Regulation of Postreceptor Signaling in the Pheromone Response Pathway of *Saccharomyces cerevisiae*

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α -Factor pheromone inhibits division of yeast a cells. After prolonged exposure to α -factor, the cells adapt to the stimulus and resume cell division. The *sst2* mutation is known to inhibit adaptation. This report examines adaptation in *scg1* (also designated *gpa1*) and *STE4^{Hpl}* (*Hpl* indicates haploid lethal) mutants that exhibit constitutive activation of the pheromone response pathway. Recovery of the *STE4^{Hpl}* mutant was blocked by the *sst2-1* mutation, whereas recovery of the *scg1-7* mutant was not completely blocked by *sst2-1*. These results indicate that both *SST2*-dependent and -independent mechanisms regulate postreceptor events in the pheromone response pathway. Down regulation of receptors in response to α -factor was independent of the signal that was generated in the *scg1* mutant.

In *Saccharomyces cerevisiae*, the conjugation of haploid a and α cells depends on the action of the peptide pheromones a-factor and α -factor (1, 17, 26, 30). The α cells secrete α -factor pheromone, which binds to specific receptor sites on the surfaces of a cells (20, 21). This causes the a-cell targets to initiate the mating program (10, 44); that is, they arrest division at G1, increase agglutinability toward α cells, and assume an elongated shape. α cells show similar responses to a-factor pheromone. The arrested a and α cells then fuse and undergo karyogamy to form diploid zygotes; these mating events are also regulated by pheromones (11, 29, 41, 47).

Genetic studies of the pheromone response have identified a number of steps in the signal transduction pathway. The *STE2* and *STE3* genes encode the receptors for α -factor and a-factor, respectively (3, 14, 20, 28). Nucleotide sequence analysis of the cloned genes suggests that both receptors span the plasma membrane seven times (4, 14, 15, 37). The *SCG1* (also designated *GPA1*), *STE4*, and *STE18* genes appear to encode G_{α} , G_{β} , and G_{γ} subunits of a GTP-binding regulatory protein (G protein) (12, 31, 35, 48). Mutations in *SCG1* cause constitutive arrest of cell division and pheromone-independent mating (2, 12, 19, 31). Recessive mutations in *STE4* and *STE18* eliminate pheromone responsiveness (18, 27, 48), whereas a special dominant allele of *STE4* leads to a constitutive response (2). The *STE5* gene product controls an unidentified step in the response pathway (18, 27). The analysis of double mutants suggests that the products of these genes function in the order *STE2*, *SCG1*, *STE4*, *STE5* (2, 12, 31, 36).

In the absence of mating, a cells will eventually recover from the effects of α -factor and resume the cell division cycle (5, 33). This recovery results from at least two different properties of a cells: secretion of a proteolytic activity that specifically inactivates α -factor (5, 9) and adaptation with time to the undegraded pheromone (33). The adaptive response could involve either modification of the cell surface receptors or inhibition of postreceptor signaling functions. The α -factor receptor is known to be phosphorylated (40) and internalized (8, 22) in response to its ligand. Mutations in

three genes are known to increase pheromone sensitivity and to inhibit the recovery process. The *bar1* mutation (also designated *sst1*) eliminates α -factor degradation (6, 7, 45). Adaptation is blocked by *sst2* mutations and by certain *ste2* mutations that remove the C-terminal domain of the receptor (24, 40). The *sst2* and *ste2* mutations probably affect two different regulatory processes, since the double mutant shows a more extreme phenotype than does either of the single mutants (24, 40).

This paper examines the steps of the signal transduction pathway that are regulated during adaptation. Mutants constitutive for the pheromone response were used to identify two postreceptor mechanisms that control adaptation. One of these mechanisms requires the action of the *SST2* gene. The effects of the postreceptor signal on the maintenance of cell surface receptor sites were also investigated.

MATERIALS AND METHODS

Strains, media, and genetic methods. The strains used are listed in Table 1. All strains are congenic to strain 381G (18). YM-1 is a rich liquid medium (16). Inhibitor medium was YM-1 containing 10 mM NaN_3 and 10 mM KF. YEPD solid medium (34) was supplemented with 40 mg of adenine per liter. Standard genetic methods were used for all strain constructions (34).

Growth curves. Cultures were inoculated in YM-1 and incubated overnight at 34°C with shaking. Exponentially growing cultures (less than 2×10^7 cells per ml) were diluted to 10^5 or 10^6 cells per ml in fresh YM-1 and transferred to 22°C. Samples of 1 ml were withdrawn at the specified times and fixed in the 3.7% formaldehyde. For cell number determinations, the samples were lightly sonicated (to disperse cell aggregates) and counted in a particle counter (Coulter Electronics, Inc.).

Binding assays. The ^3H - α -factor-binding assay was adapted from our previous procedure (23). Cultures were grown overnight in YM-1 at 34°C to final density of no more than 2×10^7 cells per ml. Cells were diluted to 5×10^6 cells per ml and shifted to a 22°C shaking water bath. α -Factor (10^{-8} M) and cycloheximide (10 $\mu\text{g/ml}$) were added to the specified cultures at the time of the shift. At the various time points, 25-ml samples were collected on a nitrocellulose filter

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TABLE 1. Strains used

Strain ^a	Genotype ^b
381G.....	<i>MATa cryl ade2-1 his4-580 lys2 trp1 try1 SUP4-3</i>
DJ164-7-1.....	381G <i>ste5-3 bar1-1</i>
DJ728-9-3.....	381G <i>ste5-3 scg1-7</i>
DJ730-2-3.....	381G <i>ste5-3 bar1-1 scg1-7</i>
DJ736-34-1.....	381G <i>ste5-3 STE4^{Hpl}</i>
DJ231-2-2.....	381G <i>ste5-3 bar1-1 ste2-10::LEU2 leu2 ura3</i>
DJ751-12-2.....	381G <i>ste5-3 ste2-10::LEU2 scg1-7 leu2 ura3</i>
5528-10-3.....	381G <i>ste5-3 sst2-1</i>
DJ750-8-1.....	381G <i>ste5-3 sst2-1 scg1-7</i>
5561-10-2.....	381G <i>ste5-3</i>
DJ761-5-3.....	381G <i>ste5-3 TYR1 scg1-7 STE4^{Hpl}</i>
DJ760-1-1.....	381G <i>ste5-3 sst2-1 STE4^{Hpl}</i>

^a All strains are congenic to strain 381G (18).

^b *ste2-10::LEU2* is a genetic substitution that removes the entire *STE2* gene. The *bar1-1* mutation (45) inhibits α -factor degradation in a cell cultures. The *scg1-7* and *STE4^{Hpl}* mutations result in constitutive activity of the pheromone response pathway (2).

(type HA; 0.45- μ m pore size; Millipore Corp.). Filters were rinsed twice with 2 ml of inhibitor medium, suspended in 10 ml of inhibitor medium, and incubated for 2 h at 25°C to dissociate the reversibly bound α -factor (20). The cells were then centrifuged and resuspended in 0.5 ml of inhibitor medium. Samples (90 μ l) of the cell suspension were incubated for 30 min (25°C) with either 10 μ l of H³- α -factor (2×10^{-7} M; active ligand) or 10 μ l of a solution containing H³- α -factor (2×10^{-7} M) and unlabeled α -factor (2×10^{-5} M). A 90- μ l sample of each reaction mixture was diluted to 2 ml. The cells were then collected on a glass fiber filter (GF/C; Whatman, Inc.) and rinsed twice with 2 ml of inhibitor medium. Filters were dried and counted in a liquid scintillation counter. Specific binding was determined as the counts per minute bound to the experimental filter (without unlabeled α -factor) minus the counts per minute bound to the control filter (with unlabeled α -factor). All measurements were performed in duplicate. ³H- α -factor was as described previously (21) except that active ligand represented only 33% of the total radioactivity.

RESULTS

Recovery from cell division arrest. α -Factor causes a cells to arrest division for only a finite period (5, 33). After prolonged exposure to the ligand, the cells recover and resume the division cycle. The *scg1-7* and *STE4^{Hpl}* (*Hpl* indicates haploid lethal) mutants, which show constitutive activity of the pheromone response pathway (2), were used to test whether postreceptor events in the response pathway are regulated during this process. The temperature-sensitive mutation *ste5-3* blocks this constitutive signal at 34°C but not at 22°C; therefore, *scg1-7 ste5-3* and *STE4^{Hpl} ste5-3* mutants will divide normally at 34°C but will arrest cell division when the cultures are shifted to 22°C (2). Figure 1B shows results for the *MATa scg1-7 ste5-3* mutant that had been cultured at 34°C and then shifted to 22°C. The mutant arrested division with kinetics similar to those observed for the control cells (*MATa ste5-3*) that had been treated with α -factor (Fig. 1A). The *scg1-7* mutant began dividing again after about 20 h, similar to results for the control cells treated with 5×10^{-9} M α -factor. At the end of the time course (30 h at 22°C), the mutant cells were cultured overnight at 34°C and then returned to 22°C. Since the cells showed the response depicted in Fig. 1B, we concluded that the recovery of the mutant cells did not result from the accumulation of revertant cells in the culture. Similar results were obtained with *MATa scg1-7 ste5-3*, *MATa scg1-4 ste5-3*, and *MATa scg1-4 ste5-3*, cells except that the α cells recovered more rapidly than did the β cells (not shown). No recovery was detectable for strains bearing the disruption mutation *scg1::lacZ6* (J. Hirschman and D. Jenness, unpublished observation), suggesting that these cells are unable to recover from the stronger signal or that the recovery mechanism requires a partially functional *SCG1* product.

Figure 1C shows the arrest and recovery of *MATa STE4^{Hpl} ste5-3* cells that were shifted from 34 to 22°C. The cells divided two to three generations before they reduced their division rate. The growth curve then passed through an inflection point, and the division rate steadily increased as the cells adapted to the postreceptor signal. The delay in

FIG. 1. Recovery of *scg1* and *STE4^{Hpl}* mutants from division arrest. Growth rates of mutant strains were examined after the cultures had been shifted from 34 to 22°C. (A) Effect of α -factor on strain DJ164-7-1 (*MATa bar1-1 ste5-3*). α -Factor was either not added (□) or added at the time of temperature shift at a concentration of 10⁻⁹ (■), 5 × 10⁻⁹ (▲), or 10⁻⁸ (◆) M. (B) Comparison of strain DJ728-9-3 (*MATa ste5-3 scg1-7*) (■) with strain 5561-10-2 (*MATa ste5-3*) (□). (C) Comparison of strain DJ736-34-1 (*MATa ste5-3 STE4^{Hpl}*) (■) with strain 5561-10-2 (□).

response to the temperature shift probably reflected instability of the mutant *STE4* product at 34°C, followed by slow accumulation of the mutant protein at 22°C. In support of this view, we have observed that *STE4^{Hpl}* mutants display improved growth and reduced pheromone responsiveness at temperatures above 34°C.

Evidence for two postreceptor recovery mechanisms. a cells regulate their sensitivity to α -factor at a number of steps in the pheromone response pathway. The *BAR1* gene controls the extracellular proteolysis of α -factor (7, 9, 45). The *SST2* gene controls a separate recovery process that is independent of α -factor proteolysis (7). The C-terminal domain of the α -factor receptor controls yet another recovery process that is independent of *BAR1* and *SST2* (24, 40). We wished to determine whether any of these recovery processes also operates on the intracellular signals that are produced in the *scg1* and *STE4^{Hpl}* mutants. Accordingly, we constructed a series of double mutants that contained the *scg1-7* or *STE4^{Hpl}* mutation together with the *bar1-1*, *sst2-1*, or *ste2-10::LEU2* mutation. These mutants were then tested for the ability to recover from arrest of cell division.

Recovery from division arrest in the *scg1* mutant did not require the *STE2*, *BAR1*, or *SST2* gene. When the *scg1-7 ste2-10::LEU2 ste5-3* mutant was shifted from 34 to 22°C (Fig. 2A), cell division arrested and the cells then recovered with the kinetics observed for the *scg1-7 ste5-3* mutant (Fig. 1B). Similar results were obtained with the *scg1-7 bar1-1 ste5-3* mutant (not shown). Thus, recovery was independent of the α -factor receptor and α -factor proteolysis. The *scg1-7 sst2-1 ste5-3* mutant recovered from division arrest at the same point in time (20 h after the temperature shift) (Fig. 2B); however, the division rate of the recovered cells was somewhat slower. We conclude that the *SST2* gene product was unnecessary for recovery of the *scg1-7* mutant, although it probably played an auxiliary role in the recovery process. This *SST2*-independent recovery mechanism was also independent of the receptor, since *scg1-7 sst2-1 ste5-3* cells and *scg1-7 sst2-1 ste2-10::LEU2 ste5-3* cells showed identical arrest and recovery profiles (not shown).

The *STE4^{Hpl}* mutant was dependent on the *SST2* gene for recovery from division arrest. The *STE4^{Hpl} sst2-1 ste5-3* mutant had not recovered 30 h after the culture was shifted from 34 to 22°C (Fig. 2C). In contrast, the *STE4^{Hpl} ste5-3* mutant began to divide after 20 h at 22°C (Fig. 1C). These results indicated that the *SST2* gene controls a postreceptor recovery process that involves the *STE4* gene product or subsequent steps in the pheromone response pathway. We were unable to detect recovery in mutants that contained both *scg1-7* and *STE4^{Hpl}*. When the *scg1-7 STE4^{Hpl} ste5-3* mutant was shifted from 34 to 22°C, the cells divided rapidly for about three generations and then divided very slowly for the remainder of the time course (Fig. 2D). This result could reflect either a stronger signal in the double mutant or a requirement for the *SCG1* product in the recovery of the *STE4^{Hpl}* mutant.

We also monitored the properties of individual cells during the recovery process. Liquid cultures growing at 34°C were spread on solid medium and cultured at 22°C. Figure 3 shows results for cells cultured at 22°C for 16 h. The *scg1 ste5-3* and *STE4^{Hpl} ste5-3* mutants (Fig. 3C and D, respectively) arrested cell division and became large and pear shaped. These mutants resembled the *ste5-3* control cells that had been exposed to α -factor for the entire 16-h period at 22°C (Fig. 3B). After 48 h at 22°C, the *scg1 ste5-3* and *STE4^{Hpl} ste5-3* mutants (Fig. 4C and D) had recovered, as judged by the appearance of microcolonies containing hundreds of small

FIG. 2. Recovery of double mutants from division arrest. Cultures were treated as described in the legend to Fig. 1. (A) Comparison of strain DJ751-12-2 (*MATa ste5-3 ste2-10::LEU2 scg1-7*) (■) with strain DJ231-2-2 (*MATa ste5-3 ste2-10::LEU2*) (□). Recovery of the *scg1* mutant was independent of the receptor. (B) Comparison of strain DJ750-8-1 (*MATa ste5-3 sst2-1 scg1-7*) (■) with strain 5528-10-3 (*MATa ste5-3 sst2-1*) (□). Recovery of the *scg1* mutant was independent of the *SST2* gene. (C) Comparison of strain DJ760-1-1 (*MATa ste5-3 sst2-1 STE4^{Hpl}*) (■) with strain 5528-10-3 (□). Recovery of the *STE4^{Hpl}* mutant required *SST2*. (D) Comparison of strain DJ761-5-3 (*MATa ste5-3 scg1-7 STE4^{Hpl}*) (■) with strain 5561-10-2 (*MATa ste5-3*) (□). Double mutant *scg1 STE4^{Hpl}* failed to recover.

budding cells. In contrast, the *STE4^{Hpl} sst2-1 ste5-3* cells produced microcolonies containing only about 10 large, severely distend cells (Fig. 4E). The *scg1 STE4^{Hpl} ste5-3* cells also remained large and unbudded after 48 h at 22°C (Fig. 4F). Taken together, these results indicate that the *STE4^{Hpl}* mutant depends on the product of the *SST2* gene for recovery and suggest that the *scg1* mutant can utilize a different recovery process.

Receptor down regulation is independent of SCG1. Down regulation of surface receptor sites is a potential mechanism for regulating the pheromone response pathway. The binding of α -factor to its receptor causes a rapid loss of receptor sites from the cell surface (23), followed by an increased rate of receptor synthesis and the reaccumulation of receptors on the cell surface. We wished to determine whether the constitutive signal that is generated in the *scg1* mutant affects the accumulation of surface receptor sites. The

FIG. 3. Morphologies of mutant and α -factor arrested cells. YM-1 liquid cultures growing exponentially at 34°C (10^6 to 2×10^7 cells per ml) were spread on YEPD plates at 22°C (about 10^6 cells per plates). Microcolonies were photographed after 16 h at 22°C. (A) Strain DJ164-7-1 (*MATa ste5-3*); (B) strain DJ164-7-1 spread on YEPD containing 5×10^{-9} M α -factor; (C) strain DJ728-9-3 (*MATa ste5-3 scg1-7*); (D) strain DJ736-34-1 (*MATa ste5-3 STE4^{Hpl}*). The four panels are phase-contrast micrographs at the same magnification.

scg1-7 ste5-3 and *ste5-3* mutants that had been cultured at 34°C were shifted to 22°C, and the binding capacity of the cells was monitored at regular intervals (Fig. 5A). Increased binding of the *ste5-3* control cells at 22°C was expected, since the number of cells in the culture was increasing and since *STE5*⁺ cells contain twice as many binding sites as do *ste5* cells (22). The *scg1-7 ste5-3* mutant exhibited a similar behavior; the somewhat greater rate of receptor accumulation was apparently due to the constitutive postreceptor signal. Both strains showed down regulation and reaccumulation of receptors when α -factor was added to the cultures at the time of the temperature shift (Fig. 5A).

We compared the turnover rates of receptors in the *scg1-7 ste5-3* and *ste5-3* mutants by monitoring the loss of surface receptor sites in the absence of new receptor synthesis (Fig. 5B). The two mutants growing at 34°C were shifted to 22°C

and cultured for 4 h to allow expression of the *scg1* phenotype. Cycloheximide was then added to block synthesis of new receptors. Both cultures showed slow loss of surface receptors that could be accelerated by addition of α -factor. Hence, the *scg1* mutation did not cause a higher constitutive rate of receptor turnover. The down regulation of receptors was apparently induced by a signal that preceded the *SCG1*-controlled events.

DISCUSSION

When procaryotic or eucaryotic cells are exposed to a persistent stimulus, the response to that stimulus often diminishes with time. This adaptive response is often complex and can result in regulation at multiple steps in the signal transduction pathway (38, 43, 46). The responsiveness

FIG. 4. Cell morphologies after recovery from division arrest. Cells were treated as described for Fig. 3 except that the plates were incubated for 48 h at 22°C. (A) Strain 5561-10-2 (*MATa ste5-3*); (B) strain 5528-10-3 (*MATa ste5-3 sst2*); (C) strain DJ728-9-3 (*MATa ste5-3 scg1-7*); (D) strain DJ736-34-1 (*MATa ste5-3 STE4^{Hpl}*); (E) strain DJ760-1-1 (*MATa ste5-3 STE4^{Hpl} sst2*); (F) strain DJ761-5-3 (*MATa ste5-3 scg1-7 STE4^{Hpl}*). All six panels are at the same magnification. Bar, 50 μ m.

of a cells to α -factor is also regulated at several levels. First, a cells degrade α -factor extracellularly (5, 7, 9, 32). This proteolytic activity is controlled by the *BAR1* gene (6, 45). The level of *BAR1* gene expression is increased in the presence of α -factor (25). A second level of regulation requires the C terminus of the pheromone receptor. The *ste2*

mutants that are missing the C-terminal domain of the α -factor receptor show increased sensitivity to pheromone and exhibit defects in adaptation (24, 41). A third level of regulation requires the *SST2* gene. Expression of *SST2* is induced by pheromone treatment (13); mutations in this gene also cause defects in adaptation and increased pheromone

FIG. 5. Down regulation of receptor sites. (A) Down regulation and reaccumulation of receptor sites in the *scg1-7 ste5-3* mutant DJ730-2-3 (Δ , $\blacktriangle$) and the *ste5-3* control strain DJ164-7-1 ($\square$, $\blacksquare$). Cultures growing exponentially at 34°C were shifted to 22°C at time zero. Cultures received no α -factor (Δ , $\square$) or 10^{-8} M α -factor ($\blacktriangle$, $\blacksquare$). Samples were withdrawn at the times indicated, and the ^3H - α -factor-binding capacity was determined. (B) Demonstration that the loss rate of surface receptors is unaffected by the *scg1* mutation. Exponentially growing cultures of the *scg1-7 ste5-3* mutant DJ730-2-3 (Δ , $\blacktriangle$) and the *ste5-3* control strain DJ164-7-1 ($\square$, $\blacksquare$) were diluted to 2.5×10^6 cells per ml and shifted to 22°C. After 4 h at 22°C (time zero), the cultures received cycloheximide (10 $\mu\text{g}/\text{ml}$) and either no α -factor (Δ , $\square$) or 10^{-8} M α -factor ($\blacktriangle$, $\blacksquare$). Binding capacity is expressed as the percentage of the initial value in the absence of α -factor.

sensitivity (6). The *SST2* gene does not appear to operate on the C terminus of the receptor, since the *sst2* mutation increases pheromone sensitivity even when the C-terminal domain of the receptor has been removed (24, 40). The results presented in this paper suggest that the *SST2* gene product affects postreceptor events in the signal transduction pathway. We also provide evidence for a second post-receptor mechanism for adaptation.

The *scg1* and *STE4^{Hpl}* mutants provided an opportunity to investigate adaptive mechanisms that operate on the post-receptor steps of the pheromone response pathway. There were three reasons to suspect that adaptive mechanisms could affect either the *SCG1* product or later steps of the pheromone response pathway: first, spores containing mutation *scg1* continue to divide slowly after germination (12); second, prolonged expression of the *scg1* phenotype leads to decreased mating efficiency (36); and third, pheromone enhances expression of the *SCG1* gene (19).

The results of this study suggest that at least two adaptive mechanisms operate on postreceptor signaling. The *STE4^{Hpl}* mutant adapted to its constitutive signal by using a mechanism that requires the *SST2* gene product. This finding indicates that *SST2* regulates postreceptor events in the response pathway and that it acts on or after the *STE4*-controlled step. In contrast, the *scg1* mutant recovered from its constitutive signal by using a mechanism that was largely independent of the *SST2* gene; however, the slower growth of *sst2 scg1* cultures in comparison with *SST2⁺ scg1* cultures suggests a role for the *SST2* gene in the recovery of *scg1* mutants as well.

The strains used for this study contained the temperature-sensitive mutation *ste5-3*, which blocked the constitutive signals at the nonpermissive temperature. At the nonpermissive temperature, mutation *ste5-3* appears to block steps in

signal transduction that are executed after the steps controlled by the *SCG1* and *STE4* genes (2). At the permissive temperature, *ste5-3* mutants show an α -factor response and recovery behavior (Fig. 1A) similar to that observed for *STE⁺* cells (23, 33). The arrest and adaptation after the *scg1-7 ste5-3* and *STE4^{Hpl} ste5-3* cultures were shifted to the permissive temperature indicated that both recovery processes were induced after the *STE5*-controlled step of the response pathway.

Adaptation to the constitutive signal in the *scg1* mutant does not require participation of the pheromone receptor. Recovery of the *scg1* mutant from division arrest was unaffected by deletion of the *STE2* gene (Fig. 1B and 2A). Furthermore, the activity of the surface receptor sites was unaffected by the constitutive signal in the *scg1* mutant (Fig. 5). We have previously demonstrated that pheromone-induced down regulation of receptor sites is not affected by mutation *ste4*, *ste5*, or *sst2* (23).

Molecular details regarding the postreceptor signal and its regulation are currently unclear. It has been proposed that the *SCG1*, *STE4*, and *STE18* genes encode the G_α , G_β , and G_γ subunits of a GTP-binding regulatory protein (G protein) that dissociates in the presence of occupied receptors; the free $G_{\beta\gamma}$ is thought to promote the subsequent events in the response pathway, which include the *STE5* gene product (2, 12, 19, 31, 48). Postreceptor adaptation mechanisms could conceivably function in such a system by promoting the association of $G_{\beta\gamma}$ with G_α or by inhibiting the subsequent steps. It is of interest that *ste4* alleles that block pheromone responsiveness are suppressed synergistically by mutation *sst2* and *cdc39*, whereas *ste5* mutants are affected by *cdc39* but not *sst2* (22, 39, 42). Specificity for suppression of *ste4* suggests that the *SST2* product is more likely to be involved with the G protein than with the subsequent events.

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