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Localization and Signaling of G_β Subunit Ste4p Are Controlled by **a**-Factor Receptor and the **a**-Specific Protein Asg7p

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Haploid yeast cells initiate pheromone signaling upon the binding of pheromone to its receptor and activation of the coupled G protein. A regulatory process termed receptor inhibition blocks pheromone signaling when the a-factor receptor is inappropriately expressed in *MAT*a cells. Receptor inhibition blocks signaling by inhibiting the activity of the G protein β subunit, Ste4p. To investigate how Ste4p activity is inhibited, its subcellular location was examined. In wild-type cells, α-factor treatment resulted in localization of Ste4p to the plasma membrane of mating projections. In cells expressing the a-factor receptor, α-factor treatment resulted in localization of Ste4p away from the plasma membrane to an internal compartment. An altered version of Ste4p that is largely insensitive to receptor inhibition retained its association with the membrane in cells expressing the a-factor receptor. The inhibitory function of the a-factor receptor required *ASG7*, an a-specific gene of previously unknown function. *ASG7* RNA was induced by pheromone, consistent with increased inhibition as the pheromone response progresses. The a-factor receptor inhibited signaling in its liganded state, demonstrating that the receptor can block the signal that it initiates. *ASG7* was required for the altered localization of Ste4p that occurs during receptor inhibition, and the subcellular location of Asg7p was consistent with its having a direct effect on Ste4p localization. These results demonstrate that Asg7p mediates a regulatory process that blocks signaling from a G protein β subunit and causes its relocalization within the cell.

The pheromone response of the yeast *Saccharomyces cerevisiae* is initiated by the binding of a peptide pheromone to its specific receptor on a responding cell. Haploid yeast cells containing the *MAT***a** allele secrete **a**-factor and express receptors for α-factor; cells containing the *MAT*α allele secrete α-factor and express receptors for **a**-factor (19, 33, 36). The **a**- and α-factor receptors are G protein-coupled receptors, and they activate a heterotrimeric G protein that is common to both cell types. Activation of the G protein by occupied receptors results in guanine nucleotide exchange on the G protein α subunit, which causes the β and γ subunits to be released from the α subunit. The free βγ complex interacts with downstream components of the pathway, resulting in activation of a mitogen-activated protein (MAP) kinase cascade. Signaling through this pathway produces a number of changes in cellular physiology, including arrest in the G_1 phase of the cell cycle, induction of gene expression, redistribution of cell surface proteins, and formation of cellular projections.

In addition to the classical G protein activation process described above, the pheromone response pathway is subject to a process that inhibits G protein signaling. This process, termed receptor inhibition, was uncovered by a mutation that causes the **a**-factor receptor to be inappropriately expressed in *MAT***a** cells. The $STE3^{DAF}$ mutation was isolated in a screen for dominant mutations that conferred resistance to pheromone-induced cell cycle arrest (4). $STE3^{DAF}$, which is an allele of the **a**-factor receptor gene *STE3*, contains a rearranged 5′ regulatory region that causes it to be expressed in *MAT***a** cells (13). Expression of *STE3* in *MAT***a** cells confers resistance to pheromone-induced cell cycle arrest by blocking signaling through the pheromone response pathway. Although *MAT***a** haploid cells do not normally express the α-specific gene *STE3*, **a**- and α-specific gene products do come into contact with each other immediately after the fusion of a *MAT***a** cell and a *MAT*α cell during the process of mating. Therefore, a potential physiological function of receptor inhibition is to inhibit signaling in mating cells that have recently undergone cell fusion. This process may function to promote recovery from mating and allow cell cycle progression to resume.

Cells that contain a $STE3^{DAF}$ allele display a characteristic pattern of signaling. In $STE3^{DAF}$ cells treated with pheromone, activation of the MAP kinase is normal early in the response but is gradually inhibited at later time points (3). This pattern suggests that prior activation of the signaling pathway must occur before the inhibitory process can function. Another characteristic of receptor inhibition is that it is independent of the G protein α subunit (4, 13). In the pheromone response pathway, the α subunit plays a negative role by sequestering the βγ complex and keeping it inactive. Deletion of *GPA1*, the G protein α subunit gene, causes constitutive signaling due to release of the βγ complex. The constitutive signaling phenotype of cells with a *GPA1* deletion is suppressed by the $STE3^{DAF}$ mutation, indicating that receptor inhibition of sig-

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naling can occur in the absence of Gpa1p. This result demonstrates that the target of receptor inhibition is a signaling component that is downstream of the G protein α subunit.

Two types of evidence suggest that the G protein β subunit, Ste4p, is the signaling component that is targeted by receptor inhibition. Initial results supporting this idea involve the phenotypes of double mutants that carry both $STE3^{DAF}$ and a constitutive signaling mutation. The $STE3^{DAF}$ allele blocks signaling in cells that contain constitutive or overexpression alleles of *STE4* (3, 13). However, $STE3^{DAF}$ does not block signaling in cells that overexpress *STE20* or that contain a constitutive allele of *STE5* (3). *STE20* encodes a kinase that activates the MAP kinase cascade, and *STE5* encodes a scaffolding protein for the MAP kinase cascade, so both of these signaling components act downstream of Ste4p. These studies are therefore consistent with the idea that receptor inhibition acts at the level of Ste4p, the G protein β subunit. This conclusion was further supported by the isolation of altered versions of Ste4p that can signal normally but that are insensitive to receptor inhibition (17). The altered residues in Ste4p are not in the regions that contact the α subunit, suggesting that they constitute part of a binding site for another protein that interacts with Ste4p. Such a protein could be a negative regulator that prevents Ste4p from interacting with its downstream targets. One potential explanation for the phenotype of the $STE3^{DAF}$ allele is that a negative regulator of Ste4p is expressed only in *MAT***a** cells.

Receptor inhibition appears to require an **a**-specific component based on the following observation. Deletion of *GPA1* causes constitutive signaling in both *MAT***a** and *MAT*α cells due to release of the βγ complex. Expression of *STE3* inhibits the constitutive signaling conferred by a *GPA1* deletion in *MAT***a** cells but has no effect in *MAT*α cells (17). One important difference between *MAT***a** and *MAT*α cells is that Ste2p, the α-factor receptor, is expressed only in *MAT***a** cells. However, receptor inhibition does not require the presence of Ste2p because the ability of $STE3^{DAF}$ to block signaling in *MAT***a** *gpa1*Δ cells is unaffected by a *ste2*Δ mutation (17). These findings indicate that an **a**-specific component other than Ste2p is required for inhibition of β-subunit activity by the **a**-factor receptor.

Here we investigate further the process by which Ste4p signaling is blocked by receptor inhibition. Examination of the subcellular location of Ste4p indicates that it undergoes altered localization during receptor inhibition. In addition, we show that altered localization of Ste4p and inhibition of signaling require an **a**-specific gene called *ASG7*.

MATERIALS AND METHODS

Plasmid construction. Plasmid YCpΔ36 was constructed by replacing the 392-bp *Hpa*I-*Xho*I fragment in plasmid YCpSTE4 (17) with a 284-bp *Hpa*I-*Xho*I fragment from a plasmid containing the *STE4* gene in which the 108 bp encoding amino acids 310 to 346 of Ste4p had been deleted by site-directed mutagenesis.

The fusion of *STE4* with the gene encoding green fluorescent protein (GFP) was constructed as follows. *STE4* was amplified by PCR using oligodeoxynucleotides 5′-CCACTAGT**GCATGC**ATGGCAGCACATCAGATG-3′ and 5′-AGGAGCTCCTA**CCCGGG**TTGATAACCTGGAGAC-3′ (the newly created *Spe*I and *Sac*I sites are underlined; the newly created *Sph*I and *Sma*I sites are in boldface) as primers and pL38 (18) as the template and cloned into the *Spe*I and *Sac*I sites of pRS316-GAL (20). The GFP^{S65T} mutant (11) was amplified by PCR using oligodeoxynucleotides 5′-CGGGATCC**GCTAGC**ATGAGTAAAGGAGAAGAAC-3′ and 5′-GCTCTAGATT**AGCATGC**ACTAGTTTTGTATAGT-3′ (the newly created *Bam*HI and *Xba*I sites are underlined; the newly created *Nhe*I and *Sph*I sites are in boldface) as primers and pRSET-B-GFP^{S65T} (obtained from R. Tsien) as the template and cloned into the *Xba*I and *Bam*HI sites of pRS316-GAL to yield plasmid pBTL32. Plasmid pBTL34 carrying *STE4* fused to the carboxyl terminus of GFP^{S65T} was created by cloning the *Sph*I-to-*Sac*I fragment of pBTL29 into pBTL32. The promoter region of *STE4* was amplified by PCR using oligodeoxynucleotides 5′-CGGAATTCAATGTTTCAGGAAGAGAT-3′ and 5′-GCGGATCCCGTAATGTGTACCTGATT-3′ (the newly created *Eco*RI and *Bam*HI sites are underlined) as primers and pL38 as the template and cloned into the *Eco*RI and *Bam*HI sites of pRS313, creating plasmid pBTL42. Plasmid pBTL60 carrying a fusion of *STE4* with the carboxyl terminus of GFP^{S65T} under the control of the *STE4* promoter was then constructed by cloning the *Bam*HI-to-*Sac*I fragment from plasmid pBTL34 into plasmid pBTL42, and pBTL49 was constructed by transferring the promoter-*GFP* fusion construct contained within the *Pvu*I fragment to plasmid pRS316. Plasmid YCpGFP-SD10 was constructed by replacing the 2.1-kb *Bam*HI-*Sac*I fragment in pBTL49, which contains *STE4*, with a 2.1-kb *Bam*HI-*Sac*I fragment that contains the $STE4^{SD10}$ allele (17).

The fragment used for construction of *asg7*::*URA3* null alleles was synthesized by two-step PCR (35). In the first step, oligonucleotides W1517 (5′-CCGCATTAGTGGGCTATCAGTAGCAC) and W1519 (5′-TATCAGTTATTACCCTATGCGGTGTGCCAAGGGTTCTCATCGTTCTCGAGGGCGC) were used as the primers and yeast genomic DNA was used as the template to generate a 427-bp fragment in which the first 401-bp region is homologous to the 5′ untranslated region of *ASG7* and the last 26-bp region is homologous to the 5′ region of the *URA3* gene. Oligonucleotides W1520 (5′-CCTTCTGTTCGGAGATTACCGAATCAGTAGATCTAAAGACAGAAAATGATATCAGCC) and W1518 (5′-CGACTGAGGTCCACTGGCAGCGACTG) were used as the primers to generate a 353-bp fragment in which the first 26 bp is homologous to the 3′ region of the *URA3* gene and the last 327 bp is homologous to the 3′ untranslated region of the *ASG7* gene. In the second step, the 427- and 353-bp fragments from the first PCRs served as the primers and plasmid pRS426, which contains the *URA3* gene, was used as the template in the PCR. The product of this reaction was used to transform yeast strains.

A plasmid containing the wild-type *ASG7* gene was isolated by complementation of the *asg7*::*URA3* mutation. DNA from yeast genomic library 2J351 (8) was transformed into a *MAT***a** $STE3^{DAF}$ *asg7*::*URA3* strain (H67-6C.a7), and transformants were screened for their ability to grow in the presence of α-factor. Plasmid pASG7-351.1, which contains the *ASG7* gene, conferred resistance to α-factor-induced cell cycle arrest. Plasmid pASG7-351.2 was constructed by subcloning the 1.6-kb *Hin*dIII-*Bgl*II fragment from pASG7-351.1 into the *Hin*dIII-*Bam*HI sites of vector YEp351. The *ASG7-GFP* fusion gene was constructed using a 1.8-kb *Hin*dIII fragment of genomic DNA from pASG7-351.1 that contains *ASG7*. A 0.7-kb fragment containing *GFP* flanked by *Not*I sites was subcloned into a *Not*I site that was inserted immediately before the stop codon in *ASG7* by site-directed mutagenesis. The 2.5-kb *Hin*dIII fragment containing the *ASG7-GFP* fusion gene was subcloned into the *Hin*dIII site of YCplac111 (10) to create plasmid pASG7-111.GFP.

Strains and media. Strains used in this study are listed in Table 1. The *asg7*::*URA3* null allele was made by transformation of strains with a 1.8-kb fragment generated by two-step PCR, as described above. The *gpa1*::*TRP1* allele was created by transformation of a strain containing the *gpa1*::*URA3* allele (7) with a 3.2-kb *Eco*RI-*Xho*I fragment from plasmid pTU10 (5). The *far1*::*LEU2* allele was created by transformation of a strain containing the *far1*::*URA3* allele (3) with a 4.6-kb *Sma*I fragment from plasmid pUL9 (5). The *asg7*::*HIS3* allele was created by transformation of a strain containing the *asg7*::*URA3* allele with a 3.6-kb *Xba*I fragment from plasmid pUH7 (5).

Strains were grown on yeast extract-peptone-dextrose (2% glucose) or yeast extract-peptone–Gal (3% galactose), and strains under selection were grown on synthetic dropout media, as described previously (32).

Yeast methods. Yeast transformations were performed by the lithium acetate method (15) modified as described previously (13). Yeast RNA was extracted from cells as described previously (6).

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

Northern blots. Cells were treated with 0.1 μM α-factor (Sigma), 60 nM **a**-factor, or 300 nM **a**-factor (generously provided by Fred Naider) for various periods of time, and RNA was isolated. RNA was transferred to a nitrocellulose membrane after formaldehyde-agarose gel electrophoresis as described previously (31). The membranes were UV cross-linked 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 using a Prime-It kit (Stratagene). The fragments used were *FUS1*, a 1.4-kb *Eco*RI-*Hin*dIII fragment from plasmid pSL589 (25), phosphoglycerate kinase gene *PGK1*, a 0.5-kb *Bam*HI-*Xba*I fragment from pPGK1, and *ASG7*, a 0.75-kb *Hpa*I-*Sac*I fragment from pASG7-351.2.

Immunoblots. For immunoblots, cells were treated with 0.1 μM α-factor (Sigma) for various periods of time, and 10-ml aliquots of cells were pelleted and washed once with 10 mM Tris-HCl (pH 7.8)–1 mM EDTA. The washed cells were resuspended in 350 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, and 1 μg each of leupeptin, aprotinin, chymostatin, and pepstatin/ml). The cell suspension was lysed by adding approximately 0.25 ml of acid-washed glass beads (0.5 mm; Biospec Products) and vortexing at high speed for 10 min at 4°C. The lysate was cleared by centrifuging for 2 min at 4°C. The protein concentrations of the samples were determined using a bicinchoninic protein assay kit (Pierce), and

TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Reference or source
W3031A	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
W3031B	*MAT*α	R. Rothstein
W3031A.Ba	*MAT***a** *sst1*::*hisG*	This study
H67-9D.Ba	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 sst1*::*hisG*	3
H67-9D.a7	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 sst1*::*hisG asg7*::*URA3*	This study
H67-6C.Ba	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 STE3*$^{DAF2.5}$ *sst1*::*hisG*	3
H67-6C.a7	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 STE3*$^{DAF2.5}$ *sst1*::*hisG asg7*::*URA3*	This study
AC17-7B	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 sst1*::*hisG ste4*::*HIS3*	17
AC17-2B	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 STE3*$^{DAF2.5}$ *sst1*::*hisG ste4*::*HIS3*	17
AC17-2B.aL	*MAT***a** *mfa1-Δ3*::*HIS3 mfa2-Δ2*::*HIS3 STE3*$^{DAF2.5}$ *sst1*::*hisG ste4*::*HIS3 asg7*::*LEU2*	This study
H125-7D	*MAT***a** *gpa1*::*TRP1 STE3*$^{DAF2.5}$	This study
H125-7D.aH	*MAT***a** *gpa1*::*TRP1 STE3*$^{DAF2.5}$ *asg7*::*HIS3*	This study

[a] All strains other than W3031A are isogenic to W3031A.

equal amounts of protein were loaded onto SDS-polyacrylamide gels (10% acrylamide). Separated proteins were transferred to nitrocellulose, and the blot was probed with anti-Ste4p rabbit antiserum (14) at a dilution of 1:1,000. Donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham) was used at a dilution of 1:10,000, and immune complexes were detected with an enhanced chemiluminescence kit (Amersham).

Microscopy. For fluorescence microscopy, cells containing GFP-Ste4p or Asg7p-GFP fusion proteins were observed on a Zeiss Axioskop microscope with a ×100 (1.3-numerical-aperture) objective and a fluorescein isothiocyanate (FITC) filter set (Chroma Technology). Digital images were captured with a Photometrics SenSys 1400-C1.cCCD camera using IPLab Spectrum image acquisition software (Scanalytics).

RESULTS

Expression of the **a**-factor receptor in *MAT***a** cells inhibits signal transduction through the pheromone response pathway by a process called receptor inhibition. In cells undergoing receptor inhibition, the signaling pathway is blocked at the level of Ste4p, the G protein β subunit (3, 17). To investigate the mechanism responsible for the signaling block, the effect of receptor inhibition on properties of Ste4p that are required for signaling was examined.

Expression of the a-factor receptor in a cells affects Ste4p localization. Previous studies have shown that association of the βγ complex with the plasma membrane is required for activation of the signaling pathway (29). Therefore, one mechanism to account for signaling inhibition in the presence of the **a**-factor receptor is that the Ste4p β subunit is not properly localized to the plasma membrane. To investigate this possibility, the localization pattern of a GFP-Ste4p fusion protein was observed in wild-type *MAT***a** cells and *STE3*^{DAF} *MAT***a** cells that were treated with pheromone for various amounts of time. *STE3* encodes the **a**-factor receptor, and the *STE3*^{DAF} allele causes expression of *STE3* in *MAT***a** cells due to an insertion in its promoter region (13). To eliminate any effects of autocrine signaling through the **a**-factor receptor, both the wild-type and *STE3*^{DAF} strains used in these experiments have deletions of the genes encoding **a**-factor (13).

Both wild-type and *STE3*^{DAF} untreated cells displayed a signal that appeared to be partially localized at the membrane and partially localized to an internal compartment (Fig. 1). This result is in agreement with cell fractionation studies done by others, which have shown that Ste4p partitions 40% with the plasma membrane, 30% with internal membranes, and 30% with nonmembrane fractions (14). After 1 h of α-factor treatment, GFP-Ste4p was concentrated at the sites of incipient mating projection formation in both strains. At this time point, the similar localization patterns of GFP-Ste4p in the two strains are expected, given the previous finding that *STE3*^{DAF} cells undergo a detectable level of signaling after 1 h of pheromone treatment (3). After 2 h of α-factor treatment of wild-type cells, GFP-Ste4p was localized predominantly at the membrane in regions where mating projections had formed (Fig. 1, 2 h). In contrast, *STE3*^{DAF} cells treated with α-factor for 2 h showed a dramatic reduction in the amount of GFP-Ste4p that was membrane associated and an increase in the amount that localized in an internal particulate pattern. This effect was observed even in cells that still contained mating projections. After 3 h of α-factor treatment, GFP-Ste4p remained at the sites of mating projections in wild-type cells but was predominantly localized to an internal compartment in *STE3*^{DAF} cells. At this time point, the *STE3*^{DAF} cells had recovered from cell cycle arrest and displayed a high percentage of budded cells. Significant inhibition of signaling occurs in *STE3*^{DAF} cells at about 2 h after pheromone treatment (3). Thus, localization of GFP-Ste4p away from the membrane correlates with the period of signaling inhibition.

Expression of the a-factor receptor in a cells does not affect Ste4p abundance. In cells undergoing receptor inhibition, the localization pattern of Ste4p resembled that of an endocytic compartment (Fig. 1, *STE3*^{DAF}, 2 h). If receptor inhibition causes Ste4p to undergo endocytosis, it is possible that the level of Ste4p would decrease under these conditions. To determine the effect of receptor inhibition on Ste4p abundance, the levels of Ste4p in wild-type *MAT***a** cells and *STE3*^{DAF} *MAT***a** cells were investigated.

Cell extracts were prepared from wild-type and *STE3*^{DAF} strains that had been treated with α-factor for various amounts of time, and immunoblots prepared from these extracts were probed with a polyclonal anti-Ste4p antibody (14). In wild-type cells treated with α-factor for 1 to 4 h, Ste4p displayed a mobility shift characteristic of the phosphorylated form (Fig. 2A, lanes 1 to 4), as described previously (2). In *STE3*^{DAF} cells, Ste4p was present in both the unmodified and phosphorylated forms after 1 h of exposure to α-factor and the unmodified form increased in abundance at later time points (Fig. 2A, lanes 5 to 8). These results are consistent with previous observations showing that *STE3*^{DAF} cells undergo an initial response to pheromone that is gradually inhibited at later times after pheromone treatment (3). There did not appear to be a significant difference between the abundance of Ste4p in wild-type cells and that in *STE3*^{DAF} cells at the 2- and 4-h time points, when signaling is inhibited in *STE3*^{DAF} cells. However, the presence of multiple forms of Ste4p made it difficult to compare its abundances in the two different strains. To eliminate this complication, the effect of the *STE3*^{DAF} allele on Ste4p abundance was investigated in a strain containing a form of Ste4p that does not undergo phosphorylation in response to

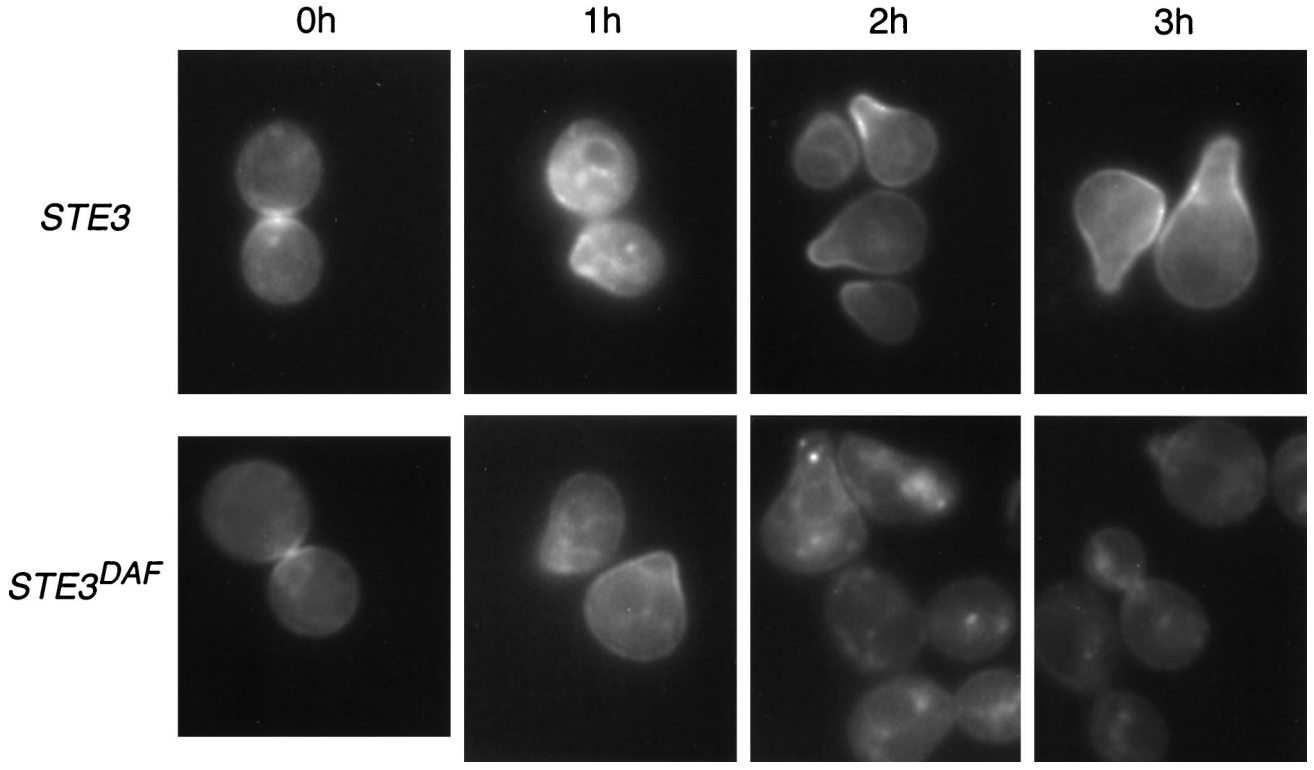

FIG. 1. Localization of GFP-Ste4p in wild-type and *STE3*^{DAF} cells. The following strains were treated with α-factor (0.1 μM) for the indicated periods of time: AC17-7B, a *MAT***a** *STE3 ste4*::*HIS3* strain (*STE3*), and AC17-2B, a *MAT***a** *STE3*^{DAF} *ste4*::*HIS3* strain (*STE3*^{DAF}). Both strains contained the low-copy-number *GFP-STE4* plasmid BTL49. The live cells were viewed by fluorescence microscopy using an FITC filter set.

pheromone (2). This form of Ste4p, which lacks residues 310 to 346, is fully capable of signal transmission because phosphorylation is not required for the Ste4p signaling function (22). Moreover, the signaling activity of Ste4p$^{\Delta 310–346}$ is capable of being inhibited by expression of *STE3* in *MAT***a** cells, indicating that phosphorylation does not play a role in receptor inhibition (J. Kim and J. P. Hirsch, unpublished data). Immunoblots of samples from wild-type and *STE3*^{DAF} strains were probed with anti-Ste4p antibody to detect the level of Ste4p$^{\Delta 310–346}$ at various times after pheromone treatment. At all time points, wild-type cells contained slightly higher levels of Ste4p$^{\Delta 310–346}$ than *STE3*^{DAF} cells (Fig. 2B, lanes 1 to 8). However, the level of Ste4p$^{\Delta 310–346}$ did not change significantly in *STE3*^{DAF} cells after treatment with pheromone. These results demonstrate that receptor inhibition does not cause a major change in the steady-state level of the Ste4p β subunit.

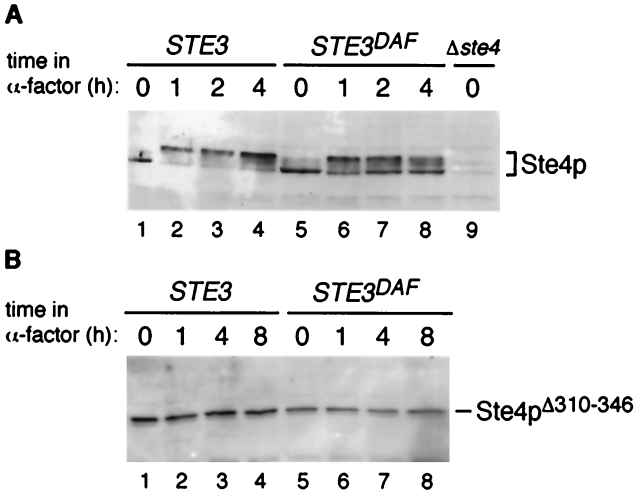

FIG. 2. Abundances of Ste4p and Ste4p$^{\Delta 310–346}$ in wild-type and *STE3*^{DAF} cells. (A) The following strains were treated with α-factor (0.1 μM) for the indicated periods of time: H67-9D.Ba, a *MAT***a** *STE3* strain (lanes 1 to 4), H67-6C.Ba, a *MAT***a** *STE3*^{DAF} strain (lanes 5 to 8), and AC17-7B, a *MAT***a** *STE3 ste4*::*HIS3* strain (lane 9). Cell extracts were prepared, and immunoblots containing these extracts were probed with anti-Ste4p polyclonal antibody. (B) The following strains were treated with α-factor (0.1 μM) for the indicated periods of time: AC17-7B, a *MAT***a** *STE3 ste4*::*HIS3* strain carrying plasmid YCpΔ36, which contains the *STE4*$^{\Delta 310–346}$ allele (lanes 1 to 4), and AC17-2B, a *MAT***a** *STE3*^{DAF} *ste4*::*HIS3* strain carrying plasmid YCpΔ36 (lanes 5 to 8). Cell extracts were prepared, and immunoblots containing these extracts were probed with an anti-Ste4p polyclonal antibody.

An altered version of Ste4p localizes normally in cells expressing the a-factor receptor. *STE4*^{SD} alleles are mutated versions of the *STE4* gene that produce Ste4p variants that are largely insensitive to inhibition by the **a**-factor receptor (17). In wild-type *MAT***a** cells, the Ste4p^{SD10} variant signals in a manner indistinguishable from that of wild-type Ste4p. In *STE3*^{DAF} *MAT***a** cells, Ste4p^{SD10} produces a much greater signal than wild-type Ste4p at late time points after α-factor treatment. To test whether signaling correlates with localization of Ste4p at the plasma membrane, the localization pattern of Ste4p^{SD10} was investigated in *MAT***a** cells expressing the **a**-factor receptor. In *STE3*^{DAF} cells treated with α-factor for 2 or 3 h, GFP-Ste4p was localized in an internal particulate pattern (Fig. 3, Ste4p). In contrast, a large proportion of the GFP-Ste4p^{SD10} signal was localized at the membrane at regions of mating projections (Fig. 3, Ste4p^{SD}). Therefore, the increased signaling conferred by the Ste4p^{SD10} variant correlates with increased membrane localization. These results suggest that inhibition of signaling by the **a**-factor receptor is effected by localization of Ste4p away from the plasma membrane.

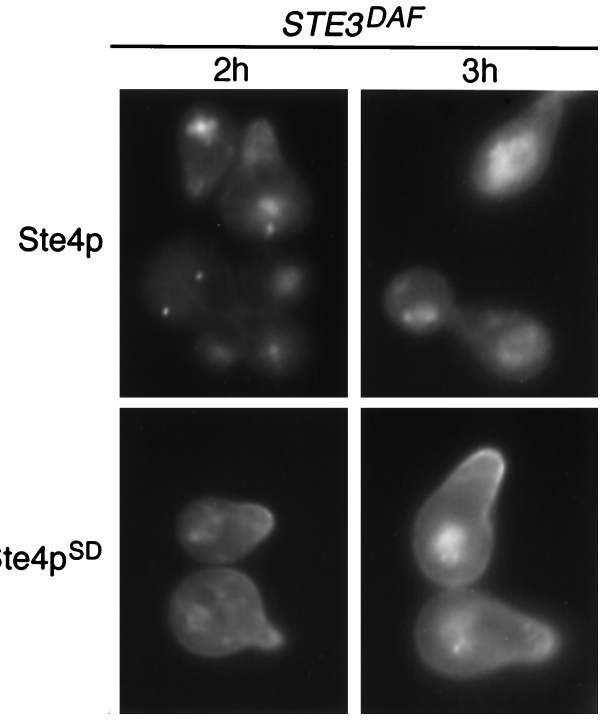

FIG. 3. Localization of GFP-Ste4p and GFP-Ste4p^{SD10} in *STE3^{DAF}* cells. Strain AC17-2B (*MAT***a** *STE3^{DAF} ste4*::*HIS3*) containing a *GFP-STE4* plasmid (pBTL49) or a *GFP-STE4^{SD10}* plasmid (YCpGFP-SD10) was treated with α-factor (0.1 μM) for the indicated periods of time. The live cells were viewed by fluorescence microscopy using an FITC filter set.

ASG7* is required for inhibition of signaling by the a-factor receptor.** Previous results indicate that receptor inhibition requires a component that is only present in *MATa** cells. However, it has been shown that inhibition of signaling does not require any of the known **a**-specific genes that might be expected to be involved in this process, including *STE2* (**a**-factor receptor gene), *MFA1* and *MFA2* (**a**-factor genes), *SST1* (α-factor protease gene), and *STE6* (**a**-factor transporter gene) (13; A. Couve and J. P. Hirsch, unpublished data). A comprehensive approach to identify **a**-specific genes was recently performed by screening the *Saccharomyces* Genome Database for Matα2p-Mcm1p binding sites in the 5′ flanking regions of open reading frames (38). The Matα2p-Mcm1p complex functions to repress transcription in *MAT*α cells, and thus these binding sites identify genes that are only expressed in *MAT***a** cells. A previously uncharacterized **a**-specific gene, *ASG7*, was identified by the screen. The requirement for *ASG7* in receptor inhibition was tested by deleting the *ASG7* gene in *MAT***a** cells containing a *STE3^{DAF}* allele.

As described previously, *MAT***a** cells containing a *STE3^{DAF}* allele did not undergo cell cycle arrest in response to pheromone as measured by a halo assay (Fig. 4A). However, deletion of *ASG7* in *STE3^{DAF}* cells completely eliminated the inhibition of cell cycle arrest seen in the *STE3^{DAF}* strain. Deletion of *ASG7* had no effect on pheromone-induced cell cycle arrest in wild-type *MAT***a** cells. These results demonstrate that a functional *ASG7* gene is required for inhibition of the cell cycle arrest response by *STE3^{DAF}*.

The *STE3^{DAF}* allele causes inhibition of pheromone-inducible transcription at late times after pheromone treatment (3). To determine the effect of *ASG7* on transcriptional induction, a time course of *FUS1* RNA expression was performed in cells containing wild-type or null alleles of *ASG7*. Deletion of *ASG7* had no effect on the level of *FUS1* RNA induced in wild-type cells (Fig. 4B, lanes 1 to 8). As described previously, *STE3^{DAF}* cells displayed a decrease in *FUS1* RNA induction that was most pronounced at the 2- and 3-h time points (Fig. 4B, lanes 9 to 12). In *STE3^{DAF}* cells containing an *asg7*Δ mutation, the levels of *FUS1* RNA were the same as those seen in wild-type cells (Fig. 4B, lanes 1 to 4 and 13 to 16). Therefore, the *asg7*Δ mutation completely eliminated the inhibitory effect of expressing *STE3* in *MAT***a** cells.

One of the characteristics of receptor inhibition is that it blocks the constitutive signaling conferred by deletion of *GPA1*, the G protein α-subunit gene, in an **a**-specific manner (4, 13, 17). In a wild-type background, a *gpa1*Δ mutation causes permanent cell cycle arrest due to constitutive activation of the pheromone response pathway by the βγ complex. In *MAT***a** cells, the *STE3^{DAF}* allele suppresses the cell cycle arrest phenotype of a *gpa1*Δ strain. If *ASG7* is required for receptor inhibition, then deletion of the *ASG7* gene would be expected to result in cell cycle arrest in *STE3^{DAF} MAT***a** cells that contain a *gpa1*Δ mutation. The effect of deleting *ASG7* in *MAT***a** *STE3^{DAF} gpa1*Δ cells was tested using a strain that contains *GPA1* under the control of the *GAL* promoter. When this strain was grown in galactose, expression of *GPA1* complemented the *gpa1*Δ mutation and both the *ASG7* and *asg7*Δ strains formed colonies (Fig. 4C). In glucose, the cell cycle arrest phenotype was suppressed by the *STE3^{DAF}* allele in the *ASG7* strain. In the *STE3^{DAF}* strain that contains an *asg7*Δ mutation, cell cycle arrest occurred in the absence of *GPA1* expression. These findings demonstrate that *ASG7* is required for the inhibitory effect of the *STE3^{DAF}* allele that occurs in cells lacking the G protein α subunit.

All of the results presented above show that *ASG7* is required for the inhibition of signaling conferred by expression of *STE3* in *MAT***a** cells. Although these results do not prove that *ASG7* is the only **a**-specific gene that mediates receptor inhibition, there is at present no evidence to indicate that other **a**-specific genes are required for this process.

Expression of *ASG7* is a specific and pheromone inducible. Previous results have shown that expression of *ASG7* RNA is **a** specific (38). To investigate further the regulation of *ASG7*, a time course of RNA expression was performed in both *MAT***a** cells treated with α-factor and *MAT*α cells treated with **a**-factor. In these experiments, *ASG7* RNA was not detectable in untreated *MAT***a** or *MAT*α cells (Fig. 5, lanes 1 and 5). Treatment of *MAT***a** cells with α-factor for 1 h caused a large increase in the abundance of *ASG7* RNA, and this increase was maintained for 3 h (Fig. 5, lanes 2 to 4). Treatment of *MAT*α cells with **a**-factor had no effect on the expression of *ASG7* RNA, although *FUS1* RNA was induced normally (Fig. 5, lanes 6 to 8). These results demonstrate that *ASG7* RNA expression is completely **a** specific and that it is pheromone inducible to a large degree.

The a-factor receptor inhibits signaling in its liganded state. In the experiments described above, the **a**-factor receptor is not occupied by a ligand because the strains used have deletions of the genes encoding **a**-factor. Therefore, it was not known whether the **a**-factor receptor could function in receptor inhibition in its liganded state. To test this idea, the effect of the *ASG7* gene on signaling that originates from the liganded **a**-factor receptor was investigated. Thus, this experiment tests whether the **a**-factor receptor can carry out its two independent functions, signal transduction and receptor inhibition, in the same cell.

To determine the effect of *ASG7* on signaling through the **a**-factor receptor, a time course of *FUS1* RNA expression was

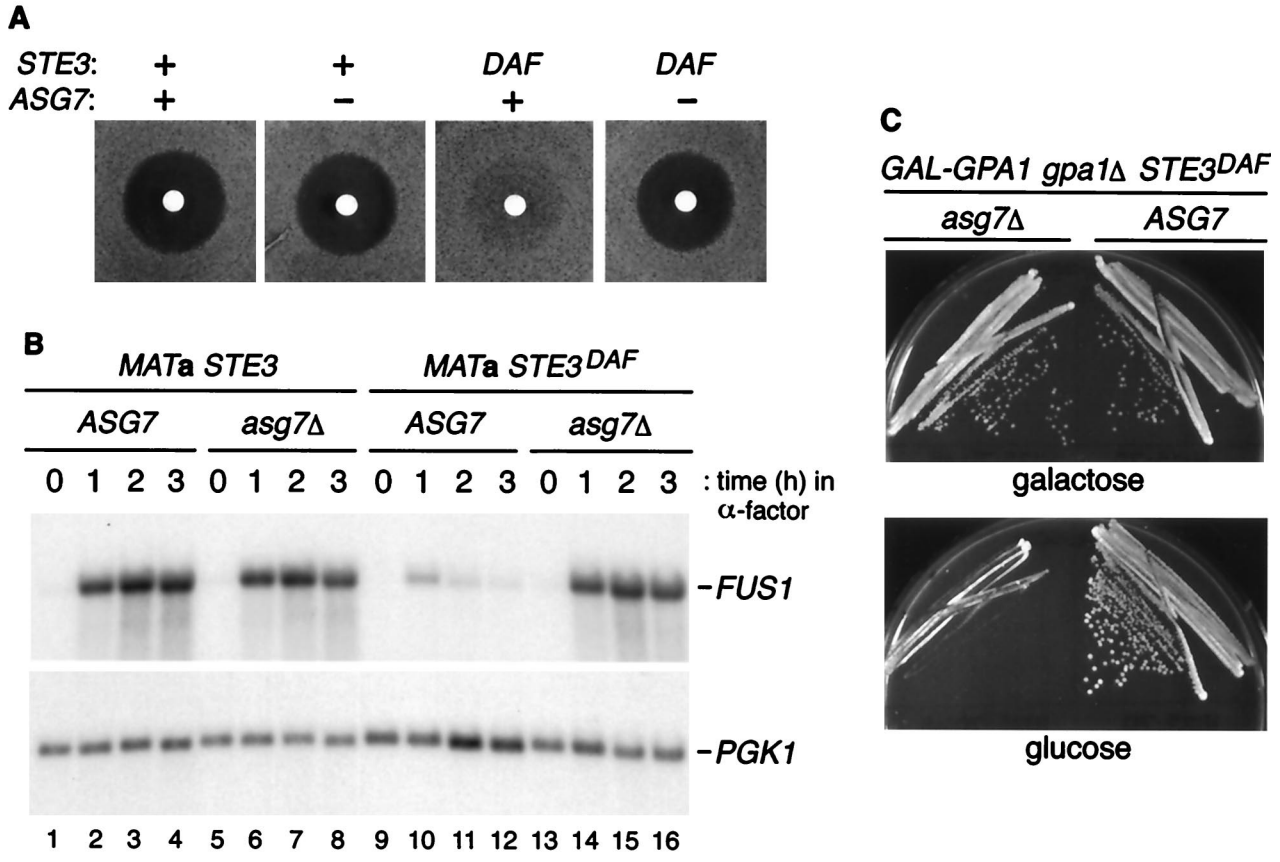

FIG. 4. Effect of *ASG7* on signaling in *STE3*^{DAF} cells. (A) Halo assays were performed with 5 μl of 1 mM α-factor using the following strains (from left to right): H67-9D.Ba, a *MAT***a** *STE3 ASG7* strain, H67.9D.a7, a *MAT***a** *STE3 asg7*::*URA3* strain, H67-6C.Ba, a *MAT***a** *STE3*^{DAF} *ASG7* strain, and H67-6C.a7, a *MAT***a** *STE3*^{DAF} *asg7*::*URA3* strain. (B) The following strains were treated with α-factor (0.1 μM) for the indicated periods of time: H67-9D.Ba, a *MAT***a** *STE3 ASG7* strain (lanes 1 to 4), H67-9D.a7, a *MAT***a** *STE3 asg7*::*URA3* strain (lanes 5 to 8), H67-6C.Ba, a *MAT***a** *STE3*^{DAF} *ASG7* strain (lanes 9 to 12), and H67-6C.a7, a *MAT***a** *STE3*^{DAF} *asg7*::*URA3* strain (lanes 13 to 16). RNA was isolated, transferred to nitrocellulose, hybridized with a *FUS1* probe, and rehybridized with a *PGK1* probe. (C) *MAT***a** *STE3*^{DAF} *gpa1*::*TRP1 ASG7* (H125-7D) and *MAT***a** *STE3*^{DAF} *gpa1*::*TRP1 asg7*::*HIS3* (H125-7D.aH) strains containing a plasmid with *GPA1* under the control of the *GAL* promoter were streaked onto galactose- or glucose-containing plates and grown at 30°C for 2 to 3 days.

performed in *MAT***a** *STE3*^{DAF} cells containing wild-type or null alleles of *ASG7*. The cells were treated with **a**-factor at a concentration of 300 nM, which is approximately 10,000-fold higher than the lowest concentration required to induce a transcriptional response in *MAT*α cells (24). Because *STE3* is expressed at comparable levels in *MAT***a** *STE3*^{DAF} cells and wild-type *MAT*α cells (13), it is expected that the majority of cell surface receptors on *STE3*^{DAF} cells would be occupied by **a**-factor at this concentration. In the strain containing wild-type *ASG7*, there was only a slight induction of *FUS1* RNA in response to treatment with **a**-factor (Fig. 6, lanes 1 to 4). In the strain containing an *asg7*Δ mutation, there was a large increase in the induction of *FUS1* RNA in response to treatment with **a**-factor (Fig. 6, lanes 5 to 8). The simplest interpretation of these results is that the **a**-factor receptor can block the signal that it initiates. In the strain that contains wild-type *ASG7*, the low level of *FUS1* RNA induction was due to the presence of both the **a**-factor receptor and Asg7p in the same cell. These results imply that the **a**-factor receptor can function in the process of receptor inhibition while it is bound to the ligand.

ASG7* affects Ste4p localization.** The results described above show that receptor inhibition results in altered localization of the Ste4p β subunit. In addition, they show that *ASG7* is required for inhibition of signaling by the **a**-factor receptor. It was therefore of interest to determine whether deletion of the *ASG7* gene would have an effect on the subcellular location of Ste4p. To investigate this possibility, the localization pattern of a GFP-Ste4p fusion protein in *MATa** *STE3*^{DAF} cells that contained the wild-type *ASG7* gene or an *asg7*Δ mutation was observed. The cells were treated with α-factor for 2 h to maximize the difference between signaling and nonsignaling cells (Fig. 2). GFP-Ste4p was localized in an internal particulate pattern in *STE3*^{DAF} cells that contained the wild-type *ASG7* gene (Fig. 7). In contrast, GFP-Ste4p was localized predominantly at the cell membrane in *STE3*^{DAF} cells that contained the *asg7*Δ mutation. These results demonstrate that, in addition to its effect on signaling, *ASG7* has an effect on Ste4p localization in cells that express the **a**-factor receptor. Moreover, localization of Ste4p to the cell membrane correlated with activation of the signaling pathway in all experiments, as would be expected for a protein that transmits a signal to membrane-associated target proteins.

Asg7p localization is consistent with a direct effect of Asg7p on Ste4p. The effect of Asg7p on Ste4p localization could occur through an indirect mechanism, such as transcriptional activation of other genes, or through a more direct mechanism, such as binding of Ste4p to a complex containing Asg7p. To determine whether the location of Asg7p in the cell is consistent with a direct effect on Ste4p, a fully functional *ASG7-GFP* fusion construct was expressed in *STE3*^{DAF} cells containing an

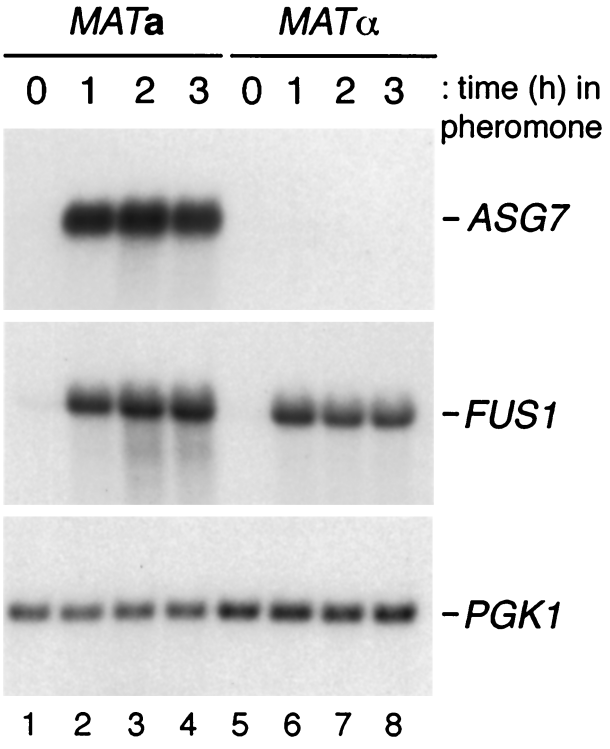

FIG. 5. Abundance of *ASG7* RNA in *MAT***a** and *MAT*α cells treated with pheromone. Strain W3031A.Ba was treated with α-factor (0.1 μM) for the indicated periods of time (lanes 1 to 4); strain W3031B was treated with **a**-factor (60 nM) for the indicated periods of time (lanes 5 to 8). RNA was isolated, transferred to nitrocellulose, hybridized with an *ASG7* or *FUS1* probe, and rehybridized with a *PGK1* probe.

*asg7*Δ mutation. In cells treated with α-factor for 2 or 3 h, Asg7p-GFP was localized in an internal particulate pattern (Fig. 8). Asg7p-GFP was observed in this pattern as soon as the signal became visible, at about 1 h after α-factor treatment,

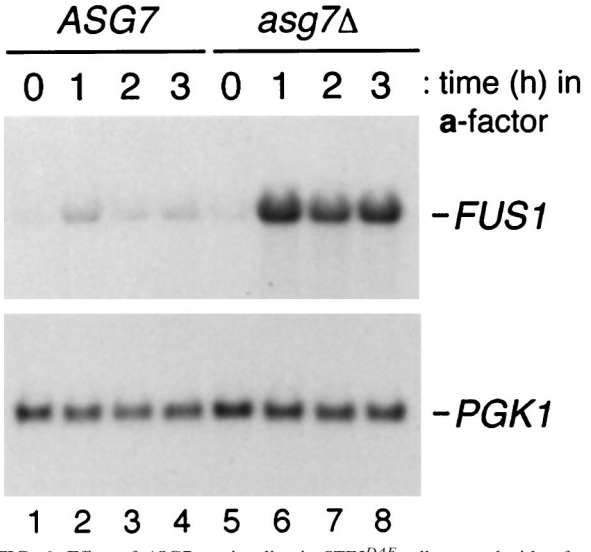

FIG. 6. Effect of *ASG7* on signaling in *STE3*^{DAF} cells treated with **a**-factor. The following strains were treated with **a**-factor (300 nM) for the indicated periods of time: H67-6C.Ba, a *MAT***a** *STE3*^{DAF} *ASG7* strain (lanes 1 to 4), and H67-6C.a7, a *MAT***a** *STE3*^{DAF} *asg7*::*URA3* strain (lanes 5 to 8). RNA was isolated, transferred to nitrocellulose, hybridized with a *FUS1* probe, and rehybridized with a *PGK1* probe.

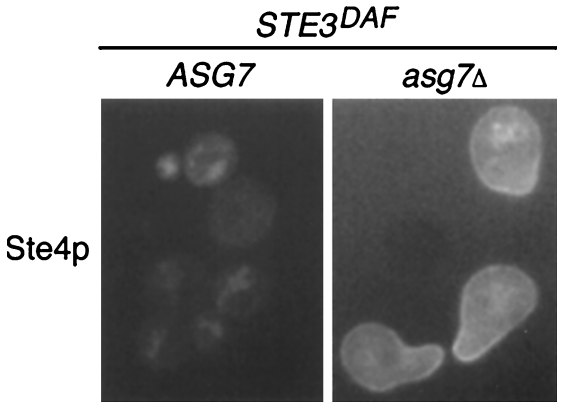

FIG. 7. Effect of *ASG7* on localization of GFP-Ste4p in *STE3*^{DAF} cells. The following strains were treated with α-factor (0.1 μM) for 2 h: AC17-2B, a *MAT***a** *STE3*^{DAF} *ste4*::*HIS3 ASG7* strain (*ASG7*), and AC17-2B.aL, a *MAT***a** *STE3*^{DAF} *ste4*::*HIS3 asg7*::*LEU2* strain (*asg7*Δ). Both strains contained the low-copy-number *STE4-GFP* plasmid pBTL49. The live cells were viewed by fluorescence microscopy using an FITC filter set.

and the pattern was the same in both *STE3*^{DAF} and wild-type cells (E. Bortz and J. P. Hirsch, unpublished data). The subcellular location of Asg7p is similar to that of Ste4p during receptor inhibition. Therefore, these results are consistent with Asg7p having a direct effect on Ste4p localization.

DISCUSSION

Localization and signaling function of Ste4p. Expression of the **a**-factor receptor in *MAT***a** cells inhibits signaling through the pheromone response pathway at the level of Ste4p, the G protein β subunit (3, 17). To investigate the way in which signaling is inhibited, the normal localization pattern of Ste4p during pheromone signaling was examined. Biochemical fractionation of cell membranes had indicated that the fraction of Ste4p that is associated with the plasma membrane does not change after pheromone stimulation (14). We used microscopic examination of live cells to show that treatment of cells with pheromone causes a redistribution of Ste4p to regions of mating projection formation. These studies were therefore able to detect a change in the location of Ste4p that had not been seen previously. Other signaling components of the pher-

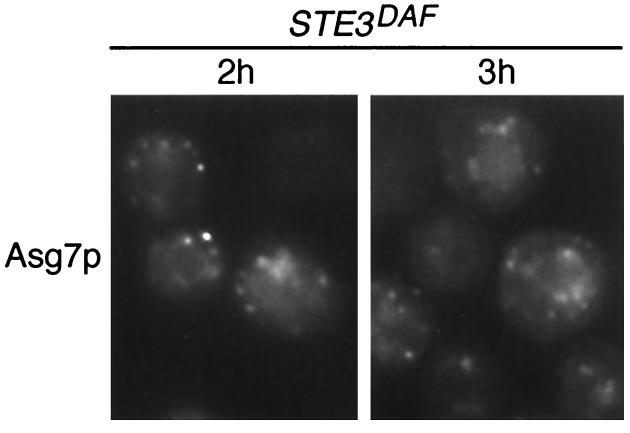

FIG. 8. Localization of Asg7p in *STE3*^{DAF} cells. Strain H67-6C.a7 (*MAT***a** *STE3*^{DAF} *asg7*::*URA3*) containing an *ASG7-GFP* plasmid (pASG7-111.GFP) was treated with α-factor (0.1 μM) for the indicated periods of time. The live cells were viewed by fluorescence microscopy using an FITC filter set.

omone response pathway, such as the pheromone receptors, the kinase Ste20p, and the scaffolding protein Ste5p, have also been shown to localize to the membranes of mating projections (16, 20, 23, 26, 29). These results are consistent with a model in which Ste5p is recruited to the plasma membrane by the released Ste4p β subunit upon activation of the G protein (29). Recruitment of Ste5p to the membrane would bring Ste11p, which is bound to Ste5p, into close proximity with the membrane-associated kinase Ste20p. Phosphorylation of the MAP kinase kinase kinase Ste11p by Ste20p would then activate the MAP kinase cascade. This model is also consistent with our observation that localization of Ste4p away from the cell membrane to an internal compartment correlates with inhibition of signaling. In this case, Ste4p would not be able to recruit Ste5p to the plasma membrane and phosphorylation of Ste11p would not occur. Therefore, sequestration of the G protein β subunit away from the plasma membrane provides an efficient post-translational mechanism for inhibition of signal transduction.

In cells undergoing receptor inhibition, Ste4p was localized to a region of the cell that resembles an endocytic compartment. However, it should be noted that the yeast βγ complex does not undergo endocytosis during pheromone signaling in wild-type cells (14) and that the steady-state level of Ste4p does not change under conditions of receptor inhibition. Therefore, although Ste4p appears to be associated with vesicles or other subcellular structures, this association probably does not result in its degradation in the lysosome.

G_β subunit binding partners and regulation of localization. *ASG7* was identified as an **a**-specific gene that is required for the inhibitory effect of the **a**-factor receptor on pheromone signaling. In addition, it was shown that the subcellular location of Asg7p and Ste4p is consistent with Asg7p having a direct effect on localization of the Ste4p β subunit. Several proteins that bind directly to βγ subunits and inhibit their signaling activity have been identified. One such protein is phosducin, which undergoes regulated phosphorylation and which binds to βγ in its unphosphorylated form. The crystal structure of the phosducin-βγ complex reveals that βγ would not be able to bind to a G protein α subunit when it is bound to phosducin (9). The binding of phosducin to βγ is also predicted to disrupt the orientation of βγ relative to the membrane (9). This idea is in agreement with experimental evidence demonstrating that the binding of phosducin to βγ causes it to shift from a membrane subcellular fraction to a soluble fraction (21, 37). The ability of phosducin to cause the translocation of βγ to a different subcellular compartment and to inhibit its signaling activity appears analogous to the action of Asg7p on the yeast βγ complex during receptor inhibition. However, unlike the phosducin-βγ complex, the yeast βγ complex remains in the pellet fraction after it has translocated away from the plasma membrane (J. Kim and J. P. Hirsch, unpublished data). Therefore, the process of receptor inhibition does not cause complete solubilization of the βγ complex.

Another example of a protein that binds to βγ subunits is the mammalian β-adrenergic receptor kinase (βARK). In this case, the βγ complex targets βARK to the membrane and facilitates phosphorylation of the receptor by the kinase (28). The direct binding of βγ subunits to a protein involved in down-regulating the response suggests a parallel with the process of receptor inhibition in yeast. However, the complex of βγ and βARK localizes to a membrane where βγ is thought to be active, unlike the altered subcellular location of βγ under conditions of receptor inhibition.

βγ subunits also bind to the mammalian protein KSR-1, a kinase that was originally identified as a regulator of the Ras signaling pathway (1). The binding of KSR-1 to βγ inhibits the ability of βγ to activate the MAP kinase ERK1. In contrast to the change in subcellular location that is associated with inhibition of the yeast βγ, the inactive complex of KSR-1 and βγ remains associated with the plasma membrane. Thus, although receptor inhibition has features in common with other systems in which βγ forms complexes with known proteins, it is not strictly analogous to any previously described process.

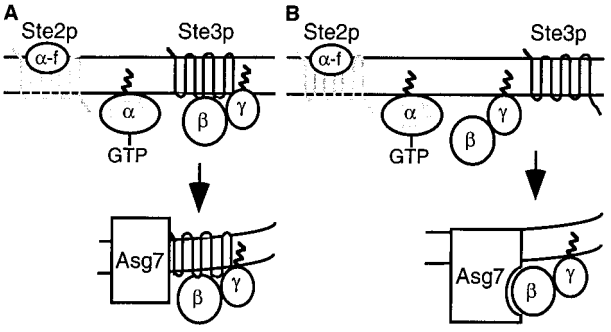

FIG. 9. Models for *ASG7* function. See text for details.

Models for Asg7p function. The **a**-specific gene *ASG7* was shown to be required for receptor inhibition by demonstrating that deletion of *ASG7* eliminated the inhibitory effects of *STE3* expression on signal transduction. Moreover, *MAT***a** cells expressing *STE3* but lacking *ASG7* displayed normal localization of Ste4p at the cell membrane. These results are consistent with several different models for the way in which Asg7p functions with the **a**-factor receptor to inhibit signaling by Ste4p. One potential model for receptor inhibition involves a direct interaction between Asg7p and the **a**-factor receptor that takes place within an intracellular membrane (Fig. 9A). The Asg7p protein is predicted to contain two hydrophobic regions that could function as transmembrane domains. In this model, the alpha-helical bundle of the receptor transmembrane domains would interact with the transmembrane domains of Asg7p. This interaction is not expected to take place in the plasma membrane because Asg7p is not observed on the cell surface. However, the pheromone receptors are associated with several different intracellular membranes during their life cycle. This model assumes that the cytoplasmic domains of the **a**-factor receptor directly associate with the βγ subunits. The interaction of the receptor with Asg7p would produce a high-affinity binding site for βγ, which would result in the association of βγ with a specific internal compartment. This model is consistent with several studies of mammalian systems, which have indicated that βγ subunits may bind directly to their associated receptors in the absence of an α subunit (12, 27, 34). Moreover, Roth and colleagues have also identified the role of *ASG7* in receptor inhibition using a genomics approach and have shown that Asg7p inhibits delivery of the Ste3p receptor to the cell surface by a process that is independent of βγ (30). This finding provides support for the idea that Asg7p and the **a**-factor receptor directly interact with each other.

The other potential model for receptor inhibition involves a direct interaction between Asg7p and the Ste4p β subunit (Fig. 9B). In this model, the presence of the **a**-factor receptor would promote loading of Asg7p into a complex that contains Ste4p. Targeting signals on Asg7p would then localize the complex to an internal compartment. This model is consistent with the observation that Ste4p and Asg7p display similar subcellular localization patterns in cells undergoing receptor inhibition. Activation of a downstream component by an unliganded G

protein-coupled receptor would represent a novel function for this class of receptors.

Altered versions of Ste4p that are resistant to receptor inhibition and thus are capable of signaling in *MAT***a** cells in which the **a**-factor receptor is expressed have been identified (17). The effect of these changes in Ste4p can be interpreted in different ways when considering the two models presented above. If the first model is correct (Fig. 9A), then these changes in Ste4p are expected to affect its ability to bind to the cytoplasmic domains of the **a**-factor receptor. If the second model is correct (Fig. 9B), then these changes in Ste4p are expected to affect its ability to bind to a protein interaction domain of Asg7p. It is also possible that additional components that have not yet been identified could transmit a signal between the receptor, Asg7p, and Ste4p.

Analysis of the kinetics of signaling in *MAT***a** cells expressing the **a**-factor receptor has shown that normal initiation of signaling is followed by a gradual inhibition of the response (3). The finding that *ASG7* is a pheromone-inducible gene provides an explanation for this pattern of signaling. When cells are first exposed to pheromone, the *ASG7* gene product is present at a low level that does not affect activation of the signal transduction pathway. As the response proceeds, the accumulation of Asg7p results in gradual inhibition of signaling activity. Induction of *ASG7* RNA by pheromone in a wild-type *MAT***a** cell, where Asg7p does not affect signaling, could function to prepare the cell for signaling inhibition after the fusion of two mating partners, as described below.

Physiological role of receptor inhibition. The results presented here document inhibition of signaling by the βγ complex when Asg7p and the **a**-factor receptor are present in the same cell. Although this situation was generated by a mutant allele in these experiments, it occurs naturally during several transient phases of the yeast life cycle. For example, homothallic strains of yeast undergo mating type switching during haploid growth. During the transition from one mating type to another, a single cell could produce both a particular pheromone receptor and the pheromone that binds to that receptor. This situation could activate the pheromone response pathway and induce the expression of *ASG7*, resulting in the presence of Asg7p and the **a**-factor receptor in the same cell. Activation of the process of receptor inhibition would then turn off the pheromone response pathway and allow the cell to resume cycling. Another example of such a situation occurs immediately after fusion of two haploid cells during the mating process. The long-term mechanism for eliminating pheromone signaling in diploids is the establishment of transcriptional inhibition of genes encoding components of the pheromone response pathway. However, it is also possible that a short-term mechanism acts to inhibit signaling in zygotes. The fusion of two mating partners could bring Asg7p from the *MAT***a** mating partner into contact with **a**-factor receptor from the *MAT*α mating partner. In the newly fused zygote, both pheromone receptors are expected to be occupied. The finding that the occupied **a**-factor receptor can participate in receptor inhibition indicates that signaling could be blocked under these conditions. The function of receptor inhibition in this case would be to prevent multiple rounds of mating or to allow the zygote to recover rapidly from cell cycle arrest. In support of this idea, Roth and colleagues have demonstrated that there is a delay in the emergence of the first mitotic bud from zygotes in which the *MAT***a** mating partner has a deletion of *ASG7* (30). Identification of *ASG7* as an **a**-specific component required for receptor inhibition provides an opportunity to test multiple physiological conditions for their ability to be affected by this unique process.

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