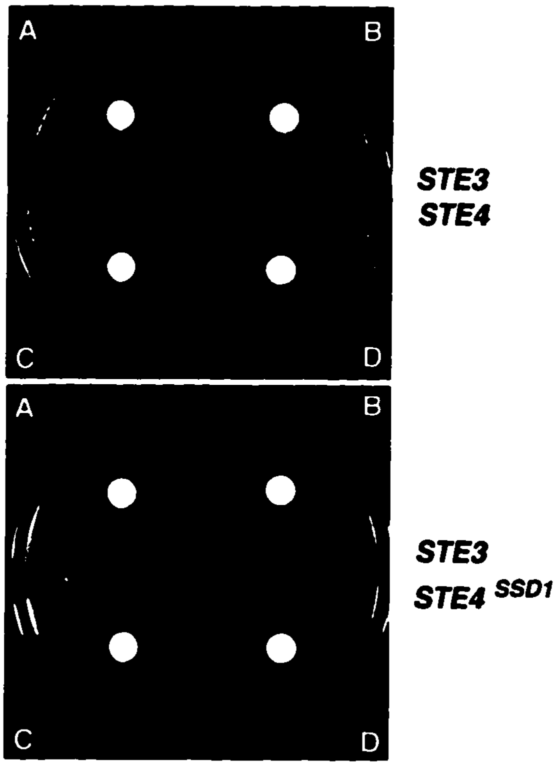
gene (Figure 21, panel c). These results clearly indicate that the *STE4-SSD1* allele is dominant over *STE4*.

g) *STE4-SSD1* confers supersensitivity to pheromone when expressed in wild type cells.

In order to have a better understanding of the effect of the *STE4-SSD1* allele, it was of interest to determine if there was any phenotype associated to expression of the allele in wild type cells. To investigate this, *STE4* and *STE4-SSD1* were introduced into a wild type *MATa* strain carrying a disruption in the *STE4* gene. Cells containing wild type and mutant *STE4* alleles were then tested for α-factor sensitivity. To achieve this, cells were exposed to serial dilutions of α-factor and formation of halos was scored for each concentration. As expected, wild type cells carrying a wild type copy of the *STE4* gene arrested under every concentration of pheromone (Figure 23, top panel). Interestingly, cells containing the *STE4-SSD1* mutant formed larger halos upon exposure to the same concentrations of α-factor (Figure 23, bottom panel). These findings indicate that the *STE4-SSD1* allele confers supersensitivity to α-factor when present in wild type cells.

To complete these studies, the three mutations present in *STE4-SSD1* were analyzed independently for their participation in supersensitivity to pheromone. Interestingly, expression of a *STE4* allele carrying the mutation corresponding to the Cys182→Arg change resulted in supersensitivity when

Figure 23. The *STE4-SSD1* allele is supersensitive to pheromone. A *MATa STE3 STE4* strain (H67-9D.Ba) was transformed either with a plasmid containing wild type *STE4* (YCpSTE4) (top panel) or with a plasmid containing the mutant *STE4-SSD1* allele (YCp-SSD1) (bottom panel). Sensitivity to pheromone was tested by analyzing the size of the halo formed in response to different concentrations of pheromone. The following amounts of α-factor were used: (A) 2× 10^{-3} μmoles, (B) 4× 10^{-4} μmoles, (C) 8× 10^{5} μmoles and (D) 1.6× 10^{-5} μmoles.


A
B
C
D
STE3
STE4
A
B
C
D
STE3
STE4 SSD1

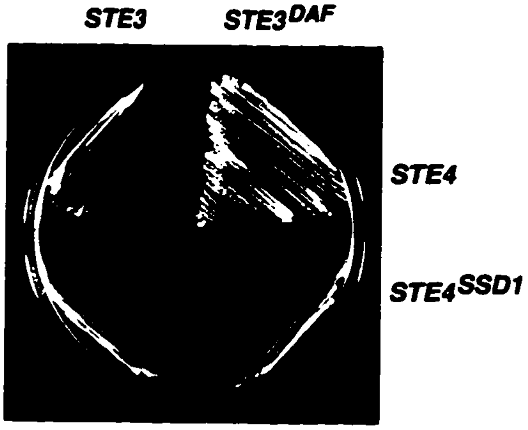
expressed in wild type cells. On the contrary, expression of *STE4* alleles carrying the mutations corresponding to Arg162→Gly or Ile195→Val did not result in supersensitivity. These results suggest that the supersensitivity to pheromone of the *STE4-SSD1* allele is the result of the Cys182→Arg change. In addition, the observations suggest that supersensitivity to pheromone and suppression of the *STE3*^{DAF} phenotype result from different mutations in *STE4-SSD1*.

Finally, it is important to mention that expression of *STE4-SSD7* in wild type cells also resulted in increased sensitivity to pheromone whereas expression of *STE4-SSD101* did not.

h) *STE4-SSD1* acts independently of Gpa1p.

Taken together, the findings described above suggest that introduction of point mutations in *STE4*, eliminate a negative adaptive function, or enhance a positive signaling activity in Ste4p. Since the *STE3*^{DAF} receptor inhibits the signal originated from pheromone binding and also inactivates the pheromone response in the absence of *GPA1* (46, 90), it was of interest to determine if the *STE4-SSD1* allele was insensitive to *STE3*^{DAF} in the absence of Gpa1p. To address this issue, the participation of the Gα subunit in the phenotype conferred by the *STE4-SSD1* allele was investigated. Absence of the Gα subunit causes constitutive activation of the mating pathway due to an excess of free Ste4p subunits (49, 144, 97). Similarly, the *gpa1*^{LYS388} allele, which contains a substitution of asparagine for lysine at position 388, abolishes the GTPase activity of Gpa1p and causes pheromone independent cell cycle arrest (114), presumably as the result of the same imbalance of free G protein subunits. To study whether the mutant Gβ subunits blocked inactivation of the mating pathway in a Gα dependent or independent fashion, an experiment was performed taking advantage of: (i) the growth arrest phenotype of the *gpa1*^{LYS388} mutation, and (ii) the ability of *STE3*^{DAF} to rescue the *gpa1*^{LYS388} arrest. Plasmids carrying wild type *STE4* and *STE4-SSD1* were introduced into wild type and *STE3*^{DAF} strains containing the *gpa1*^{LYS388} mutation. These strains also contained a plasmid carrying a copy of the *GPA1* gene under the control of the *GAL* promoter, and a disruption in the *STE4* gene. Cells were transformed, grown on galactose and afterwards transferred to glucose containing media to deplete the cells of wild type Gpa1p. As expected, *STE3*^{DAF} cells carrying the wild type *STE4* gene were able to grow in the absence of wild type Gpa1p (Figure 24, top right). Also as expected, wild type cells carrying *STE4* arrested in the absence of Gpa1p (Figure 24, top left). These results confirm previous findings concerning the epistatic relationship between *STE3*^{DAF} and *gpa1* (46, 90). Wild type cells carrying *STE4-SSD1* did not grow in absence of functional Gpa1p (Figure 24, bottom left). Interestingly, *STE3*^{DAF} cells carrying the *STE4-SSD1* allele did not grow either upon depletion of wild type Gpa1p (Figure 24, bottom right). These results provide a clear indication that the *STE4-SSD1* allele blocks

Figure 24. Suppression of the *STE3*^{DAF} phenotype by *STE4-SSD1* is independent of Gpa1p. *MAT**a** STE3 ste4::HIS3 gpa1*^{LYS388} (H100-10C) (left) and *MAT**a** STE3*^{DAF} *ste4::HIS3 gpa1*^{LYS388} (H100-10D) (right) strains were transformed with a plasmid containing *GPA1* under the control of the *GAL* promoter (pGAL-SCG1). The strains were then transformed either with a plasmid containing wild type *STE4* (YCpSTE4) (top) or with a plasmid containing the mutant allele *STE4-SSD1* (YCpSSD1) (bottom). Cell cycle arrest in response to depletion of wild type Gpa1p was tested by streaking the cells onto glucose containing media, and allowing them to grow for 2-3 days.


STE3
STE3DAF
STE4
STE4SSD1

receptor-mediated inactivation of the mating pathway in the absence of functional Gpa1p as well as during exposure to α-factor. However, it is important to mention that the *gpa1*^{LYS388} mutation does not eliminate Gpa1p. Thus, the potential interference of the *gpa1*^{LYS388} gene product remains to be studied in a strain carrying a complete gene disruption in *GPA1*.

i) Concluding remarks.

The results presented above demonstrate that *STE4* alleles bypass receptor-mediated inactivation, and strongly suggest that an interaction between the pheromone receptor and Ste4p has been disrupted. They are also encouraging for the further utilization of the *STE3*^{DAF} receptor in the investigation of the complex interactions that mediate the activation of G protein-coupled receptor signaling systems.

Discussion

1. General overview of signaling inactivation due to *STE3*^{DAF}.

Each of the two mating type cells in yeast expresses an individual pheromone receptor that binds the diffusible pheromone secreted by the opposite mating type cell during conjugation. Interestingly, *MAT**a*** cells that abnormally express the Ste3p receptor are resistant to pheromone-mediated cell cycle arrest. The receptor initiates an inactivation of the pheromone response approximately 30-60 minutes after the signal has been generated. This inactivation affects many of the components and events in the pathway, including a cell cycle inhibitor, a MAP kinase, cell cycle arrest and mating specific transcription. *STE3*^{DAF}-mediated inactivation is prevented if functional, but altered, alleles of the Gβ subunit (Ste4p) of the heterotrimeric G protein are expressed in these cells. Since Gβ is involved in signaling and adaptation after pheromone exposure, it is tempting to speculate that the *STE3*^{DAF} phenotype results from an enhanced physiological adaptive mechanism.

2. Implications of *STE3*^{DAF} for signaling.

a) Significance of receptor-mediated inactivation during mating.

In *S. cerevisiae*, sexual reproduction is initiated by fusion of two haploid cells of opposite mating types. In response to pheromone, cells polarize their secretion machinery and form a projection in the direction of the pheromone gradient (181, 27, 176). At the same time, they arrest in the G_1 phase of the cell cycle and increase expression of genes that are required or advantageous for mating (132, 87). As a consequence, two cells actively secreting pheromone grow towards each other and fuse their plasma membranes at the projection tips. Karyogamy and formation of a zygote result from the fusion of the two haploid cells. Importantly, soon after the process is completed the newly formed zygote must resume growth and enter mitotic division if the conditions are favorable. Thus, the specific alterations that prepare the cells for mating must be reversed. This implies that the signaling pathway that was turned on by receptor-bound pheromone must be inactivated as a consequence of mating. Adaptation of the mating pathway, therefore, becomes essential for the production of viable diploids. As discussed above, several adaptation mechanisms have been described in the yeast mating response and proteins at many different levels are potentially important for a complete desensitization of the pathway (132). However, the physiological relevance, individual contribution and time of action of each of these mechanisms are poorly understood.

The relevance of *STE3*^{DAF} for wild type cells is evident when the phenotype is considered in the context of the requirements for growth of the newly formed zygote. Probably, during cell fusion, termination of the signal and presence of the Ste2p and Ste3p receptors in the membrane of the zygote occur simultaneously. Thus, it is reasonable to suggest that the *STE3*^{DAF} mutation serves as a model to study reintroduction of early zygotes to the normal cell cycle and that inactivation of the mating pathway in mutant cells resembles a physiological receptor-mediated adaptation mechanism. An assumption of the model presented above is that pheromone receptors are present at the cell surface until fusion is initiated. Two observations from receptor localization experiments and cell fusion experiments suggest that this is indeed the case. First, pheromone receptors localize to the projection tip prior to cell fusion and probably remain in the membrane early during the formation of the zygote (96). When cells were assayed for the presence of the Ste2p receptor 2.5 hours after mating had started, few receptors were observed at the surface of the zygote (96). However, earlier stages were not analyzed in the study, and by 2.5 hours the cells might be expressing the characteristic set of genes of diploid cells. Second, export of **a**-factor, high concentrations of **a**-factor and the presence of Ste6p are required for fusion to take place (59). Since pheromone receptors mediate all known functions of the mating peptides, these results strongly suggest that the receptors themselves are required at least until the fusion process is started (59). Therefore, the model of receptor-mediated inactivation seems an attractive and feasible mechanism of adaptation in the mating pathway.

An alternative model explains inactivation of the mating pathway in terms of an adaptive function of the *STE3*^{DAF} receptor that reflects a similar but weaker activity of the appropriate wild type receptor during the mating response in haploid cells. Inappropriate expression of the receptor could cause the early activation of a desensitization event that normally occurs later or slower in haploid wild type cells.

b) *STE3*^{DAF}-mediated inactivation meets the requirements of a physiological response.

It is important to illustrate the difference between adaptation and overall reduction of signaling during the activation of signal transduction pathways. An adaptive response requires a period of normal activity followed by a decrease in signaling. Many negative elements of signal transduction pathways, such as the dominant negative Ste4p-D62N mutant protein, prevent activation altogether (119). According to the results presented above, the *STE3*^{DAF} receptor, on the contrary, inhibits the mating signaling cascade only after the early normal response is generated. Therefore, inactivation of the signal in *STE3*^{DAF} cells fits the properties of an adaptation mechanism.

If the pheromone receptor is involved in adaptation, the kinetics of inactivation of the mating pathway must correlate with the length in time it takes to achieve mating and resume growth, or entry into the normal cell cycle. Thus, it is worth considering the timing of the response in more detail. Phosphorylation of the MAP kinase Kss1p is detected as early as 2 minutes after α-factor exposure and reaches a peak after 5 minutes (126). In agreement with these findings, phosphorylation of Fus3p, the other MAP kinase, reaches maximal phosphorylation 2.5 minutes after pheromone addition (71). These observations correlate with complete phosphorylation of the transcription factor Ste12p by 5 minutes (189) and of the cell cycle inhibitor Far1p by 15 minutes (26). Furthermore, the transcriptional response, as measured by mRNA accumulation of the mating specific gene *FUS1*, appears to reach a plateau after 15 minutes of pheromone exposure (136, 208). Therefore, the full activation of the mating pathway takes approximately 15 minutes to reach completion. Interestingly, by 20 minutes of α-factor exposure, α-factor receptors are hyperphosphorylated and internalized from the cell surface in a mating cascade activation-independent fashion (32, 100, 224). However, the α-factor binding activity does not disappear from the cell surface, since new receptors are synthesized even during the process of adaptation which starts approximately 100 minutes after pheromone exposure (100).

The results described above, regarding inactivation of the mating cascade by the $STE3^{DAF}$ receptor, show that receptor-mediated inhibition starts to takes place around 60 minutes of pheromone exposure, as indicated by the decrease in Far1p phosphorylation, the decrease in Fus3p MAP kinase activity and the decrease in *FUS1* mRNA accumulation. Thus, inactivation of the pathway in *STE3*^{DAF} occurs at a time when other inhibitory events are taking place, and this correlation suggests that the inhibitory effect may contribute significantly to the termination of the mating signal.

STE3^{DAF}-mediated inactivation operates independently of *SST2* (90) the carboxyl-terminus of the wild type Ste2p receptor (90), the Fus3p specific phosphatase Msg5 and the recovery mediated by the MAP kinase homolog Kss1p. Therefore, the considerations presented above, and evidence accumulated so far suggest that inactivation of the mating cascade by the *STE3*^{DAF} receptor constitutes a novel inhibitory mechanism that acts late in this signaling pathway.

c) Signaling pathway inactivation during mating type switch.

Haploid yeast strains have the ability to change their mating type, a phenomenon termed mating type switching, soon after budding is completed (201, 202). The process is under the control of the HO endonuclease, and evidence indicates that an inhibitory mechanism exists that allows mating type switch to occur exclusively in the mother cell and not in the daughter (152, 16). Switching the mating type enables formation of diploid cells within a haploid population originated from a single mother. As new cells emerge, change their mating type and undergo mating, it is easy to visualize the importance of a receptor-mediated inhibitory mechanism to prevent activation of the signaling pathway while the cell type change is underway.

Newly synthesized receptors (from the new mating type) may block signaling from the old receptors (from the original mating type) and ensure proper switching without the interference of the mating signal. This model is also based on the assumption that a haploid cell will carry the products of both specific mating types for a determined period of time during the process of mating type switch. The physiological role of the receptor in this process is attractive and the reflection of the phenomenon in the response of *STE3*^{DAF} cells remains to be determined.

d) Inhibition of signaling in cells expressing multiple receptors.

Ligand binding triggers the activation of G protein-coupled receptors in a wide variety of systems (6, 40). Interestingly, different receptors may be activated simultaneously by different ligands to produce an integrated response in a single cell (200, 40, 6). In addition, the same ligand may bind related receptors, or even splice variants of a single receptor (149), and activate distinct signaling pathways due to the different G protein specificity of each receptor (40). In these complex systems, the importance of cross-talk between receptors and signaling pathways is evident, but the understanding of these regulatory mechanisms is not understood in detail. However, recruitment and blocking of G proteins are likely to participate in the production of coherent responses. Considering the relative simplicity of yeast, the *STE3*^{DAF} model represents an ideal system to investigate these kinds of interactions.

3. Potential interference of cell cycle elements in the *STE3^{DAF}* phenotype.

In yeast and mammals, cell cycle control represents a complex machinery that regulates passage from one stage of the cell cycle to the next. In *S. cerevisiae*, one cell cycle dependent kinase, Cdc28p, is the main regulator of the transitions that take place during a round of cell division (171). In addition, a large collection of mutants have been studied in relation to cell cycle control and a large number of proteins have been implicated in the process (81). Essentially, most gene disruptions in yeast result in subtle defects in growth, thus, many pathways interfere at different levels with cell cycle control. *DAF* mutants were originally isolated as cells that did not undergo cell cycle arrest in response to pheromone (46, 45). Interestingly, alleles of the *CLN2* and *CLN3* genes were found in the screen, indicating that the pheromone pathway and cell cycle control shared at least some components, or affected each other for an integrated response (45). These findings were confirmed when *DAF2-2* was found to be an allele of *STE3*, the **a**-factor receptor gene (90).

An interesting question was, therefore, whether Ste3p (in the inappropriate mating type) and Cln2p or Cln3p belonged to a single or separate signaling pathways that controlled cell cycle arrest under certain circumstances. Cln3p controls the G_1 to S transition by inducing the expression of other G_1 cyclins (Cln1p and Cln2p), and therefore, by activating Cdc28p right before START (222, 210, 203). Since the *CLN* genes represent an important regulatory mechanism of cell cycle progression, any stimulus that affects Clnp activity may in turn affect the cell cycle. The inhibition of the mating response that occurs when Cln1p and Cln2p are overexpressed confirms these predictions and indicates that cell cycle elements and mating components participate in the same signaling event (212, 157). Thus, it was of interest to study cell cycle components in order to find out the relationship between *STE3*^{DAF} and the G_1 to S transition, and to examine the signaling route of receptor-mediated inhibition of the mating pathway. Since Cdc28p and G_1 cyclins participate directly in cell cycle control, the *STE3*^{DAF} receptor could inhibit these components directly, without the participation of mating pathway elements. To test this possibility, several approaches were taken. All of them explored the ability of the inappropriately expressed receptor to maintain the activity of cell cycle control elements. If this were the case, in the absence of an individual component, pheromone binding would trigger cell cycle arrest even under *STE3*^{DAF} conditions. A first round of experiments was designed to test whether the receptor caused escape from cell cycle arrest by maintaining the activity of the G_1 cyclins. This possibility was eliminated by the fact that deletion of individual cyclins, or *CLN1* and *CLN2*, had no effect on escape from cell cycle arrest. The small amount of arrest observed in the *STE3*^{DAF} *cln3* strain may be explained by the fact that Cln3p promotes the expression of *CLN1* and *CLN2*, and therefore, in the absence of Cln3p the lower levels of Cln1p and Cln2p may disappear faster and cell cycle arrest may be achieved easier. Thus, in summary, these studies ruled out the possibility that *STE3*^{DAF} acted directly through one G$_1$ cyclin, or through Cln1p and Cln2p, to promote cell cycle progression in the presence of α-factor.

Since additional components are required to regulate cell cycle arrest and progression during the pheromone response (159, 160), the relationship between *STE3*^{DAF} and the cell cycle was studied further. Far1p is the most important cell cycle inhibitor that acts during the mating response (160). However, cell cycle arrest in a *Δcln2 Δfar1* strain reveals the existence of a different and Far1p-independent mechanism (25). Cell cycle progression of *STE3*^{DAF} cells in the absence of individual G$_1$ cyclins and Far1p, demonstrated that the Far1p-independent mechanism was also affected in the *STE3*^{DAF} strain.

Taken together, these findings were extremely important in that they revealed a mechanism of inhibition that affected all three G$_1$ cyclins simultaneously and influenced the two known mechanisms of arrest. The most obvious interpretation of these experiments was, therefore, that inhibition was taking place upstream and was probably independent of the cell cycle control elements.

Although other potential G$_1$ cyclins have been identified in yeast (67, 139, 105), their role in the G$_1$ to S transition has not been clearly established, and their participation in the *STE3*^{DAF} phenotype was not investigated.

4. The *STE3*^{DAF} phenotype is caused by inactivation of mating specific elements.

a) The MAP kinase module is inactivated in *STE3*^{DAF}.

The time course of Far1p phosphorylation revealed that Far1p was phosphorylated early in response to pheromone but that the modification was not sustained in *STE3*^{DAF} cells. These findings were in agreement with the idea that *STE3*^{DAF} acted upstream of the G_1 cyclins and suggested that the inhibitory effect was transmitted through the components of mating pathway.

If the inability of *STE3*^{DAF} cells to sustain phosphorylation of Far1p is related to an inactivation of the mating cascade, the same inactivation event should be observed upstream of Far1p. Interestingly, the Fus3p kinase activity decreased after approximately 60 minutes of pheromone treatment in *STE3*^{DAF} cells. These results were in agreement with an inactivation of the mating cascade upstream of Far1p, and explained the ability of *STE3*^{DAF} to affect the Far1p-independent mechanism of arrest. In addition, they demonstrated that to achieve cell cycle arrest, signaling has to persist for prolonged periods of time. Furthermore, the observations provided an explanation for the kinetics of cell cycle arrest in *STE3*^{DAF} cells. Briefly, *STE3*^{DAF} cells arrested transiently and resumed growth at a time when wild type cells were completely arrested (46). Inactivation of Fus3p is likely to be the cause of cell cycle progression. Finally, the results provided additional evidence to suggest that an adaptive response cannot operate before 1-2 hours of pheromone signaling. A potential reason for this delay would be to prevent a premature inactivation of the mating pathway and reentry into the cell cycle at a critical stage of conjugation.

Since Fus3p and other kinases in the mating response are activated by phosphorylation, dephosphorylation represents an ideal inactivation mechanism for the signaling cascade (215). The study of the participation of phosphatases in the generation of the *STE3*^{DAF} phenotype has not yielded clear insights into the mechanisms of inhibition. However, their role could be essential and remains to be explored in more detail.

Inactivation of Far1p and down-regulation of the Fus3p kinase response presented somewhat different kinetics. While Far1p phosphorylation decreased significantly after 30 minutes, Fus3p kinase activity remained high for 60 minutes and only started to diminish from that point on. Several interpretations of these findings are plausible. First, Far1p phosphorylation assays and Fus3p kinase measurements were performed in strains that shared the same genotypic background, but were slightly different in some components important for signaling. Thus, the Far1p experiments were carried out in a strain that overexpressed *FAR1* from a *GAL* promoter, while Fus3p kinase assays were performed in a strain that contained an extra copy of *FUS3*. It is possible, therefore, that an excess of Far1p accelerated the kinetics of cell cycle arrest in response to pheromone and modified the response to *STE3*^{DAF} inactivation. Alternatively, the subtle differences could reflect a real property of the inhibition process, and may result from a feedback mechanism in which an initial and small deactivation of Far1p is transmitted upstream to amplify the inactivation of the signaling cascade.

Analysis of *FUS1* mRNA in mutant cells indicated that inactivation of Fus3p affected transcriptional induction as well as cell cycle arrest. Contrary to the slight differences observed with Far1p phosphorylation, accumulation and disappearance of *FUS1* mRNA correlated perfectly with Fus3p kinetics of activity. These findings indicate that $STE3^{DAF}$ is responsible for down-regulating the entire signaling cascade at the level of, or upstream of the MAP kinase homolog Fus3p. In addition, they confirm that sustained MAP kinase activation is associated with cell cycle arrest, whereas transient activation is sufficient for early expression of mating specific genes.

b) Early and late stages of inactivation.

Sustained versus transient activation of signaling pathways may be explored either by defining the causes that generate such a brief activation or by scoring the outcome of the short lived signal. At least two mechanisms to generate a short term signal may be envisioned. In the first one, a signal generated by the direct effector of the receptor never reaches appropriate levels to induce a full response. In other words, the signaling pathway requires a threshold of activity to trigger a sustained response due to positive feedback mechanisms or silencing of normal negative regulators. A prediction of this possibility is that lower concentrations of ligand elicit a subthreshold signal that, in turn, produces a transient activation of the signaling pathway. In the mating pathway, lower concentrations of α-factor failed to trigger an activation of the cascade and the conditions that reproduced a transient activation were not found. Therefore, the experiments suggested an alternative inactivation mechanism, in which the signal is generated normally by ligand binding and is only inhibited at a later stage. These findings are in agreement with previous observations that ruled out the possibility that *STE3*^{DAF} inhibited the signaling cascade by interfering with the Ste2p receptor directly in the plasma membrane (90) (e.g. due to inability of Ste2p to aggregate in response to pheromone), since this mechanism of interference would most likely result in the inability of the receptor to activate normally.

The *STE3*^{DAF} receptor allows the initial stages of signaling to take place, but interferes with a second, late stage activation of the signaling cascade. Several kinases in the pheromone response pathway phosphorylate upstream signaling components, and although the consequences of these modifications are unknown, the potential for a feedback loop clearly exists in the pathway (65, 228). In addition, several MAP kinase cascades exist in yeast, and the potential for cross-talk among the pathways represents an attractive possibility to affect different stages of signaling (66, 3, 133). An example of this is the activation of the MAP kinase homolog, Mpk1p, as a result of the cell integrity cascade in *S. cerevisiae*. Here, the cell wall construction cascade is activated after pheromone exposure and its full response requires activation of Ste12p through the mating pathway (18). One interpretation of these observations is that early activation of the cell wall integrity pathway results from Ste12p regulated transcription, while a second stage of activation depends on a different input, probably from the morphology regulator Spa2p (73, 18). A similar diphasic profile may exist in the mating pathway, and sustained activation could be coupled to a second input that enhances signaling after a prolonged period of exposure to pheromone. Early and late activation phases are characteristic of systems which require different gene products during the different stages of the response.

c) A similar example of early stage activation.

A striking parallel exists between the profile of the pheromone response during receptor inhibition and the differential responses of the PC12 pheochromocytoma cell line to extracellular growth and differentiation factors. Treatment of PC12 cells with nerve growth factor (NGF) causes them to undergo cell cycle arrest and differentiate into sympathetic neuron-like cells, whereas treatment with epidermal growth factor (EGF) causes them to proliferate (134). The NGF and EGF receptors are tyrosine kinases that activate a ras-dependent signal transduction pathway and result in activation of MAP kinases. NGF stimulation causes an increase in MAP kinase activity that persists for several hours, while EGF stimulation produces an equal initial burst of MAP kinase activity that decreases to near basal level by one hour (74, 83, 168, 207). Stimulation of PC12 cells by the physiologically relevant factor NGF thus parallels stimulation of wild type yeast cells with pheromone, which caused an increase in Fus3p MAP kinase activity that persisted for up to 4 hours. Both systems respond to sustained activation of MAP kinase activity by cell cycle arrest and alteration of cellular morphology. In the case of EGF stimulation of PC12 cells, transient MAP kinase activation results in cell division without the changes in morphology that accompany differentiation. The onset of cell division probably involves transcriptional induction of early genes involved in proliferation (89). As described above, pheromone stimulation of cells expressing an inappropriate receptor also caused a transient activation of MAP kinase which produced a transient transcriptional induction response with only minor changes in morphology. One differential effect of the duration of MAP kinase activation in PC12 cells is that only sustained activation results in nuclear translocation of MAP kinase (156, 207). The subcellular distribution of the yeast Fus3p MAP kinase has not been investigated, although the location of Kss1p is predominantly nuclear and does not appear to change after activation of the signaling pathway (126). Therefore, the downstream events that result in cell cycle arrest probably take place in the nucleus in both the mammalian and yeast systems.

d) Kss1p activity is also transient in *STE3*^{DAF}.

Two MAP kinase homologs, Fus3p and Kss1p, participate in the mating pathway in *S. cerevisiae*. Even though they share similar functions, their redundancy is not complete. While Fus3p mediates cell cycle and transcriptional responses, Kss1p is primarily involved in the activation of the transcription factor Ste12p (62, 61, 71). One prediction of these observations is that both MAP kinase homologs are required for early activation of the pathway, but only Fus3p is required for late stage signaling. In other words, activation of Kss1p could be short-lived. If this interpretation is correct, duration of the mating signal in a *fus3* strain should resemble *STE3*^{DAF}. However, transcriptional induction was sustained for several hours in the absence of Fus3p. Thus, even though Kss1p is mainly involved in activation of transcription, its activity persists for prolonged periods of time and does not correlate with the changes observed in the *STE3*^{DAF} phenotype. These results suggest that the ability of Kss1p to supply the transcriptional induction function, but not the cell cycle arrest function, is a consequence of substrate specificity and not duration of activation. Finally, since inactivation of Fus3p alone is not responsible for the transient activation of transcription in *STE3*^{DAF}, this information supports the notion that *STE3*^{DAF} affects the signaling pathway upstream of the MAP kinase module.

e) Upstream inhibition of *STE3*^{DAF}.

A signaling cascade with as many steps as the mating pathway may have different inactivation mechanisms at different levels. These mechanisms may include intrinsic half life of activation of the components, GTPase activities, inhibition of protein kinases by phosphatases, etc. The mechanism of interference of *STE3*^{DAF} might be very different depending on where it impinges its effect on the signaling cascade. Thus, elucidation of the site of action of *STE3*^{DAF} was essential to find out the mechanism of inhibition.

A hyperactive allele of *STE5, STE5H* (82), suppressed inactivation mediated by the inappropriate receptor. Suppression of the *STE3*^{DAF} phenotype by *STE5H* indicates that inhibition occurs upstream of Ste5p. Moreover, in light of the tight physical association of the MAP kinase module (30, 167) these findings strongly suggest that inactivation takes place upstream of Ste11p, the first enzyme directly implicated in the cascade. To confirm these results, an experiment was performed using a hyperactive allele of *STE11, STE11ΔN*. This constitutively active allele lacks the N-terminal regulatory portion of the protein kinase and causes cell cycle arrest when expressed under the control of the *GAL* promoter (20). Unfortunately, the growth defect caused by *STE11ΔN* resulted from non-specific toxicity, as implied by control experiments in diploid strains and strains lacking essential components for signal transmission (42). A complementary approach to answer this question was taken using the serine threonine protein kinase Ste20p. Ste20p is required for activation of the mating pathway (118, 169) and one piece of evidence suggests that Ste11p is a substrate for Ste20p *in vitro* (223). Although an excess of this protein did not lead to cell cycle arrest, overexpression prevented *STE3*^{DAF} cells from progressing through the cell cycle in the presence of pheromone. In other words, an excess of Ste20p partially suppressed the *STE3*^{DAF} phenotype in an α-factor dependent manner. These findings indicate that abundance of Ste20p is critical for activation of the mating pathway in the mutant cells, and suggest that an inhibitory component, activated in *STE3*^{DAF}, is competed out by activation and excess of Ste20p. They also suggest that the receptor inhibits the mating pathway upstream of Ste20p. However, since suppression of the *STE3*^{DAF} phenotype in an excess of Ste20p was dependent on α-factor, the possibility that *STE3*^{DAF} acted by inhibiting a downstream component still remained and further experiments were required to clarify this point.

Ste20p is a phosphoprotein and its phosphorylation levels fluctuate during the cell cycle (158). In *STE3*^{DAF} cells, sustained phosphorylation of Ste20p probably reflects a secondary modification in response to changes in Cdc28p-Clnp activity and is therefore a consequence of cell cycle progression. Nevertheless, it is important to evaluate the phosphorylation state of the molecule in light of the findings concerning partial suppression of the *STE3*^{DAF} phenotype. In wild type cells, activation of the mating pathway correlated with the appearance of a single, fast migrating form of Ste20p (158). In *STE3*^{DAF} cells, slower migrating forms of Ste20p were present even during prolonged exposure to pheromone. However, the fast migrating, and possibly less phosphorylated form of Ste20p was present as well at the end of the time course. It is tempting to speculate that under normal conditions (i.e. no overexpression of *STE20*) *STE3*^{DAF} cells will contain exclusively the slow migrating forms of the protein after 2 hours of pheromone exposure. If this interpretation is correct, the presence of the fast migrating form of Ste20p at the end of the time course experiment is a consequence of overexpression. Alternatively, persistence of the fast migrating form could reflect the slow kinetics of regaining the completely inactivated state of the mating pathway in *STE3*^{DAF}.

These results do not address the issue of which proteins are responsible for Ste20p phosphorylation. However, it is likely that Cdc28p participates in this process, and that regulation of Ste20p represents an intermediate step in the regulation and synchronization of polarization and budding by Cln1p and Cln2p (158). Thus, phosphorylation of Ste20p in *STE3*^{DAF} is in full agreement with recent findings concerning the connection between the mating pathway and cell cycle control. Moreover, the results support the idea that cell cycle elements do not cause the mutant phenotype, but rather respond to it.

5. The role of Ste4p in the *STE3*^{DAF} phenotype.

a) The pheromone receptor and Ste4p may participate in the same inactivation event.

Over the past few years, Gβ subunits of heterotrimeric G proteins have been implicated in the regulation of several molecules and signal transduction pathways (195). These Gβ effectors include PLCβ2 (98), adenylyl cyclase (204, 205), MAP kinase pathways (43, 69), Ca^{2+} channels (86, 94), phosphatidylinositol 3-kinase (145), K^+ channels (174, 92) and Tsk and Btk kinases (117). In addition, the positive role of Gβ during the yeast mating response has been clearly established (217, 218, 35, 119, 220). Finally, molecular and mutational analyses of Ste4p have suggested that the subunit is actively involved in adaptation to pheromone exposure (34, 75). Taken together, these findings provide unequivocal evidence for the importance of Gβ in signal transduction in a variety of systems.

Three basic observations suggested that Ste4p participated in the production of the *STE3*^{DAF} phenotype. First, overexpression of Ste4p and disruption of *GPA1* were suppressed by *STE3*^{DAF} (46, 90). Thus, the site of action of receptor inhibition appeared to be at the level of, or downstream of Ste4p, and the mechanism of action independent of Gpa1p. Second, expression of *STE5H* and overexpression of *STE20* suppressed the *STE3*^{DAF} phenotype. Therefore, the site of action had to be upstream of these components and in close proximity to the G protein. Third, expression of dominant negative mutants of Ste4p, with very similar phenotypes to *STE3*^{DAF}, were completely dependent on the presence of a pheromone receptor (38). Thus, the receptor and Ste4p were likely to participate in the same inactivating event.

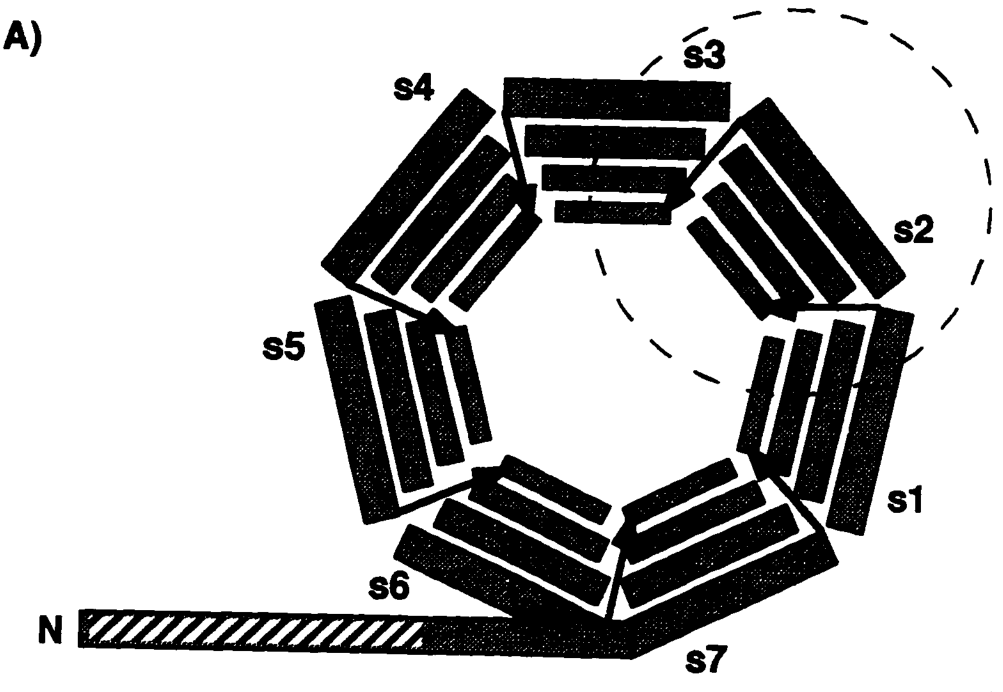
Although Ste4p is an attractive candidate to mediate inactivation of the mating pathway in *STE3*^{DAF}, the results cannot be explained by sequestration of Ste4p from its receptor/heterotrimer complex, or by dilution of the Ste4p pool, since i) overexpression of Ste2p clearly demonstrated that inhibition of the mating signal does not occur in excess of a single species of receptor (42), ii) overexpression of Ste4p does not overcome the *STE3*^{DAF} receptor-mediated inhibition and iii) resistance to cell cycle arrest is maintained when the *STE3*^{DAF} receptor is activated by **a**-factor and, presumably, the Ste4p subunits released from it. Thus, direct competition for Ste4p does not seem to explain receptor-mediated inhibition. However, it could contribute to other receptor-mediated inactivation events different from *STE3*^{DAF} (182, 165).

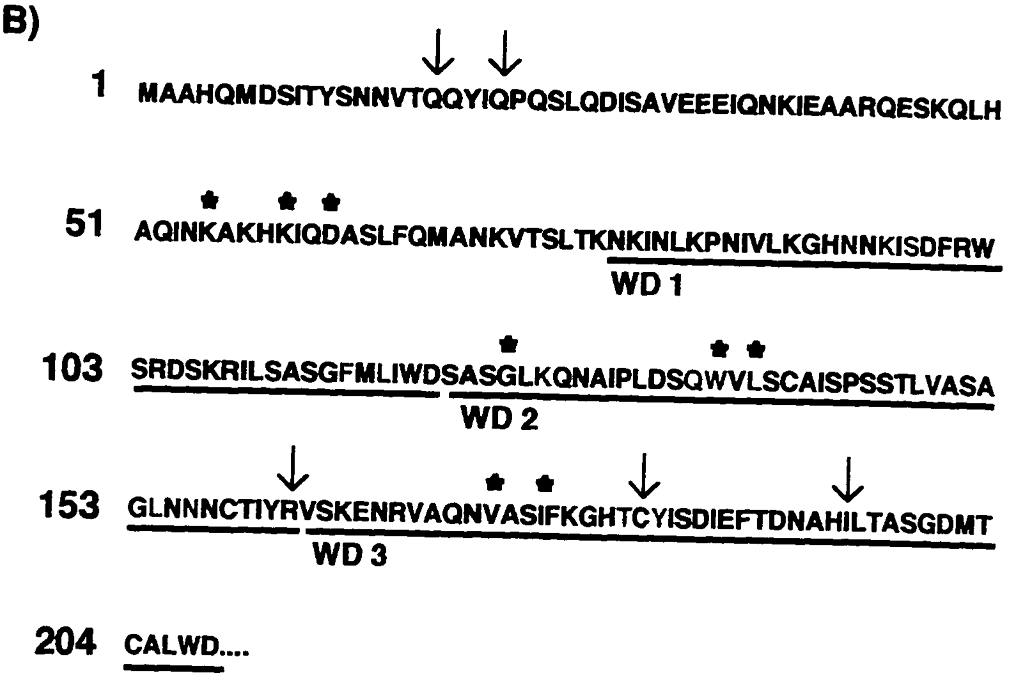
b) Mutations in Gβ uncover an effector for *STE3*^{DAF}.

The screen for Ste4p suppressors of the *STE3*^{DAF} phenotype yielded 3 alleles of *STE4* that when expressed in a *STE3*^{DAF} strain restored prolonged signaling and bypassed, partially or completely, receptor-mediated inactivation. Two of the alleles, *STE4-SSD1* and *STE4-SSD101*, showed a strong influence on cell cycle arrest and little or no signs of constitutive activation. Considering that even excess of Ste4p does not result in cell cycle arrest in *STE3*^{DAF} cells, the effects of these mutations are likely to represent a change in the quality, and not quantity, of the signal propagated by Ste4p. The third allele, *STE4-SSD7*, suppressed *STE3*^{DAF}-mediated inhibition partially and cell cycle arrest in response to pheromone was almost undetectable. However, isolation of *STE4-SSD7* can be explained by the fact that the screen was not based on cell cycle arrest exclusively, but that in addition, it selected colonies that presented elevated levels of transcription of the *FUS1*-β-galactosidase fusion gene. Enhanced basal and inducible β-galactosidase levels were evident when mating-specific transcription was monitored in cells expressing *STE4-SSD7*. Interestingly, in *STE3*^{DAF} cells, two constitutive forms of Ste4p, *STE4*^{Hpl} (46) and excess of Ste4p, had no effect in the cell cycle arrest response of the mutant cells. However, the levels of mating-specific transcription during expression of these alleles remain to be determined to compare the similarity of those results to the *STE4-SSD7* allele and to clarify the influence of pheromone independent activation of Ste4p in the *STE3*^{DAF} phenotype.

STE4 has extensive homology to a wide variety of Gβ subunits from distantly related organisms (187). A common characteristic of these subunits is that most of the residues comprise a repeated motif, WD repeats, found seven times along the length of the protein. These WD repeats are responsible for the seven-fold symmetry and the β-propeller structure of the Gβ subunits (187) (Figure 25A). Besides the conserved domains, the yeast Ste4p presents unique features in its sequence, as it contains two large insertions not found in other Gβ subunits. One of these sequences lies at the extreme N-terminus of the protein and the second interrupts the sixth WD repeat (217, 187). Surprisingly, the two point mutations in the *STE4-SSD101* allele that partially suppress the *STE3*^{DAF} phenotype mapped to the large N-

Figure 25. (A) Schematic representation of the three dimensional structure of the Gβ subunit of the heterotrimeric G_A protein from the transducin system. The protein is organized as a seven-bladed propeller, which contains the seven WD repeat motifs, and an N-terminal helix. Blades are designated s1-s7. The unique Gα and effector binding domain is shown by a dashed circle. Taken from (187), and modified. (B) Sequence of the N-terminal portion and the first three WD repeat motifs of *STE4*. Residues in Ste4p corresponding to mammalian Gβ/Gα contact sites are shown in red. Residues of Ste4p which when substituted disrupt Ste4p/Gpa1p interaction are indicated by red stars. Residues that interfere with Ste4p function are indicated by blue stars. Relevant substitutions in *STE4-SSD1* and *STE4-SSD101* are indicated by arrows.




B)

1 MAAHQMDSITYSNNVTQQYIQPQSLQDISAVEEEIQNKIEAARQESKQLH

51 AQINKAKHKIQDASLFQMANKVTSLTKNKINLKPNIVLKGHNNKISDFRW
WD 1

103 SRDSKRILSASGFMLIWDSASGLKQNAIPLDSQWVLSCAISPSSTLVASA
WD 2

153 GLNNNCTIYRVSKENRVAQNVASIFKGHTCYISDIEFTDNAHILTASGDMT
WD 3

204 CALWD....

terminal insertion in the *STE4* sequence (Figure 25B). Since this region is not represented in the three dimensional structure of the mammalian Gβ subunit, it is difficult to predict the residues in this region that make contact with the heterotrimer and the seven transmembrane receptor. Therefore, the mechanism by which *STE4-SSD101* bypasses receptor-mediated inhibition remains to be determined. However, the ability of altered Ste4p proteins to suppress the receptor inhibitory effect provides the first evidence to link this particular domain to a function of Ste4p. Together with the participation of the 310-346 domain in supersensitivity and recovery from pheromone exposure (34), these results emphasize the importance of the non-conserved regions of Ste4p in the activation and inactivation of the mating pathway.

Sequencing of the *STE4-SSD1* allele also provided an excellent opportunity to relate a novel function of Ste4p to the recently reported three-dimensional structure of mammalian heterotrimeric G protein complexes (214, 116, 187, 72). Only two of the mutations in *STE4-SSD1* suppressed, partially, the *STE3*^{DAF} phenotype when expressed independently. Interestingly, the mutation that resulted in the Cys182→Arg change did not block the inhibitory effect of the receptor but resulted in supersensitivity to pheromone when expressed in wild type cells. Since this mutation affects the immediate region of a Gα binding site, it is reasonable to believe that supersensitivity is caused by disruption of Gα/Gβ interactions. The fact that *STE3*^{DAF} cells escape cell cycle arrest when this particular mutation is present is in agreement with the ability of the receptor to suppress the growth defect caused by overexpression of *STE4* or absence of *GPA1* (46). The other two mutations in *STE4-SSD1*, that resulted in Arg162→Gly and Ile195→Val changes, appear to be responsible for receptor insensitivity. Thus, the distribution of mutations in the *STE4-SSD* alleles suggest that replacements that affect Gα binding sites directly, as in Cys182→Arg (from *STE4-SSD1*) or *STE4-SSD7* do not bypass the $STE3^{DAF}$ phenotype completely but cause supersensitivity to pheromone in wild type cells. On the contrary, mutations that map farther form Gα binding sites, as in Arg162→Gly and Ile195→Val (from *STE4-SSD1*) or Gln17→Leu and Gln21→Arg (from *STE4-SSD101*) appear to correlate with suppression of the $STE3^{DAF}$ phenotype by an unknown mechanism.

The three-dimensional structure of Gβ suggests that residues in one particular domain of the molecule are involved in Gβ/Gα and Gβ/effector interactions (187, 80, 72) (Figure 25A). Such predictions are unambiguously supported by recent studies in mammalian cells, which confirm that the Gβ subunit binds the regulator phosducin via the same domain that interacts with Gα and that some overlapping residues are implicated in both associations (72). These interactions involve mainly WD repeats 1, 2 and 3, and the corresponding outer blades of the β-propeller that they comprise, according to the crystal structure of the subunit (187) (Figure 25A). Thus, activation of a signaling pathway that propagates the signal through Gβ is likely to occur by exclusive binding of Gβ to an effector molecule immediately after displacement of Gα upon GTP exchange. Similarly, inactivation is likely to occur by the reverse reaction, or enhanced by the exclusive binding of a third inhibitory element to Gβ. Since the mutations in *STE4-SSD1* mapped to WD repeats 2 and 3 (Figure 25B) the existence of the allele allows the construction of a simple model to explain the rescue of the *STE3*^{DAF} phenotype. The model proposes that mutated Ste4p subunits are unable to bind an inhibitory element present in *STE3*^{DAF} cells. The most obvious candidate to perform such inhibitory function is, undoubtedly, Gpa1p (Gα). However, two observations suggest a different mechanism: i) the *STE3*^{DAF} receptor inhibits the mating pathway in the absence of *GPA1* (46, 90). Thus, the putative inhibitor is still present in *gpa1* cells, and this observation supports the existence of an inhibitor different from Gα. ii) According to the mammalian three-dimensional structure of Gβ, mutations in *STE4-SSD1* mapped to a shared domain between Gα and effector molecules (72), but did not coincide with Gα binding residues (Figure 25A and 25B). An alternative model takes these observations into account. In this model, the G protein-coupled receptor functions as a third inhibitory element in the system.

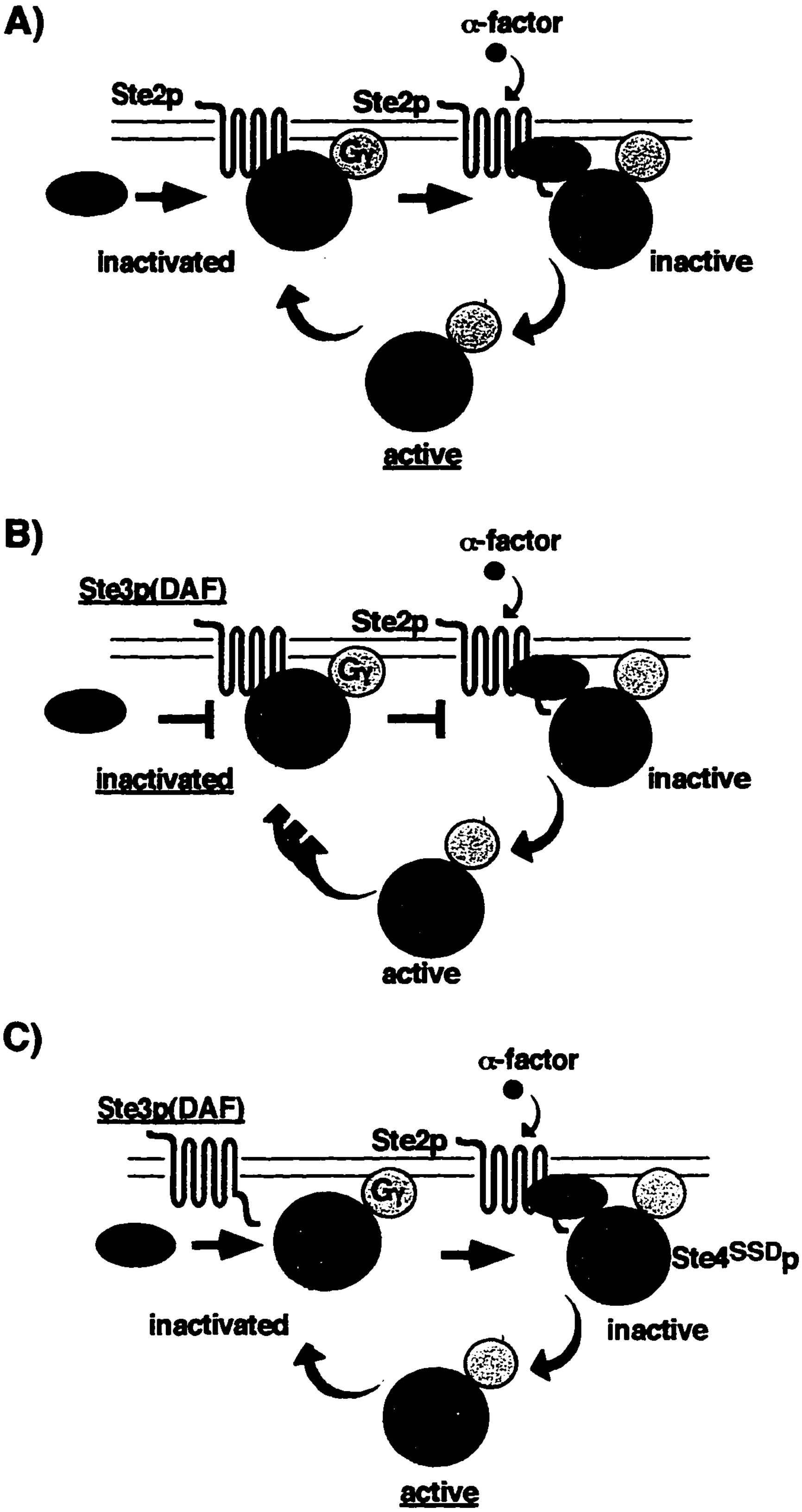
c) The Ste4p/receptor interaction model.

Accumulation of information suggests that a region in Ste4p is involved in direct association with the seven transmembrane G protein-coupled receptor (84, 163, 206). First, evidence from mammalian systems indicates that Gβ is necessary to target βARK, the adaptation protein kinase, to the β-adrenergic receptor (164). Second, Gβ is required for membrane localization of regulatory protein kinases in the muscarinic acetylcholine and rhodopsin systems (195). Third, fluorescence spectroscopy experiments using different components of the heterotrimeric G protein system suggest, but do not demonstrate, a direct interaction between the seven transmembrane receptor and the Gβ subunit (163, 84). Furthermore, studies using seven transmembrane receptor derived peptides have demonstrated that the Gβ subunit binds specific portions of the β–adrenergic receptor (206). An attractive model can explain these observations. Briefly, after a cycle of activity caused by GDP-GTP exchange and GTP hydrolysis by the Gα subunit, direct Gβ/receptor associations could, in principle, enhance the ability of GDP-Gα to return and re-form the inactive heterotrimer-receptor complex, thereby terminating the signal (21, 80). Increased affinity of Gα for the receptor in the presence of Gβ agrees with this proposition (22, 21, 163, 80). Thus, modification of the association-dissociation equilibrium of the receptor and the G protein subunits could affect the activation of downstream events. A similar phenomenon may exist in the yeast pheromone pathway (Figure 26A).

Interestingly, another piece of information supports the notion that the G protein coupled receptor is a target for Gβ regulation, at least in terms of activation and inactivation of signaling pathways (21). Addition of Gβγ subunits to a GTPase assay for transducin Gα-GTPase activity demonstrated that Gβ promoted a 40 fold increase in the rate of light stimulated GTPase activity (70). When examined as a function of the concentration of Gβ, half maximal GTPase activities occurred at a ratio Gα:Gβ of 20-25:1. Two alternative models explain these results. First, Gβ could have a catalytic function with respect to Gα, or second, Gβ could increase GTPase activity by directly acting on the G protein coupled receptor and not on Gα. Since an important amplification step is achieved between the receptor and the Gαβγ complex, the last interpretation provides a better description of the GTPase increase in response to Gβ (21).

The possibility that Gβ facilitated adaptation of the mating signaling cascade by way of a receptor interaction is intriguing. The presence of *STE3* at the surface of *MATa* cells could specifically deplete the pool of activatable Gβ and decrease the rate of Gβ/effector binding, producing, as a result, a terminal non-activatable species and locking the system in an inactive state. The

Figure 26. Model for the *STE3*^{DAF} phenotype and the action of the *STE4-SSD* alleles. For details see "Discussion". Briefly, the model proposes that in wild type cells Ste4p facilitates inactivation of the signaling cascade by directly binding the pheromone receptor and allowing the reformation of the inactive G protein heterotrimer/receptor complex (A). In the presence of the *STE3*^{DAF} receptor the pathway is inactivated as a result of receptor-mediated Ste4p inhibition (B). This interpretation is supported by the isolation of the *STE4-SSD* alleles which are not inhibited by the *STE3*^{DAF} receptor (C).


A)
α-factor
Ste2p
Ste2p
Gγ
inactivated
inactive
active
B)
α-factor
Ste3p(DAF)
Ste2p
Gγ
inactivated
inactive
active
C)
α-factor
Ste3p(DAF)
Ste2p
Gγ
Ste4^{SSD}p
inactivated
inactive
active

existence of the *STE4-SSD* alleles supports these observations. The mutations may create Ste4p subunits that are not susceptible to *STE3*^{DAF} receptor inhibition because residues that participate in receptor association, within a protein interacting domain of Gβ, have been modified (Figure 26C). The plausibility of this model is supported by the fact that *STE2* and *STE3* do not share extensive primary sequence similarity (148), and therefore, could bind the heterotrimeric G protein through different residues and with different affinities. In addition, the fact that the receptor inhibition phenotype is not identical in both mating types agrees with this conclusion (46, 90).

The structure and binding properties of mammalian Gβ subunits imply that there are different, and probably overlapping, surfaces for protein interaction in Gβ. In Ste4p, one of these protein binding surfaces could be responsible for activating a downstream effector in the yeast mating cascade, while a second one could mediate inactivation through the pheromone receptor. In these terms, the *STE3*^{DAF} phenotype could simply be explained as an accelerated inactivation mechanism that normally operates with different kinetics to inhibit the mating pathway in haploid cells after pheromone exposure (Figure 26B). Isolation of the *STE4-SSD* alleles suggests that the signaling and adaptive functions of Ste4p can be uncoupled.

In summary, the findings regarding the isolation of *STE4* mutations that interfere with the *STE3*^{DAF} phenotype and information from mammalian G protein systems suggest that suppression of the *STE3*^{DAF}

phenotype is caused by disruption of the interaction between Ste4p and a key inhibitory molecule, probably the Ste3p receptor, in the yeast mating pathway. Furthermore, since mutations conferring very different functions to Ste4p have been mapped to nearby domains of the protein (119, 75), the results indicate that alteration of a protein interacting surface in Gβ may have different effects depending on the exact position of the modification.

d) Different Ste4p mutants and the receptor association model.

Besides the *STE4-SSD* mutants, a few observations provide support for the idea that the pheromone receptor and Ste4p participate in the same inactivation event. First, recent findings indicate that deletion of last 6 amino acids of the carboxyl-terminus of Ste4p (ΔC6) results in resistance to pheromone. Interestingly, this dominant negative effect is entirely dependent on the presence of the pheromone receptor (38). It is tempting to speculate that the observations indirectly reflect a receptor/Gβ interaction. However, the phenotype is also dependent on Gα (38). This piece of evidence tends to contradict the receptor/Gβ interpretation, but it does not rule out the possibility that Gβ acts as an enhancer for inactivation in a Gα-dependent manner.

The ΔC6 phenotype is remarkably similar to $STE3^{DAF}$-mediated inactivation, and a relationship between the two mutations cannot be excluded. If they indeed represent the same phenomenon, a model would predict that incorporation of the two mutations in the same cell would not result in an additive effect. The accuracy of this prediction remains to be determined.

A second group of mutations provides more evidence for the possible participation of Ste4p and the pheromone receptor in the same inactivation mechanism. *STE4* dominant negative mutants cause resistance to pheromone induced cell cycle arrest (119, 75), and it was suggested that the dominant negative effects resulted from enhanced Gα binding or reduced effector binding (119) (Figure 25B). However, the resemblance of these alleles to the *STE3*^{DAF} phenotype are intriguing. One subset of these mutations lies in the N-terminus of *STE4*, approximately 30 amino acids downstream of the two mutations obtained for *STE4-SSD101*. A second group mapped to the second and third WD repeats, as some of the changes in *STE4-SSD1* and *STE4-SSD7* did. Nevertheless, when duration of the pheromone response in cells containing one of these alleles, *STE4-D62N*, was compared to the *STE3*^{DAF} effect, the kinetics of the response were found to be very different, and the dominant negative mutation did not mimic the *STE3*^{DAF} phenotype. While transcription in the *STE3*^{DAF} cells was short lived and reached an approximately normal peak within the appropriate time, the overall level of signal was very low and persisted for extended periods of time in *STE4-D62N* cells. *STE4-D62N* is only one of several mutations that caused inactivation of the pathway and none of the others have been investigated in terms of activation kinetics.

Suppression of the *STE3*^{DAF}-mediated inactivation by the *STE4-SSD* alleles reveals an inhibitory function of Ste4p, mediated by residues in close proximity to the mutations that produce the dominant negative forms of Ste4p. These observations support the existence of different binding sites, for effectors and regulators, within the protein-interacting surfaces of Ste4p. They also indicate that a high degree of specificity is determined by different residues within these regions (80). The precise relationship between other dominant negative mutations and the *STE4-SSD* alleles, and the possible involvement of the pheromone receptor in the negative effect remain to be investigated.

Finally, the existence of a third allele has implicated Ste4p in the adaptation process, though in this case receptor dependency seems less likely. As mentioned above, deletion of a stretch of residues between positions 310 and 346 of the protein (Ste4p-Δ36) results in the loss of protein phosphorylation, a 6-fold increased sensitivity to pheromone, increased basal and pheromone-induced levels of transcription and delayed recovery from pheromone exposure. Absence of Gpa1p (Gα) or Ste18p (Gγ) impairs the ability of Ste4p to be phosphorylated in the 310-346 region (35). It is interesting to point out that the 310-346 domain lies in the middle of the sixth, well conserved, WD repeat of Gβ, and corresponds to a large insertion of 36 residues in one of the antiparallel β-sheets of the β-propeller structure of Gβ (187). This region is positioned in close proximity to Gγ, making it attractive to postulate that phosphorylation interferes with Gβ/Gγ interactions thereby modifying Gβ activity. Since the domain is missing from other Gβ subunits, the crystal structure did not reveal its three dimensional structure (214, 187). However, the sixth WD repeat contains residues important for effector interaction (187). Thus, the participation of the receptor in the generation of the supersensitivity cannot be excluded.

The results concerning *STE3*^{DAF} indicate that the inhibition mechanism involving *STE4-310-346* is not responsible for the *STE3*^{DAF} effect. These results argue against receptors being involved in the production of the *STE4-Δ310-346* phenotype.

e) An alternative model also explains the effect of the *STE4-SSD* alleles.

The Gβ/receptor interaction model favored above is not, of course, the only possible one. The modification of a binding surface in Ste4p may affect the interaction of this molecule with an effector protein, and this altered association could explain most of the findings concerning the *STE4-SSD* alleles. For example, the alleles may interact with higher affinity with the scaffolding protein Ste5p. The fact that a hyperactive allele of *STE5* suppressed the *STE3*^{DAF} phenotype suggests that activation of Ste5p is sufficient to bypass the inhibitory effect. In addition, since two hybrid and co-immunoprecipitation analyses indicate that Ste4p and Ste5p interact *in vitro* (220), an alternative model explains suppression of the *STE3*^{DAF} phenotype by the *STE4-SSD* alleles in terms of an increased ability of Ste4p to activate Ste5p, or an increased ability to target Ste5p to the appropriate location for activation. The significance of the model will largely depend on whether the residues involved in Ste5p binding correspond to the domains affected by the *STE4-SSD* mutations. Finally, since other Ste4p effectors are likely to exist in the mating pathway, the newly found interaction between Gβ and phosducin may provide additional evidence to support this alternative model (72).

6. The mating type context of the *STE3*^{DAF} phenotype remains to be explored.

Mating type in *S. cerevisiae* is determined by the *MAT* locus and a number of proteins that regulate its expression. *MATα* encodes two factors, Matα1p and Matα2p, that function as regulators of mating specific genes (194, 192, 153). Matα1p activates transcription of α-specific genes while Matα2p inhibits expression of **a**-specific ones. Thus, expression of the *MATα* locus results in the production of the α mating type. On the contrary, production of the **a** mating type results from absence of *MATα* locus regulators (193). In haploid cells, therefore, expression of *MATα* is determinant in establishing the mating type. An interesting consequence of this organization is that deletion of *MATα2* results in induction of expression of both *MATα* and *MAT**a***-specific genes.

Although many of the factors that participate in mating have been characterized (132), the mechanism by which the specificity of the mating type is achieved remains somewhat obscure. As mentioned above, *matα2* cells express genes from both mating types and mutations in *matα1* result in lack of expression α-specific genes. In addition, pheromones and pheromone receptors constitute the primary determinants of the mating specificity (10). In a previous study, expression of *STE3* (the **a**-factor receptor gene), and *MFα1* (the gene that encodes α-factor) in a *matα1* mutant strain resulted in the generation of artificial α mating type cells (10). Similarly, expression of *STE3* and *MFα1* in a *MAT**a*** *ste2* (α-factor receptor deficient) *ste6* (**a**-factor secretion deficient) strain resulted in the production of an artificial α cell, but this time in a *MAT**a*** background (10). Interestingly, artificial α cells mated much better, with a mating type **a** partner, when the *MAT**a*** background was not present in the artificial α cells (10). These findings suggest the existence of a *MAT**a***-dependent mating inhibitor, present or activated in the mating type **a** cells whenever the *MAT**a*** locus, inappropriate pheromone and inappropriate pheromone receptor are expressed simultaneously (10).

The implications of these experiments for the *STE3*^{DAF} phenotype are worth considering. *STE3*^{DAF} receptor-mediated inhibition takes place exclusively when the **a**-factor pheromone receptor is expressed in the inappropriate mating type. These observations are supported by the fact that overexpression of the appropriate mating type receptor (*STE2* in *MATa* cells) does not result in such inactivation. As seen in the observations discussed above, these findings are in agreement with the existence of an unknown **a**-specific product, present as a consequence of *MATa* expression, that inhibits signaling as a result of the inappropriately expressed pheromone receptor. In other words, receptor-mediated inactivation of the mating pathway could be explained in terms of mating type-specific products that respond to the mating type context and inhibit signaling, at a step downstream of the pheromone receptor, if the incorrect receptor is expressed. It is inviting to speculate that a mechanism of mating type sensors could enable the signaling pathways derived from the two mating partners to cross-talk during cell fusion and promote inactivation of the signaling cascades and integration of the newly made diploid into future rounds of division. As mentioned above, the pheromone receptor and the Ste4p subunit could account for the existence of a mating type sensing mechanism. Another attractive candidate for the mating-type sensor was Ste6p. This ABC transporter localizes to the plasma membrane and is exclusively expressed in *MATa* cells (140). Absence of *STE6* in a *STE3*^{DAF} strain had no effect on signal inactivation. These results demonstrated that Ste6p does not participate as a sensor in the *STE3*^{DAF} phenotype. Thus, the identification of mating type-sensors remains to be accomplished. Importantly, the *STE3*^{DAF} phenotype provides an ideal system to study this topic, in part because the inactivation of the mating cascade by inappropriate mating type receptors is not equivalent in both mating types (90).

7. Concluding remarks.

The utilization of the *STE3*^{DAF} mutation provided an excellent model to study inactivation mechanisms of the mating pathway. Starting from the observation that the receptor was responsible for cell cycle arrest resistance (90), the phenotype lead to the identification of an interesting set of events that were associated with a possible adaptive function. The last molecule to emerge as a key effector of the *STE3*^{DAF} receptor, Ste4p, was also one of the first ones to be studied (46). However, the complexity of the system masked the implications of Ste4p action and generated serious contradictions that were solved by means of powerful genetic, biochemical and molecular biological tools.

As these experiments were being performed, the Gβ subunit of the heterotrimeric G protein received considerable attention, and its involvement in key regulatory mechanisms demonstrated the importance of all the subunits of the heterotrimer. In addition, the crystal structure of the G protein in its different activation states significantly advanced the understanding of the properties of the molecules. The investigation of the *STE4-SSD* alleles is, undoubtedly, an exciting branch of the original *STE3*^{DAF} project. In the near future it should be possible to provide more definitive clues as to the exact effect of the individual mutations that cause receptor insensitivity, and it will be challenging to tackle the receptor/Gβ interaction model directly, by way of obtaining complementary mutations in the receptor that suppress the insensitivity of the *STE4-SSD* alleles to *STE3*^{DAF}, or by analyzing the *in vitro* interaction of receptor derived peptides and wild type and mutant Ste4p molecules.

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