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Receptor Inhibition of Pheromone Signaling Is Mediated by the Ste4p G_β Subunit

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The pheromone response pathway of the yeast *Saccharomyces cerevisiae* is initiated in *MAT*a cells by binding of α-factor to the α-factor receptor. *MAT*a cells in which the a-factor receptor is inappropriately expressed exhibit reduced pheromone signaling, a phenomenon termed receptor inhibition. In cells undergoing receptor inhibition, activation of the signaling pathway occurs normally at early time points but decreases after prolonged exposure to pheromone. Mutations that suppress the effects of receptor inhibition were obtained in the *STE4* gene, which encodes the β-subunit of the G protein that transmits the pheromone response signal. These mutations mapped to the N terminus and second WD repeat of Ste4p in regions that are not part of its G_α binding surface. A *STE4* allele containing several of these mutations, called *STE4^{SD13}*, reversed the signaling defect seen at late times in cells undergoing receptor inhibition but had no effect on the basal activity of the pathway. Moreover, the signaling properties of *STE4^{SD13}* were indistinguishable from those of *STE4* in wild-type *MAT*a and *MAT*α cells. These results demonstrate that the effect of the *STE4^{SD13}* allele is specific to the receptor inhibition function of *STE4*. *STE4^{SD13}* suppressed the signaling defect conferred by receptor inhibition in a *MAT*a strain containing a deletion of *GPA1*, the G protein α-subunit gene; however, *STE4^{SD13}* had no effect in a *MAT*α strain containing a *GPA1* deletion. Suppression of receptor inhibition by *STE4^{SD13}* in a *MAT*a strain containing a *GPA1* deletion was unaffected by deletion of *STE2*, the α-factor receptor gene. The results presented here are consistent with a model in which an a-specific gene product other than Ste2p detects the presence of the a-factor receptor and blocks signaling by inhibiting the function of Ste4p.

Cells respond to their external environment by recognizing an extracellular signal, transmitting the signal across the cell membrane, and eliciting a response through activation of the appropriate signal transduction pathway. The binding of a secreted peptide pheromone to its cell surface receptor initiates mating in the budding yeast *Saccharomyces cerevisiae*. Mating occurs between cells of opposite mating types; haploid yeast cells may be either **a** or α mating type and produce the secreted peptide pheromone **a**-factor or α-factor, respectively (reviewed in references 20 and 33). These pheromone ligands bind to the appropriate receptor located on the surface of cells of the opposite mating type; the **a**-factor receptor (encoded by *STE3*) is present on the surface of *MAT*α cells, and the α-factor receptor (encoded by *STE2*) is present on the surface of *MAT***a** cells. The pheromone receptors are members of the G protein-coupled receptor family and are coupled to a heterotrimeric G protein composed of α-, β-, and γ-subunits (encoded by *GPA1*, *STE4*, and *STE18*, respectively). In addition to the G protein, the yeast pheromone response pathway utilizes another common eukaryotic signaling module, a mitogen-activated protein (MAP) kinase cascade (13). Transmission of the signal from the G protein βγ complex to the downstream kinase cascade probably occurs through activation of the PAK kinase homologue Ste20p (21). Specificity of the kinases that are sequentially activated during pheromone signaling is thought to be maintained by the scaffold protein Ste5p (38). The ultimate responses to pheromone signaling include arrest in the G_1 phase of the cell cycle, which is mediated by the cyclin-dependent kinase inhibitor Far1p (3, 27, 28). Other responses to pheromone signaling include morphological changes leading to projection formation and transcriptional induction of genes involved in mating.

The differential expression of mating type-specific genes is controlled by regulatory proteins encoded by the mating-type (*MAT*) locus (reviewed in reference 32). Haploid *MAT***a** cells normally express Ste2p, the α-factor receptor, and undergo cell cycle arrest and transcriptional induction in response to α-factor stimulation. However, *MAT***a** cells containing the *STE3^{DAF}* mutation inappropriately express the **a**-factor receptor and exhibit resistance to pheromone-induced cell cycle arrest, a phenomenon termed receptor inhibition (14). The *STE3^{DAF}* mutation, originally named *DAF2* (for dominant α-factor resistance), was isolated in a screen for mutations that resulted in resistance to α-factor-induced cell cycle arrest in *MAT***a** cells (7). The *STE3^{DAF}* allele contains a rearrangement in the 5′ flanking region of the *STE3* gene which permits expression of wild-type *STE3* in all cell types (14). The abundance of *STE3* RNA in cells containing *STE3^{DAF}* is comparable to the normal level of *STE3* RNA in *MAT*α cells, so the *STE3^{DAF}* phenotype is not the result of overexpression of the receptor. In addition to conferring resistance to cell cycle arrest, the *STE3^{DAF}* allele also causes an increase in the basal expression of a pheromone-inducible gene, *FUS1*. The increase in *FUS1* basal expression is eliminated in *STE3^{DAF}* cells that contain deletions of the genes that encode **a**-factor. This finding demonstrates that the increase in *FUS1* basal expression is caused by the presence of both **a**-factor and the **a**-factor receptor in the same cell. Thus, expression of a pheromone receptor and its ligand in the same cell causes autocrine stimulation of the pheromone signaling pathway. However, inhibition of cell cycle arrest by the *STE3^{DAF}* allele is not affected by deletion of the **a**-factor genes, indicating that autocrine stimulation does not play a role in this phenotype. *STE3^{DAF}*-mediated receptor inhibition can sup-

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TABLE 1. Strains used in the study

Strain	Genotype	Source
W3031A	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
Strains are isogenic to W3031A		
AC17-7B	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3 sst1::hisG ste4::HIS3*	This study
AC17-2B	*MAT***a** *mfa1-Δ3::HIS3 mfa2-Δ2::HIS3* $STE3^{DAF2.5}$ *sst1::hisG ste4::HIS3*	This study
AC18-9C	*MATα mfα1::LEU2 mfα2::LEU2 ste4::HIS3*	
K39-23B	*MAT***a** *ste4::HIS3* $STE3^{DAF2.5}$ *gpa1::TRP1*	This study
K39-23B.f	*MAT***a** *ste4::HIS3* $STE3^{DAF2.5}$ *gpa1::TRP1 far1::URA3*	This study
K39-23D.f	*MATα ste4::HIS3* $STE3^{DAF2.5}$ *gpa1::TRP1 far1::URA3*	This study
K39-23B.s2	*MAT***a** *ste4::HIS3* $STE3^{DAF2.5}$ *gpa1::TRP1 ste2::LEU2*	This study

press the constitutive cell cycle arrest caused by deletion of *GPA1*, the G protein α-subunit gene, and thus does not require the α-subunit for function (7, 14). Expression of *STE2*, which encodes the α-factor receptor, is not required for receptor inhibition; deletion of the *STE2* gene does not affect the ability of $STE3^{DAF}$ to suppress constitutive cell cycle arrest in cells containing null alleles of *GPA1* (14). In addition to inhibition of cell cycle arrest, cells containing the $STE3^{DAF}$ mutation exhibit a block in pheromone-mediated signaling at late time points after pheromone treatment (6). In $STE3^{DAF}$ cells, initial activation of the pheromone response pathway is similar to that observed in wild-type cells, as measured by Fus3p MAP kinase activity and *FUS1* RNA levels. However, at later time points after pheromone induction, $STE3^{DAF}$ cells display a decrease in signaling when compared to wild-type levels (6). Furthermore, epistasis experiments suggest that $STE3^{DAF}$ acts at the level of either *STE5* or *STE4* (6).

In this work, we have isolated mutations in the G protein β-subunit gene, called $STE4^{SD}$ mutations, that suppress receptor inhibition resulting from expression of $STE3^{DAF}$ in *MAT***a** cells. The $STE4^{SD}$ mutations encode Ste4p proteins that reverse the effects of $STE3^{DAF}$ on both pheromone-mediated cell cycle arrest and transcriptional activation. The effects of the $STE4^{SD}$ mutations are specific to cells undergoing receptor inhibition, suggesting that these mutations may define a region of Ste4p that is the target of the signaling block that occurs as a result of receptor inhibition.

MATERIALS AND METHODS

Plasmid construction. A centromeric *URA3* plasmid containing *STE4* was constructed by cloning the 5-kb *Sph*I-*Bam*HI fragment from plasmid M81p12 (5) into YCplac33 (11) to create YCpSTE4. A pUC19 plasmid containing *STE4* was constructed by cloning the 5-kb *Sph*I-*Bam*HI fragment from plasmid M81p12 into pUC19 to create pUC-STE4.1. A centromeric *LEU2* plasmid containing *STE4* was constructed by cloning the 5-kb *Sph*I-*Bam*HI fragment from plasmid M81p12 into YCplac111 (11) to create YCpLSTE4. Centromeric *LEU2* plasmids containing other $STE4^{SD}$ alleles (see Table 2) were constructed by cloning the 5-kb *Sph*I-*Bam*HI fragment from pUC-STE4.1 plasmids that had been subjected to site-directed mutagenesis (Transformer Site-Directed Mutagenesis kit; Clontech) into YCplac111. The *FUS1-lacZ* reporter plasmid was constructed by cloning the 6-kb *Pst*I fragment from pSB234 (kindly provided by E. Elion) into YCplac111 to create YCpF1-LZ.

Strains and media. The strains used in this study are listed in Table 1. The *gpa1::TRP1* null allele was made by transformation of a strain that contains a *gpa1::URA3* allele (10) with a 3.8-kb *Sma*I fragment from marker swap plasmid pUT11 (8). The *FAR1* gene was disrupted by transformation with a 3.8-kb *Xho*I-*Sac*I fragment from pfar1-U1 (6) to create *far1::URA3*. The *ste2::LEU2* allele was made by transformation with a *Bam*HI fragment from pAB506. All strain constructions involving transformations were confirmed by Southern blotting.

Strains were grown on yeast extract-peptone-dextrose (2% glucose) or yeast extract–peptone–3% galactose, and strains under selection were grown on synthetic dropout medium, as described previously (30).

PCR mutagenesis and screen for $STE4^{SD}$ alleles. $STE4^{SD}$ alleles were isolated by cotransformation of yeast with a pool of PCR-generated mutagenized linear fragments containing *STE4* and with a gapped *STE4* plasmid, allowing recombination to occur in vivo as described previously (2, 24). Error-prone PCR was performed with YCpSTE4 as a template in 30 cycles of PCR with oligonucleotide primers oMSTE4.1 (5′-AAGAGTACACTAGATCCATTC-3′) and oMSTE4.3 (5′-AAAGGAAGCAAATGACAATGC-3′). Error-prone incorporation of nucleotides was achieved by performing PCRs under a nucleotide imbalance (80 μM dATP, 400 μM dCTP, 400 μM dGTP, and 400 μM dTTP or 400 μM dATP, 400 μM dCTP, 80 μM dGTP, and 400 μM dTTP) and by using modified *Taq* polymerase buffer containing 100 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.25 mM $MnCl_2$. Approximately 10% of the mutated fragments produced nonfunctional Ste4p proteins as determined by their ability to complement a Δ*ste4* mutation.

Strain AC17-2B(YCpF1-LZ) was cotransformed with the pooled products of the error-prone PCRs and with the 9-kb *Xho*I-*Afl*II fragment of YCpSTE4, which lacks the *STE4* coding region. Transformants were replica plated to selective plates (pH 7.0) that had been spread with 15 μl of 1 mM α-factor (Sigma) and 80 μl of a 40-mg/ml concentration of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Jersey Lab Supply). The strain used in this screen contains a deletion of the *SST1* gene, which encodes an extracellular protease that degrades α-factor, to ensure that the α-factor on the plates remained intact. A total of approximately 24,000 replicated colonies were scored for the absence of growth and for the development of blue color. Plasmids that retained the ability to confer the phenotype after retransformation were sequenced by the dideoxy chain termination method (Sequenase kit; Amersham). Mutagenized plasmids YCpSD1, YCpSD2, and YCpSD3 were isolated by this procedure.

Yeast methods. Yeast transformations were performed by the lithium acetate method (16), modified as described previously (14). Yeast RNA was extracted from cells as described previously (9).

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. The plates were then incubated at 30°C for 1 to 2 days.

The percentage of unbudded cells was determined by growing cells to log phase, treating them with α-factor (0.1 μM) for 3 h, and then fixing them for 1 h with 3.7% formaldehyde. The cell suspension was then sonicated, and the number of budded cells in a total of approximately 200 cells was counted.

Northern (RNA) blots. Cells were treated with either 0.1 μM α-factor (Sigma) or 40 ng of **a**-factor (generously provided by Fred Naider) per ml for various periods of time, and RNA was isolated. RNA was transferred to a nitrocellulose membrane after formaldehyde-agarose gel electrophoresis as described previously (22). The membranes were UV cross-linked by using a Stratalinker UV box. Prehybridization and hybridization were done at 65°C in a buffer containing 0.9 M NaCl, 0.09 M sodium citrate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 33 mM sodium pyrophosphate, and 50 mM sodium phosphate monobasic. The probes used were gel-purified DNA restriction fragments ^{32}P-labeled by random primer labeling with a Prime-It kit (Stratagene). The fragments used were as follows: for *FUS1*, a 1.4-kb *Eco*RI-*Hin*dIII fragment from plasmid pSL589 (26); for phosphoglycerate kinase gene *PGK1*, a 0.5-kb *Bam*HI-*Xba*I fragment from pPGK1.

RESULTS

The pheromone response signal transduction pathway is inhibited in *MAT***a** cells that contain the $STE3^{DAF}$ allele, which causes inappropriate expression of *STE3*, the **a**-factor receptor gene (6). Receptor inhibition is specific for the late phase of the response and only occurs at times after 1 h of exposure to pheromone. Under conditions of receptor inhibition, the signaling pathway is blocked at a step upstream of MAP kinase cascade activation and at or downstream of activation of the Ste4p G_β subunit. These results suggest that Ste4p is a likely

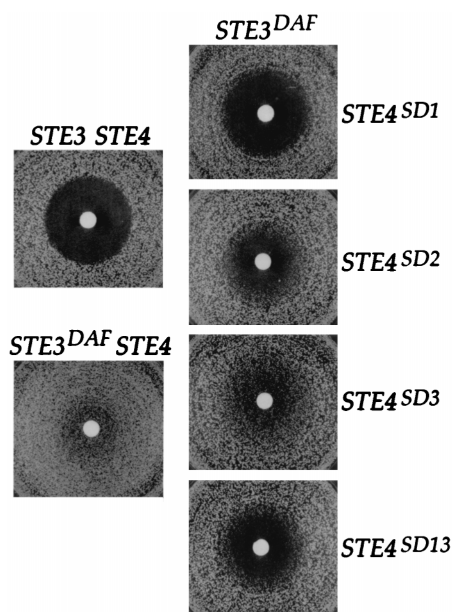

FIG. 1. Mutations in *STE4* suppress receptor inhibition. Halo assays were performed with 5 μl of 1 mM α-factor. Top left, a *MAT***a** *STE3 ste4::HIS3* strain (AC17-7B) containing a wild-type *STE4* plasmid (YCpLSTE4); bottom left, a *MAT***a** *STE3^{DAF} ste4::HIS3* strain (AC17-2B) containing a wild-type *STE4* plasmid (YCpLSTE4); right, top to bottom, a *MAT***a** *STE3^{DAF} ste4::HIS3* strain (AC17-2B) containing plasmids with *STE4^{SD1}*, *STE4^{SD2}*, *STE^{SD3}*, and *STE4^{SD13}* alleles (YCpSD1, YCpSD2, YCpSD3, and YCpSD13), respectively.

target for the inhibitory effect of *STE3^{DAF}*. A screen was therefore performed to identify altered versions of Ste4p that can signal normally but are insensitive to receptor inhibition.

Mutations in *STE4* suppress receptor inhibition. A screen was performed to obtain mutations in *STE4* that have the potential to be specific for its receptor inhibition function. This screen was designed to isolate alleles of *STE4* that suppress the signaling defect of *MAT***a** cells containing the *STE3^{DAF}* allele. *MAT***a** *STE3^{DAF}* cells exposed to α-factor do not arrest and do not sustain pheromone-inducible transcription. Therefore, mutations in *STE4* that caused cell cycle arrest and sustained transcription to occur in a *MAT***a** *STE3^{DAF}* strain were sought. This phenotype requires that the altered versions of Ste4p retain the ability to transmit the pheromone response signal. Thus, this screen has the potential to generate mutations in *STE4* that are specific to receptor inhibition.

The strain used to screen for *STE4* mutations contains the *MAT***a** allele and the *STE3^{DAF}* mutation. Previous studies have shown that *MAT***a** *STE3^{DAF}* cells, which express both **a**-factor and the **a**-factor receptor, display a low level of constitutive signaling due to autocrine stimulation (14). Therefore, the strain used in this screen (AC17-2B) was constructed to contain deletions of the **a**-factor genes, *MFA1* and *MFA2*. Deletion of these genes eliminates any contribution to signaling due to autocrine stimulation of the **a**-factor receptor (14). A *STE3^{DAF}* strain containing *MFA1* and *MFA2* deletions thus displays the normal basal level of signaling, which facilitates the ability to score for increased signaling.

A fragment containing the *STE4* gene was subjected to error-prone PCR, and the PCR products were cotransformed with a gapped *STE4* plasmid into *MAT***a** *STE3^{DAF}* cells to allow recombination in vivo (2, 24). Transformed cells were transferred to plates containing α-factor and X-Gal to assay their ability to undergo cell cycle arrest and to activate a pheromone-inducible *lacZ* reporter construct. Three *STE4* alleles that caused cells to arrest and activate the reporter construct in response to α-factor were isolated, and each of them suppressed the *STE3^{DAF}* phenotype to a different degree. The *STE4^{SD}* alleles (designated *STE4^{SD}* for suppressor of *STE3^{DAF}*) were tested for their ability to confer cell cycle arrest by a halo assay, which measures the density of cell growth in an area surrounding a filter disk containing α-factor. The *STE4^{SD1}* allele caused *STE3^{DAF}* cells to arrest at a level comparable to that of wild-type cells; the *STE4^{SD2}* and *STE4^{SD3}* alleles caused *STE3^{DAF}* cells to undergo partial arrest (Fig. 1). To quantify the level of G_1 arrest conferred by these mutations, *STE3^{DAF}* strains carrying each of the *STE4^{SD}* alleles were treated with pheromone and the percentage of unbudded cells was determined. After treatment with α-factor for 3 h, *STE3^{DAF}* cells carrying the *STE4^{SD1}* allele were 92% unbudded, whereas *STE3^{DAF}* cells carrying wild-type *STE4* were 60% unbudded (Table 2). The *STE4^{SD2}* allele conferred a modest increase in the percentage of unbudded cells, in agreement with the halo

TABLE 2. Level of G_1 arrest conferred by mutations in *STE3^{DAF}* cells and degree of supersensitivity conferred by mutations in wild-type cells

STE4 allele	Plasmid	Mutation(s) present	% Unbudded cells[a]	Halo size (mm)[b]
STE4	YCpSTE4		60 (56, 63)	39.0
STE4^{SD1}	YCpSD1	R162G, C182R, I195V	92 (91, 93)	**44.0**
STE4^{SD2}	YCpSD2	Q17L, Q21R, M283V	70 (68, 72)	**42.5**
STE4^{SD3}	YCpSD3	L132I, N155D, D270G	66 (66, 66)	**44.0**
STE4^{SD13}	YCpSD13	Q17L, Q21R, R162G	71 (67, 75)	40.5
STE4	YCpLSTE4		51 (47, 55)	35.5
STE4^{SD4}	YCpLSD4	R162G	43 (43, 44)	36.0
STE4^{SD5}	YCpLSD5	C182R	50 (52, 48)	**43.0**
STE4^{SD7}	YCpLSD7	Q17L	51 (50, 52)	37.0
STE4^{SD8}	YCpLSD8	Q21R	49 (46, 52)	35.5
STE4^{SD9}	YCpLSD9	R162G, I195V	62 (65, 59)	35.0
STE4^{SD10}	YCpLSD10	Q17L, R162G	65 (61, 69)	36.0
STE4^{SD11}	YCpLSD11	Q21R, R162G	61 (63, 59)	36.5
STE4^{SD12}	YCpLSD12	Q17L, Q21R	59 (62, 56)	37.0
STE4^{SD13}	YCpLSD13	Q17L, Q21R, R162G	71 (69, 72)	36.5
STE4^{SD14}	YCpLSD14	Q17L, Q21R, R162G, I195V	62 (60, 64)	37.5

[a] *MAT***a** *STE3^{DAF}* strains (AC17-2B) containing the indicated plasmids were treated with 0.1 μM α-factor for 3 h, and the percentage of unbudded cells was determined. The experiment was performed with two independent transformants, and the average of the two determinations is presented, followed by the values for each experiment in parentheses.

[b] Halo assays were performed on a single transformant each of a *MAT***a** strain (AC17-7B) containing the indicated plasmids using 5 μl of 1 mM α-factor. Boldfacing indicates supersensitive responses.

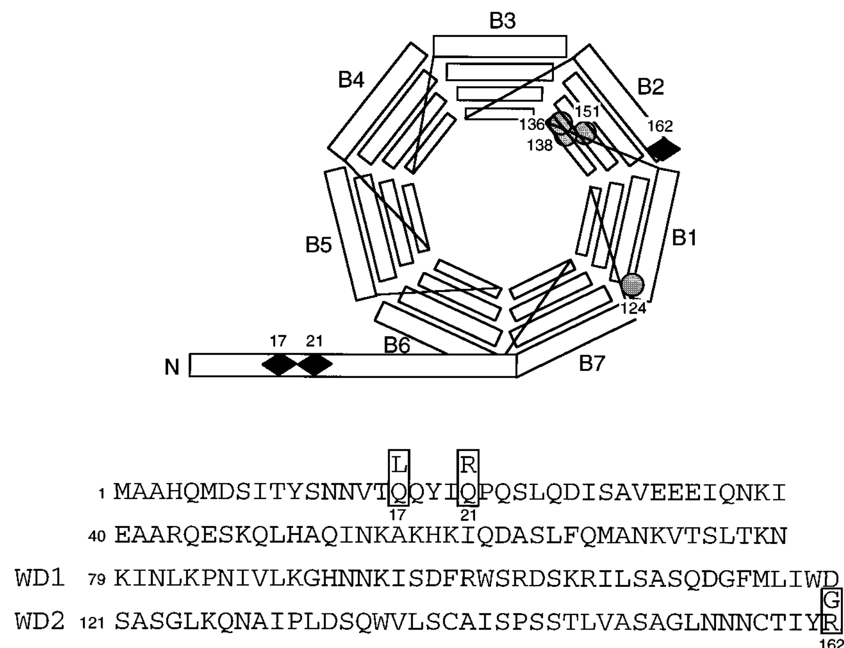

FIG. 2. Location of mutations on G_β structure. The diagram is a schematic drawing representing the complete Ste4p sequence of 423 amino acids as a seven-bladed β-propeller structure. The product of the *STE4^{SD13}* allele contains the Q17L and Q21R mutations encoded by *STE4^{SD2}* and the R162G mutation encoded by *STE4^{SD1}*, all of which are represented by filled diamonds in the diagram. Mutations that disrupt the interaction between the α- and β-subunits and result in constitutive signaling (36) are represented as grey circles. The amino acid sequence shows amino acids 1 to 162 of Ste4p.

assays; the *STE4^{SD3}* allele had little or no effect on cell cycle arrest in this assay.

Isolation of these suppressors demonstrates that the block to cell cycle arrest seen in *STE3^{DAF}* cells can be reversed by mutations in *STE4*. However, these mutations could alter residues of Ste4p that are unrelated to receptor inhibition. For example, if they confer an increase in the ability of Ste4p to transmit the pheromone response signal, then the increased signal might overcome the inhibition caused by expression of *STE3*. Further tests of the phenotype conferred by the *STE4^{SD}* alleles were therefore necessary to determine if they specifically affect the receptor inhibition function of *STE4*.

Mutations conferring supersensitivity enhance the *STE4^{SD}* phenotype. To determine whether the *STE4^{SD}* alleles cause increased signaling in wild-type cells, plasmids containing each of the three alleles were transformed into *MAT***a** Δ*ste4* cells that did not contain the *STE3^{DAF}* mutation. Cells carrying each of the *STE4^{SD}* alleles displayed increased sensitivity to α-factor, as shown by production of halos larger than those produced by cells containing a wild-type *STE4* plasmid (Table 2). Therefore, some component of the suppression of *STE3^{DAF}* by the *STE4^{SD}* mutations is probably due to an increase in the signaling capacity of the encoded Ste4p proteins.

The *STE4^{SD}* alleles were sequenced to identify the nucleotide changes that were responsible for the *STE4^{SD}* phenotype. Each allele harbored three nucleotide changes that resulted in three alterations in the coding sequence (Table 2). Because the *STE4^{SD3}* allele displayed the smallest degree of *STE3^{DAF}* suppression and conferred a supersensitive phenotype, it was not studied further. Mutations present in the *STE4^{SD1}* and *STE4^{SD2}* alleles were tested individually to assess their contribution to the *STE4^{SD}* phenotype. *STE3^{DAF}* cells containing *STE4^{SD}* alleles with single mutations did not display significant pheromone-induced cell cycle arrest, as demonstrated by the finding that only 43 to 51% of the cells were unbudded after α-factor treatment (Table 2). This percentage is similar to the fraction of G_1 cells in cycling populations. The C182R mutation (*STE4^{SD5}*) originally encoded by *STE4^{SD1}* was found to confer supersensitivity to α-factor in wild type cells but did not suppress the *STE3^{DAF}* phenotype. Therefore, it is likely that this mutation contributed to the phenotype of the *STE4^{SD1}* allele, although it is not specific to the receptor inhibition function of *STE4*.

To obtain a *STE4^{SD}* allele that conferred a high degree of *STE3^{DAF}* suppression but did not cause supersensitivity to α-factor in wild-type cells, *STE4* genes containing different combinations of *STE4^{SD}* mutations were tested in both *STE3^{DAF}* and wild-type cells. *STE4^{SD}* alleles encoding the R162G mutation and either the I195V, Q17L, or Q21R mutation conferred a partial cell cycle arrest response to *STE3^{DAF}* cells, resulting in 61 to 65% unbudded cells after α-factor treatment (Table 2). The greatest degree of arrest was seen in cells containing the *STE4^{SD13}* allele, which were 71% unbudded after α-factor treatment. The product of this allele contains the Q17L and Q21R mutations encoded by *STE4^{SD2}* and the R162G mutation encoded by *STE4^{SD1}*. The *STE4^{SD13}* allele did not confer a complete cell cycle arrest response on *STE3^{DAF}* cells, because wild-type cells become 90 to 97% unbudded under these conditions (17). However, this allele clearly suppressed the phenotype of *STE3^{DAF}* cells to a significant degree and did not cause supersensitivity in wild-type cells, suggesting that it does not encode a version of Ste4p that has an increased ability to transmit the pheromone response signal. The *STE4^{SD13}* allele was therefore chosen for further studies because of the high probability that it encodes a version of Ste4p that has a specific defect in receptor inhibition.

***STE4^{SD}*-encoded mutations map to the N terminus and second WD repeat.** The crystal structure of mammalian G protein β-subunits has revealed that their seven WD domains fold into a symmetric structure in the form of a seven-bladed β-propel-

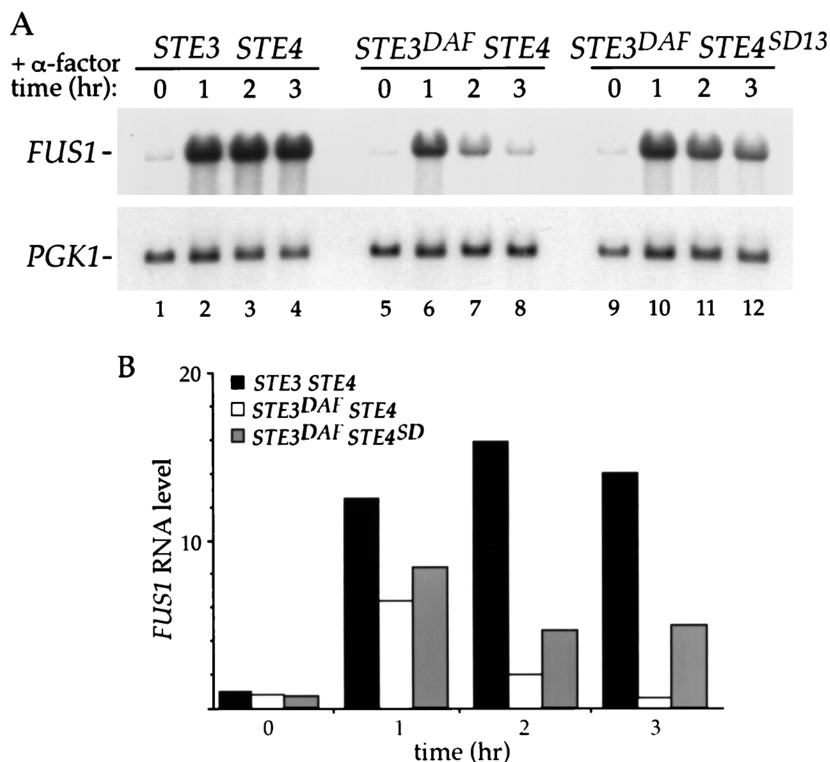

FIG. 3. Effect of $STE4^{SD13}$ on pheromone-induced transcription. (A) The following strains were treated with α-factor (0.1 μM) for the indicated periods of time: a *MAT***a** *STE3 ste4::HIS3* strain (AC17-7B) containing a wild-type *STE4* plasmid (YCpLSTE4) (lanes 1 to 4) and a *MAT***a** $STE3^{DAF}$ *ste4::HIS3* strain (AC17-2B) containing either a wild-type *STE4* plasmid (YCpLSTE4) (lanes 5 to 8) or a plasmid containing the $STE4^{SD13}$ allele (YCpLSD13) (lanes 9 to 12). RNA was isolated, transferred to nitrocellulose, and hybridized with a *FUS1* probe. The blot was rehybridized with *PGK1* to determine the amount of RNA per lane. (B) The data were quantified by PhosphorImager analysis, and the level of *FUS1* RNA was normalized to the control *PGK1* RNA level. Values from the *STE3 STE4* strain are represented by black bars; values from the $STE3^{DAF}$ *STE4* strain are represented by open bars; values from the $STE3^{DAF}$ $STE4^{SD13}$ strain are represented by grey bars. The graph shows the average values from duplicate experiments.

ler (31, 35). The yeast Ste4p β-subunit also has seven WD domains that are expected to form a similar structure. Mutations in *STE4* that suppress the $STE3^{DAF}$ phenotype were positioned on a diagram of a β-subunit that is based on the crystal structure (Fig. 2). The Q17L and Q21R mutations map to the extreme N terminus of Ste4p, which is composed of a 30-amino-acid extension that is present only in the yeast protein and thus is not represented in the crystal structure. The R162G mutation maps to the turn between the third and fourth strands of the β-sheet of the second blade, at the end of WD repeat 2. This residue is not in either of the two regions of the β-subunit that are in direct contact with the α-subunit (19, 35). The region of the β-subunit that makes the most extensive contacts with the α-subunit is the base of the β-propeller domain (Fig. 2, surface facing away from viewer). Mutations that alter amino acids on this surface, such as changes at residue 136, disrupt the interaction between the α- and β-subunits and result in constitutive signaling (36). The other region of contact between the two subunits is along the sides of the first and seventh blades of the β-propeller. A mutation that changes the amino acid at position 124, which is in the first blade, also disrupts the interaction between the α- and β-subunits and results in constitutive signaling (36). The finding that the changes encoded by the $STE4^{SD13}$ allele are not in the regions of the β-subunit that contact the α-subunit suggests that the altered residues could constitute part of a binding site for another protein that interacts with the β-subunit.

$STE4^{SD13}$ promotes sustained transcriptional activation in $STE3^{DAF}$ cells. Expression of *STE3* in *MAT***a** cells inhibits signaling during the late phase of the pheromone response (6). It was therefore of interest to determine whether the $STE4^{SD13}$ allele specifically affects the late phase of the response or whether it affects signaling at other times. To assess the duration of signaling in cells containing $STE4^{SD13}$, a time course of RNA accumulation from the pheromone-inducible *FUS1* gene was performed. The strains used in this experiment contain deletions of the *MFA1* and *MFA2* genes, which encode **a**-factor, to eliminate any contribution to signaling due to autocrine stimulation of the **a**-factor receptor in *MAT***a** cells.

Wild-type cells treated with α-factor exhibited an increase in *FUS1* RNA levels that remained high for 3 h (Fig. 3A, lanes 1 to 4). As observed previously (6), *FUS1* RNA levels in $STE3^{DAF}$ cells increased the first hour and then decreased gradually to basal level at 3 h (Fig. 3A, lanes 5 to 8). In $STE3^{DAF}$ cells containing $STE4^{SD13}$, the *FUS1* RNA level was higher at late time points than it was in $STE3^{DAF}$ cells containing wild-type *STE4* (Fig. 3A, lanes 8 and 12). Quantification of the results from duplicated experiments showed that there was an approximately sevenfold increase in *FUS1* RNA at the 3-h time point in a $STE3^{DAF}$ strain containing $STE4^{SD13}$ compared to the same strain containing wild-type *STE4* (Fig. 3B, 3 h). Thus, although the *FUS1* RNA level in $STE3^{DAF}$ $STE4^{SD13}$ cells was not equal to the induced level seen in wild-type cells, it was significantly higher than that seen in $STE3^{DAF}$ *STE4* cells. The $STE4^{SD13}$ allele did not affect the basal level of *FUS1* RNA in $STE3^{DAF}$ cells (Fig. 3B, 0 h), indicating that it does not cause constitutive activation of the pheromone response pathway. In addition, the $STE4^{SD13}$ allele

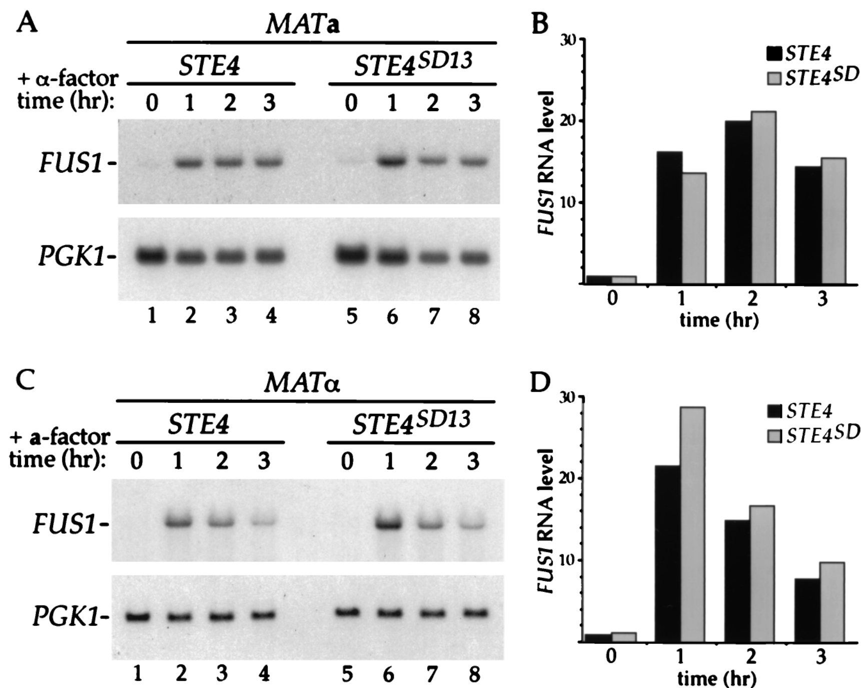

FIG. 4. Ability of $STE4^{SD13}$ to signal in wild-type *MAT***a** and *MAT*α cells. (A) A *MAT***a** *STE3 ste4::HIS3* strain (AC17-7B) containing either a wild-type *STE4* plasmid (YCpLSTE4) (lanes 1 to 4) or a plasmid containing the $STE4^{SD13}$ allele (YCpLSD13) (lanes 5 to 8) was treated with α-factor (0.1 μM) for the indicated periods of time, and RNA was isolated. RNA blots were prepared and hybridized as described in the legend to Fig. 3. (B) The data from the experiment shown in panel A were quantified by PhosphorImager analysis, and the level of *FUS1* RNA was normalized to the control *PGK1* RNA level. Values from the *STE4* strain are represented by black bars; values from the $STE4^{SD13}$ strain are represented by grey bars. (C) A *MAT*α *STE3 ste4::HIS3* strain (AC18-9C) containing either a wild-type *STE4* plasmid (YCpLSTE4) (lanes 1 to 4) or a plasmid containing the $STE4^{SD13}$ allele (YCpLSD13) (lanes 5 to 8) was treated with **a**-factor (40 ng/ml) for the indicated periods of time, and RNA was isolated. RNA blots were prepared and hybridized as described in the legend to Fig. 3. (D) The data from the experiment shown in panel C were quantified by PhosphorImager analysis, and the level of *FUS1* RNA was normalized to the control *PGK1* RNA level. Values from the *STE4* strain are represented by black bars; values from the $STE4^{SD13}$ strain are represented by grey bars.

did not have a significant effect at 1 h of pheromone treatment in $STE3^{DAF}$ cells (Fig. 3B, 1 h), indicating that it does not cause a supersensitive response to pheromone. These results demonstrate that the $STE4^{SD13}$ allele specifically suppresses the late inhibition of signaling caused by $STE3^{DAF}$ and does not alter other characteristics of the response.

$STE4^{SD13}$ has no effect in wild-type cells. If the $STE4^{SD}$ mutations only affect the receptor inhibition function of *STE4*, then they should have no effect in *MAT***a** cells that do not express the **a**-factor receptor. This idea was tested by expressing $STE4^{SD13}$ as the only copy of *STE4* in a wild-type strain and assaying its ability to respond to pheromone. Accumulation of *FUS1* RNA in cells treated with α-factor was very similar at all time points in *MAT***a** cells expressing either $STE4^{SD13}$ (Fig. 4A, lanes 5 to 8) or *STE4* (Fig. 4A, lanes 1 to 4). Quantification of these results showed that the levels of *FUS1* RNA differed by less than 20% in *MAT***a** cells expressing $STE4^{SD13}$ and *STE4*, even after 3 h of pheromone treatment (Fig. 4B). These experiments demonstrate that the $STE4^{SD13}$ allele is capable of transmitting the pheromone signal in a manner indistinguishable from that of wild-type *STE4* in *MAT***a** cells that do not express *STE3*.

The $STE4^{SD13}$ allele affects signaling in *MAT***a** cells that inappropriately express *STE3* but does not affect signaling in wild-type *MAT***a** cells. It was therefore of interest to determine the effect of $STE4^{SD13}$ in *MAT*α cells, which normally express *STE3*. *MAT*α cells expressing either $STE4^{SD13}$ or *STE4* were treated with **a**-factor for different lengths of time, and the level of *FUS1* RNA was determined. Basal *FUS1* RNA levels were very similar in *MAT*α cells expressing either *STE4* or $STE4^{SD13}$ (Fig. 4C, lanes 1 and 5). In *MAT*α *STE4* cells treated with **a**-factor, *FUS1* RNA was induced to a high level at 1 h of pheromone treatment and then gradually decreased for the next 2 h (Fig. 4C, lanes 1 to 4). A similar result was seen in *MAT*α $STE4^{SD13}$ cells (Fig. 4C, lanes 5 to 8). Quantification of these results showed that the levels of *FUS1* RNA differed by less than 35% in *MAT*α cells expressing $STE4^{SD13}$ and *STE4* (Fig. 4D). The decrease in signaling at late time points in wild-type cells may be due to degradation of **a**-factor by an extracellular protease (25). However, the observation that *FUS1* RNA levels are essentially the same in *MAT*α cells expressing either *STE4* or $STE4^{SD13}$ demonstrates that $STE4^{SD13}$ does not affect pheromone signaling in the presence of the **a**-factor receptor when it is expressed in the appropriate cell type.

The $STE4^{SD13}$ phenotype is independent of *GPA1* and is specific to *MAT*a cells. Deletion of *GPA1*, which encodes the G_α subunit that functions in the pheromone response pathway, causes constitutive signaling due to free βγ-subunits. Therefore, cells containing a Δ*gpa1* mutation undergo cell cycle arrest and induction of *FUS1* expression. The constitutive signaling seen in a *MAT***a** Δ*gpa1* strain is blocked by the $STE3^{DAF}$ allele, demonstrating that receptor inhibition is independent of *GPA1* (7, 14). This result supports the idea that inappropriate *STE3* expression inhibits a step that is downstream of α-subunit activation. If the $STE4^{SD13}$ allele produces a β-subunit that is only partially inhibited by expression of *STE3*, then the $STE4^{SD13}$ allele should increase signaling in a *MAT***a** Δ*gpa1* strain that expresses *STE3*. To test this idea, the level of *FUS1* RNA was determined in the absence of pheromone in a *MAT***a**

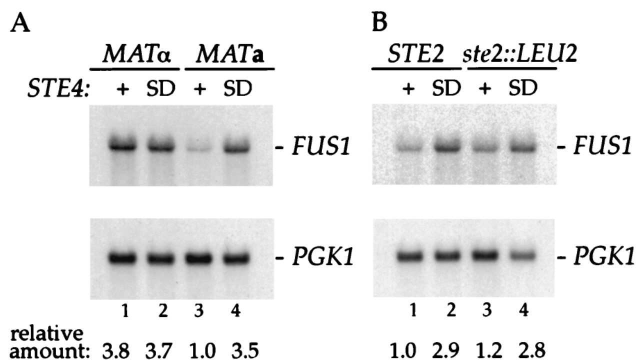

FIG. 5. Effect of $STE4^{SD13}$ in cells lacking the G_α and α-factor receptor genes. (A) RNA was isolated from the following strains: a *MAT*α $STE3^{DAF}$ *ste4::HIS3 gpa1::TRP1 far1::URA3* strain (K39-23D.f) containing either a wild-type *STE4* plasmid (YCpLSTE4) (lane 1) or a plasmid containing the $STE4^{SD13}$ allele (YCpLSD13) (lane 2), and a *MAT***a** $STE3^{DAF}$ ste4::HIS3 gpa1::TRP1 far1::URA3 strain (K39-23B.f) containing either a wild-type *STE4* plasmid (YCpLSTE4) (lane 3) or a plasmid containing the $STE4^{SD13}$ allele (YCpLSD13) (lane 4). (B) RNA was isolated from the following strains: a *MAT***a** $STE3^{DAF}$ *ste4::HIS3 gpa1::TRP1* strain (K39-23B) containing either a wild-type *STE4* plasmid (YCpSTE4) (lane 1) or a plasmid containing the $STE4^{SD13}$ allele (YCpSD13) (lane 2), and a *MAT***a** STE^{DAF} ste4::HIS3 gpa1::TRP1 ste2::LEU2 strain (K39-23B.s2) containing either a wild-type *STE4* plasmid (YCpSTE4) (lane 3) or a plasmid containing the $STE4^{SD13}$ allele (YCpSD13) (lane 4). RNA blots were prepared and hybridized as described in the legend to Fig. 3. The data were quantified by PhosphorImager analysis, and the level of *FUS1* RNA was normalized to the control *PGK1* RNA level. The relative level of *FUS1* RNA is shown below each lane.

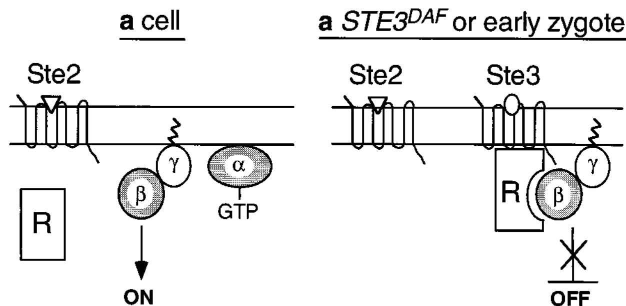

FIG. 6. Model for receptor inhibition. See text for details.

Δ*gpa1* $STE3^{DAF}$ strain expressing either $STE4^{SD13}$ or wild-type *STE4*. A *MAT***a** Δ*gpa1* $STE3^{DAF}$ strain does not undergo cell cycle arrest due to inhibition of signaling by the $STE3^{DAF}$ allele; however, to prevent the possibility of cell cycle arrest under conditions where $STE3^{DAF}$ is suppressed by $STE4^{SD13}$, this strain was constructed to contain a Δ*far1* mutation. The *FAR1* gene encodes a cyclin-dependent kinase inhibitor that is required for cell cycle arrest in response to pheromone (3, 27, 28).

$STE4^{SD13}$ caused an increase in the level of *FUS1* RNA of about 3.5-fold compared to wild-type *STE4* in a *MAT***a** Δ*gpa1* $STE3^{DAF}$ strain (Fig. 5A, lanes 3 and 4). This experiment rules out the possibility that the $STE4^{SD13}$ allele causes an increase in signaling due to decreased binding of its encoded β-subunit to the Gpa1p α-subunit. This cannot be the case because $STE4^{SD13}$ has an effect in the absence of Gpa1p. Expression of $STE4^{SD13}$ was not able to confer cell cycle arrest on a *MAT***a** Δ*gpa1* $STE3^{DAF}$ *FAR1* strain (17). This finding is consistent with results presented above indicating that suppression of $STE3^{DAF}$ by $STE4^{SD13}$ is not complete.

The increase in signaling conferred by $STE4^{SD13}$ in a Δ*gpa1* strain provides an opportunity to test whether the **a**-factor receptor can have an inhibitory effect in *MAT*α cells, where it is normally expressed. Because deletion of *GPA1* activates the pathway in a pheromone-independent manner, the pheromone receptors and their ligands are not required for generating the signal under these circumstances. Therefore, the negative role of *STE3* can be assayed in the absence of its positive role in signal generation. If expression of *STE3* causes equivalent inhibitory effects in *MAT***a** and *MAT*α cells, then the $STE4^{SD13}$ allele would be expected to increase signaling in Δ*gpa1* strains of both cell types. For this experiment, a *MAT*α strain that contains the $STE3^{DAF}$ allele was constructed to ensure that expression of *STE3* in this strain was comparable to its expression in a *MAT***a** $STE3^{DAF}$ strain. The strain was also constructed to contain a Δ*far1* mutation to prevent the possibility of constitutive cell cycle arrest. The *MAT*α Δ*gpa1* $STE3^{DAF}$ strain expressing wild-type *STE4* contained about 3.8-fold more *FUS1* RNA than the *MAT***a** Δ*gpa1* $STE3^{DAF}$ strain expressing wild-type *STE4* (Fig. 5A, lanes 1 and 3). This result suggests that $STE3^{DAF}$ inhibits signaling to a greater degree in *MAT***a** cells than it does in *MAT*α cells. Moreover, expression of either $STE4^{SD13}$ or wild-type *STE4* resulted in essentially identical levels of *FUS1* RNA in a *MAT*α Δ*gpa1* $STE3^{DAF}$ strain (Fig. 5A, lanes 1 and 2). Therefore, expression of *STE3* in a *MAT*α strain probably does not have an inhibitory effect on signaling, because all of the $STE3^{DAF}$ phenotypes in *MAT***a** cells are affected by $STE4^{SD13}$. One interpretation of these results is that there is a cell type-specific factor in *MAT***a** cells that is required for the inhibitory function of *STE3*. The absence of this factor in *MAT*α cells would explain why the normal expression of *STE3* in these cells does not cause receptor inhibition.

One difference between *MAT***a** and *MAT*α strains is that *MAT***a** strains express *STE2*, which encodes the α-factor receptor. It was therefore of interest to determine whether *STE2* is the cell type-specific gene that allows *STE3* to function as an inhibitor in *MAT***a** cells. To test this idea, *FUS1* expression was assayed in *MAT***a** Δ*gpa1* $STE3^{DAF}$ cells that contained either a *STE2* or a Δ*ste2* allele. Quantification of *FUS1* RNA isolated from these strains showed that expression of $STE4^{SD13}$ caused an approximately threefold increase in *FUS1* RNA abundance in both the *STE2* and Δ*ste2* strains (Fig. 5B, lanes 1 to 4). These results indicate that expression of the α-factor receptor has no effect on the inhibitory function of *STE3*. This finding is in agreement with a previous result showing that $STE3^{DAF}$ suppression of the cell cycle arrest phenotype of a Δ*gpa1* strain is unaffected by *STE2* expression (14). Therefore, a different cell type-specific gene is probably responsible for the receptor inhibition function of *STE3*.

DISCUSSION

The $STE4^{SD}$ mutations were isolated based on their ability to restore pheromone-induced cell cycle arrest in *MAT***a** cells expressing *STE3*. The following evidence confirms the idea that the $STE4^{SD13}$ allele is specific for the receptor inhibition function of *STE4*. First, expression of $STE3^{DAF}$ in *MAT***a** cells inhibits signaling only at late times during the response, and $STE4^{SD13}$ causes an increase in *FUS1* RNA levels only at late times after pheromone treatment in *MAT***a** $STE3^{DAF}$ cells. Second, $STE4^{SD13}$ does not cause an increase in either the basal or induced level of *FUS1* RNA in wild-type *MAT***a** or *MAT*α cells. Third, the phenotype conferred by $STE4^{SD13}$ is independent of *GPA1*, the G_α subunit gene. And, fourth, the effect of $STE4^{SD13}$ is cell type specific. These findings rule out

the possibility that the Ste4p protein encoded by $STE4^{SD13}$ increases signaling by a mechanism that is unrelated to receptor inhibition, such as that it has an increased affinity for a downstream activator of the signaling pathway. Isolation of an allele of *STE4* that is specific for suppression of receptor inhibition confirms that this phenomenon is an active cellular process that plays a physiological role in some aspect of the yeast life cycle.

G protein βγ-subunits have been shown to interact with a wide variety of other proteins (4). Structural and functional studies of these interactions demonstrate that the binding surfaces for α-subunits and downstream effectors are partially overlapping, suggesting that these interactions are mutually exclusive. The Ste4p β-subunit has been shown to interact with the Gpa1 α-subunit (36) and with the potential downstream effectors Ste20p (21) and Ste5p (15, 37). The binding surfaces on Ste4p that mediate these interactions are probably not altered by the mutations present in $STE4^{SD13}$ because the protein encoded by this allele behaves identically to wild-type Ste4p when it is expressed in wild-type cells. It is therefore likely that the mutations present in $STE4^{SD13}$ identify a binding surface for a novel β-subunit binding partner. Two of the mutations in $STE4^{SD13}$ change residues in the N-terminal extension of Ste4p that is not present in the mammalian β-subunits that have been crystalized; the other mutation changes an amino acid that faces outward at the turn between the third and fourth strands of the second blade of the propeller. Because the structure of the Ste4p N terminus is unknown, the two regions of Ste4p identified by the $STE4^{SD13}$ mutations could be quite close together, thus forming a unique binding surface for a protein that has not yet been identified.

STE4 alleles that confer prolonged signaling resulting in a defect in adaptation to pheromone have been isolated by Li et al. (23). These alleles contain mutations in the first, second, and seventh WD repeats of Ste4p. The mutations present in the *STE4* Adp^- alleles have been proposed to identify a target site for a negative regulator other than the Gpa1p α-subunit. The *STE4* Adp^- alleles, like the $STE4^{SD}$ alleles described here, are thought to confer an increase in signaling at late times during the pheromone response. However, it appears that the *STE4* Adp^- and $STE4^{SD}$ alleles do not affect the same process, for the following reasons. First, whereas the effects of *STE4* Adp^- alleles are observed in wild-type *MAT***a** cells, the effects of the $STE4^{SD13}$ allele are only observed in *MAT***a** cells containing a $STE3^{DAF}$ mutation. Second, whereas all of the *STE4* Adp^- alleles confer an increase in the basal and induced levels of signaling, resulting in supersensitivity to pheromone, the $STE4^{SD13}$ allele confers normal basal and induced levels of signaling in wild-type cells. And finally, the effects of *STE4* Adp^- alleles are dependent on a process initiated by Gpa1p, but the effects of the $STE4^{SD13}$ allele are independent of Gpa1p function.

Some evidence suggests that β-subunits have the potential to bind directly to their associated receptors in the absence of an α-subunit. For example, fluorescence energy transfer experiments have demonstrated potential interactions between βγ-subunits and the β-adrenergic receptor (12) and between βγ-subunits and rhodopsin (29). In addition, one study has demonstrated direct photoaffinity labeling of a β-subunit by a peptide derived from the third cytoplasmic loop of the α-adrenergic receptor (34). Direct binding of the Ste4p β-subunit to the **a**-factor receptor may play a role in receptor inhibition; however, a model in which inhibition of signaling is caused by binding of Ste4p to Ste3p does not account for the observation that Ste3p only inhibits signaling in *MAT***a** cells. One explanation for the cell type specificity of receptor inhibition is that a component required for this process is expressed only in *MAT***a** cells, as shown in the model presented in Fig. 6 and described below.

The results presented here are consistent with a model in which the activity of the βγ-subunit complex is inhibited by a *MAT***a** cell type-specific regulatory protein (factor R) that binds to or is activated by Ste3p, the **a**-factor receptor (Fig. 6). The observation that $STE3^{DAF}$ only affects signaling at late times after pheromone treatment could be due to the fact that factor R is induced by pheromone. Thus, factor R would not be present before pheromone treatment but would gradually increase in abundance during the time course of the response. In a wild-type *MAT***a** cell, the presence of factor R would have no effect because Ste3p is not expressed (Fig. 6, **a** cell). In a *MAT***a** $STE3^{DAF}$ cell, detection of Ste3p by factor R would produce a change in factor R that would cause it (or another factor activated by it) to block signaling by the βγ-subunits (Fig. 6, **a** $STE3^{DAF}$). In the normal life cycle, factor R and Ste3p would be present in the same cell immediately after the fusion of **a** and α haploid cells that are in the process of mating (Fig. 6, early zygote) because factor R is an **a**-specific gene product and Ste3p is an α-specific gene product. Detection of Ste3p by factor R in early zygotes would result in inhibition of pheromone signaling by the same mechanism as that seen in *MAT***a** $STE3^{DAF}$ cells. Factor R could function by directly binding to the βγ complex and preventing the activation of downstream effectors or it could affect βγ complex activity by an indirect mechanism, such as altering its association with the plasma membrane. In this model, the effect of the $STE4^{SD}$ mutations would be to reduce the affinity of Ste4p for factor R, causing less Ste4p to interact with factor R. Thus, more Ste4p would be available to activate the pheromone response pathway, resulting in a higher signal. Preliminary studies on a newly identified **a**-specific gene suggest that its product is a good candidate for factor R (18).

The putative regulatory factor is expected to be specific to *MAT***a** cells because inhibition of signaling by *STE3* occurs in a *MAT***a** Δ*gpa1* strain but does not occur in a *MAT*α Δ*gpa1* strain. However, factor R cannot be the α-factor receptor, which is specific to *MAT***a** cells, because a null allele of *STE2* does not affect inhibition of signaling by *STE3*. Previous studies by Bender and Sprague support the concept of a novel inhibitor of mating that is active in *MAT***a** cells that express *STE3* (1). These studies showed that expression of a given combination of receptor and pheromone had different effects depending on the *MAT* allele of the cell in which they were expressed. The experiment was performed by expressing *STE3* and *MF*α*1* from mating type-independent promoters in either *mat*α*1* cells or *MAT***a** *ste2 ste6* cells. Both of these strains are designed to express only α-factor and the **a**-factor receptor (in the *MAT***a** strain, the *ste2* mutation eliminates expression of the α-factor receptor and the *ste6* mutation prevents secretion of **a**-factor). However, the *mat*α*1* strain mated with 10-fold greater efficiency than the *MAT***a** strain did. The only difference between the two strains is that **a**-specific genes are not expressed in *mat*α*1* cells due to the presence of the Matα2p inhibitor. Therefore, this result supports the existence of an **a**-specific component that inhibits mating when *STE3* is expressed. The **a**-specific component cannot be Ste2p and therefore is likely to be our proposed factor R.

*MAT***a** cells do not express *STE3* at any time during their normal life cycle, so the function of this putative regulatory factor is probably not relevant to vegetative haploid growth. However, a potential physiological function of this regulatory factor is to inhibit signaling in mating cells that have recently undergone cell fusion. Fusion of cells would allow factor R to

come into contact with Ste3p, which would block the signaling function of Ste4p. This process may function to promote recovery from mating and allow cell cycle progression to resume. Our attempts to demonstrate an effect of the *STE4*^{SD13} allele on recovery from mating have been hampered by an inability to obtain cultures that undergo synchronous mating. It is difficult to observe short-term effects in unsynchronized mating mixtures because recovery from mating is aided by the long-term process of transcriptional inhibition of haploid-specific genes by the Mat**a**1p/Matα2p complex. Further experiments in which cell fusion and recovery can be precisely controlled will allow definitive testing of the model of receptor inhibition.

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