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The Mating-Specific G_α Protein of *Saccharomyces cerevisiae* Downregulates the Mating Signal by a Mechanism That Is Dependent on Pheromone and Independent of $G_{\beta\gamma}$ Sequestration

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It has been inferred from compelling genetic evidence that the pheromone-responsive G_α protein of *Saccharomyces cerevisiae*, Gpa1, directly inhibits the mating signal by binding to its own βγ subunit. Gpa1 has also been implicated in a distinct but as yet uncharacterized negative regulatory mechanism. We have used three mutant alleles of *GPA1*, each of which confers resistance to otherwise lethal doses of pheromone, to explore this possibility. Our results indicate that although the G322E allele of *GPA1* completely blocks the pheromone response, the E364K allele promotes recovery from pheromone treatment rather than insensitivity to it. This observation suggests that Gpa1, like other G_α proteins, interacts with an effector molecule and stimulates a positive signal—in this case, an adaptive signal. Moreover, the Gpa1-mediated adaptive signal is itself induced by pheromone, is delayed relative to the mating signal, and does not involve sequestration of $G_{\beta\gamma}$. The behavior of N388D, a mutant form of Gpa1 predicted to be activated, strongly supports these conclusions. Although N388D cannot sequester βγ, as evidenced by two-hybrid analysis and its inability to complement a *gpa1* null allele under normal growth conditions, it can stimulate adaptation and rescue a *gpa1Δ* strain when cells are exposed to pheromone. Considered as a whole, our data suggest that the pheromone-responsive heterotrimeric G protein of *S. cerevisiae* has a self-regulatory signaling function. Upon activation, the heterotrimer dissociates into its two subunits, one of which stimulates the pheromone response, while the other slowly induces a negative regulatory mechanism that ultimately shuts off the mating signal downstream of the receptor.

In the life cycle of the budding yeast *Saccharomyces cerevisiae*, two haploid cells of opposite mating type, *MAT***a** and *MAT*α, can conjugate to form a *MAT***a**/α diploid cell. Each mating type constitutively secretes a peptide mating pheromone that activates surface receptors on cells of the opposite type. Binding of pheromone to receptor triggers a genetic program that transforms vegetatively growing cells into gametes. In preparation for cell and nuclear fusion, cells stimulated by pheromone induce mating-specific genes, arrest in the G_1 phase of the cell cycle, reorganize their cytoskeletons, and elongate toward their mating partners. The molecular mechanisms by which pheromone elicits these responses have been studied by numerous laboratories, and the yeast pheromone response is currently one of the best-understood eukaryotic signaling pathways (for reviews, see references 27 and 44). Moreover, it has gradually become apparent that the pheromone signal of *S. cerevisiae* is propagated by many of the same types of signaling elements found in higher eukaryotes. The pheromone receptors, for example, belong to the seven-transmembrane-domain class of receptors and, as such, are coupled to a heterotrimeric G protein. When occupied by ligand, these receptors are thought to activate the pheromone-responsive protein G_α (encoded by *GPA1*) via guanine nucleotide exchange and the concomitant dissociation of G_α-GTP from the βγ dimer (encoded by *STE4* and *STE18*). Through an as yet unidentified effector, the $G_{\beta\gamma}$ subunit stimulates a mitogen-activated protein (MAP) kinase cascade, which ultimately leads to dramatic changes in gene expression and the inactivation of the cell division kinase, Cdc28, late in G_1.

In contrast to the relatively detailed outline of how the mating signal is transduced, a unified picture of how the mating signal is turned off has yet to be established. As is true for all sensory systems, the mating signal transduction pathway of *S. cerevisiae* must strike a balance between response and recovery. Haploid yeast cells must be sufficiently sensitive to detect and orient toward potential mating partners yet be able to terminate the mating signal and resume vegetative growth if mating is unsuccessful. While the signal transduction elements must be constitutively expressed to ensure a rapid response, the basal activity of the pathway must be modulated so as to avoid inappropriate induction of the signal. Yeast cells rely on a variety of adaptive mechanisms, operating at distinct levels of the pathway, to provide this balance. The known adaptive phenomena include pheromone degradation (32, 33), receptor desensitization (25, 26, 40), phosphorylation of G_β (8), dephosphorylation of the Fus3 MAP kinase by a phosphatase encoded by *MSG5* (14), and less well characterized mechanisms that depend on the products of *SST2* (12, 13) and *MOT2*/*SIG1* (4, 22, 30) and the protein kinase homolog encoded by *SGV1* (21). In addition, the pheromone-responsive G_α protein, Gpa1, has been hypothesized to stimulate recovery from pheromone treatment (35, 46), although this idea is not universally accepted (28).

On the basis of strong genetic evidence, and by analogy to

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mammalian G proteins, it is generally agreed that the inactive (GDP-bound) form of Gpa1 negatively regulates the mating signal by sequestering the Ste4-Ste18 ($G_{\beta\gamma}$) dimer. There is disagreement, however, as to whether Gpa1 has an additional role in regulating the mating response. We, and others, have suggested that the active form of Gpa1, like the GTP-bound forms of mammalian G_{α} proteins, interacts with an effector and stimulates a positive signal. We believe the Gpa1-mediated signal is an adaptive one, acting to downregulate the mating response. This hypothesis is based on the observation that mutations predicted to activate Gpa1 constitutively confer resistance to high levels of pheromone. It is also possible, however, that the mutant forms of Gpa1, presumed to be activated, confer an inability to respond to pheromone rather than an augmented ability to recover from pheromone treatment. In this study we attempted to resolve this issue. We present evidence in support of the idea that activated Gpa1 stimulates recovery. Our data also suggest that the Gpa1-mediated adaptive signal is induced by pheromone, is delayed relative to the mating signal, and does not depend on sequestration of $G_{\beta\gamma}$.

MATERIALS AND METHODS

Yeast strains, media, and microbiological techniques. All strains used in this study except those used for the two-hybrid analysis were derived from strain 15Dau (*MAT***a** *bar1*Δ *ade1 his2 leu2-3,112 trp1 ura3*Δ), which is congenic with strain BF264-15D, described previously (39). Unless otherwise noted, the designation of cells as wild type, E364K, G322E, or N388D refers to strain 15Dau transformed with the centromeric vector YCplac111 or YCplac22 (16) containing the wild-type, E364K, G322E, or N388D allele of *GPA1* (46). Thus, these strains express a plasmid-borne allele of *GPA1* as well as the native copy of *GPA1*. For experiments requiring the overexpression of *GPA1* alleles, 15Dau cells were transformed with plasmids containing the *GPA1* coding sequence fused to attenuated *GAL1* promoters (see below). For experiments requiring the expression of *GPA1* alleles in a *gpa1*Δ background, strain 15Dau *gpa1*::*ura3* *GAL1*^{EG43}-*GPA1* was grown in galactose-based medium, transformed with the test alleles, and plated on selective glucose-based medium. The Ste4 overexpression strains used for the Ste4 sequestration assay (Fig. 5) were constructed as follows: the attenuated *GAL1-STE4* fusion plasmid, GAL1^{EG43}-STE4, was integrated at the *LEU2* locus of strain 15Dau, and a single representative transformant was used as a recipient for the final set of vectors, YCplac22, YCplac22/GPA1$^{wild\text{-}type}$, and YCplac22/E364K. All yeast transformations were carried out by the method of Ito et al. (23).

Yeast growth media were prepared as described by Sherman et al. (42). Leucine, tryptophan, and uracil were omitted as necessary from synthetic media in order to maintain plasmids. For experiments requiring the induction of the *GAL1*-regulated genes, cells were grown to mid-log phase in selective sucrose medium and galactose was added to a final concentration of 2%, or the cells were pelleted and resuspended in selective galactose medium.

Plasmids. Recombinant DNA techniques were essentially as described by Sambrook et al. (41) and by Ausubel et al. (1).

To facilitate construction of the *GAL4*$^{1\text{-}148}$::*GPA1* hybrid genes, we altered the polylinker of the Gal4$^{1\text{-}148}$ expression plasmid, pGBT9, kindly provided by S. Fields. Two synthetic oligomers, 5′ AAT TGG ATC CTC TAG AAT TC 3′ and 5′ GAT CGA ATT CTA GAG GAT CC 3′, were annealed, creating a double-stranded molecule with 5′ overhangs corresponding to *Bam*HI and *Eco*RI sticky ends. Plasmid pGBT9 was then digested with *Bam*HI and *Eco*RI, and the new polylinker was inserted. The polylinker of the new plasmid, pGBT9-BXE, contains restriction sites for *Sal*I, *Bam*HI, *Pst*I, *Xba*I, and *Eco*RI. The *GAL4*$^{1\text{-}148}$::*GPA1* translational fusions used in this study were created by subcloning the entire coding regions of three *GPA1* alleles (wild type, E364K, and G322E) into the *Bam*HI and *Eco*RI sites of pGBT9-BXE. Directed subcloning of the *GPA1* alleles into a polylinker designed specifically to preserve their translational reading frames allowed us to avoid the problem of PCR-induced mutagenesis. The Gal4$^{768\text{-}881}$::STE4 plasmid used in our two-hybrid analysis was graciously provided by Karen Clark and has been described elsewhere (7).

The YCplac22/GPA1 plasmids used in the Ste4 sequestration assay (Fig. 5) were constructed by moving the *Xho*I-*Sst*II fragment containing *GPA1* alleles from various YCplac111/GPA1 plasmids (46) into the polylinker of YCplac22 (16).

The plasmid designated GAL1^{EG43}-STE4 is a *LEU2* integrative vector (YIplac128) (16) containing a *GAL1* promoter fragment, weakened by partial deletion (17), joined to the *STE4* coding region just upstream of the *STE4* ATG. This attenuated *GAL1* promoter is approximately 1% as active as the native *GAL1* promoter (17). The plasmid designated GAL1^{EG43}-GPA1 was constructed by replacing the coding region of *STE4* on plasmid YIplac128/GAL1^{EG43}-STE4 with the coding region of *GPA1*. The plasmid designated GAL1^{EG28}-E364K is a *TRP1* integrative vector (YIplac204) (16) containing a *GAL1* promoter fragment, also weakened by partial deletion (17), joined to the E364K coding region just upstream of the *GPA1* ATG. This attenuated *GAL1* promoter is approximately 18% as active as the native *GAL1* promoter (17). The plasmid designated GAL1^{EG28}-GPA1 was constructed by replacing the E364K allele on plasmid YIplac128/GAL1^{EG28}-E364K with the coding region of *GPA1*. The attenuated *GAL1* promoter fragments were amplified by PCR from plasmids described by Giniger and Ptashne (17), pEG43 and pEG28. The PCR products were then ligated into the polylinker of YCplac33 (16), using two naturally occurring restriction sites, *Nsi*I and *Bam*HI. Finally, the attenuated *GAL1* promoter fragments were moved to the polylinkers of YIplac128 and YIplac204 (16) as *Sph*I-*Bam*HI fragments.

In vitro mutagenesis. The N388D mutant allele of *GPA1* was created in vitro by using the Chameleon Mutagenesis system (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. A subclone of wild-type *GPA1* on the YCplac22 vector (YCplac22/GPA1) was used as the template. The selection primer, 5′ CCA TGC AGT TGG ACT ATG TCA ATG CCG TAA TCA TTG ACC 3′, was designed to destroy the unique *Eco*RV site in YCpac22/GPA1. The mutagenic primer, 5′ CCG TTT ATT TTG TTT TTA GAT AAA ATT GAT TTG TTC GAG G 3′, was designed to change the adenine at nucleotide position 1162 of the *GPA1* coding region to guanine.

Pheromone response assays. Strains were tested for pheromone-induced growth inhibition in standard halo assays as previously described (9). After growth to mid-log phase, approximately 10^5 cells were diluted into 7 ml of top agar (0.7%) and spread onto plates. Various doses of synthetic α-factor (Multiple Peptide Systems, San Diego, Calif.) were then dotted onto the surface of the plates in 4-μl aliquots. The plates were incubated for 2 days at 30°C prior to photographing.

Recovery assays were performed by treating either asynchronous mid-log phase cells or early-G_1 cells collected from late-log-phase cultures with 0.5 μg of synthetic α-factor and growing them at 30°C. At the indicated times, aliquots of each culture were placed in 1/10 volume of 37% formaldehyde for a total volume of 200 μg. The fixed cells were then sonicated briefly (six 0.5-s pulses with a Braun-sonic sonicator set on low) and examined at a magnification of ×400 in a Leitz Laborlux S phase-contrast microscope. Budding indices were determined with the aid of a hemacytometer. At least 300 cells were counted for each time point. Photomicrographs of cells in liquid medium (Fig. 1B, 2B, and 7C) were taken with a Zeiss Axioskop microscope fitted with differential interference contrast (Nomarski) optics. Photomicrographs of cells on plates (Fig. 7A and B) were taken with an Axiovert 100 microscope, using bright-field illumination.

Centrifugal elutriation. Cells to be elutriated were grown in selective sucrose medium to late log phase (~4×10^7 cells per ml). A half liter of culture was then chilled in an iced 2-liter beaker and exposed to the full power of a Braun-sonic sonicator for two 1.5-min intervals. The sonicated cells were slowly loaded into an elutriation rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 2,000 rpm and 4°C, and the speed of the rotor was adjusted until an equilibrium at which only the newly abscised daughter cells flowed through the chamber and into an iced collection flask was established. When approximately 5% of the cells had been collected, they were concentrated by centrifugation and resuspended in cold YEPD at a density of 4×10^6/ml. The elutriated cells were kept on ice until α-factor was added; the cultures were then grown and sampled as described above.

Two-hybrid analysis. The two-hybrid analysis was carried out in strain Y526 (*leu2 his3 trp1 GAL1-lacZ* Δ*GAL4* Δ*GAL80*), kindly provided by S. Fields. To prevent induction of the pheromone response by the Gal4$^{768\text{-}881}$::Ste4 chimeric protein, a *MAT***a**/α diploid version of this strain was isolated by transient expression of a *GAL1-HO* fusion and screened for competence to sporulate. The Y526 **a**/α strain was then cotransformed with the Gal4$^{768\text{-}881}$::STE4 and GAL4$^{1\text{-}148}$::GPA1 plasmids. After growth to mid-log phase in selective medium, six cotransformants of each type were assayed for β-galactosidase activity as previously described (43).

Immunoblots. Crude cell lysates were prepared by harvesting 2×10^8 mid-log cells and resuspending them in 200 μg of 2× sample buffer (0.24 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol, 20% glycerol). After the cells were boiled for 4 min, acid-washed glass beads (0.45-mm diameter) were added to the suspension. The mixture was vortexed vigorously at 4°C until lysis was complete and then boiled again for 2 min. The lysates were spun in a clinical centrifuge at 2,000 rpm to remove the glass beads and then for 10 min in a microcentrifuge (12,000 × *g*) to pellet the cell debris. The resulting lysates were normalized by measuring A_{280}, and approximately 40-μg samples (where 1 A_{280} unit is assumed to equal 1 mg of protein per ml) were electrophoresed on a discontinuous SDS-polyacrylamide gel (4%/10%) and electroblotted to a PVDF-Plus transfer membrane (Micron Separations, Inc., Westboro, Mass.) according to the protocol supplied with the semidry blotter (Fisher Scientific, Pittsburgh, Pa.). Blots were then blocked with 5% nonfat dry milk in Tris-buffered saline (25 mM Tris-HCl [pH 8.0], 135 mM NaCl, 3 mM KCl) overnight. The blots were subsequently incubated with dilute strip-purified antiserum raised against full-length Gpa1 (45) in 0.2× blocking solution in Tris-buffered saline for 4 h and processed with an ECL immunoblot detection kit (Amersham, Buckinghamshire, England) as instructed by the manufacturer. Strip purification of the Gpa1 antiserum was performed as described by Harlow and Lane (18).

RESULTS

E364K, a mutant form of Gpa1, stimulates recovery from pheromone-induced cell cycle arrest. In previous studies designed to elucidate the relationship between the structure and function of Gpa1, two classes of mutant *GPA1* alleles were identified (46). The first class consists of alleles that confer insensitivity to pheromone. Mutations analogous to those that lock mammalian $G_{\alpha s}$ in an inactive conformation, or mutations in the carboxy-terminal region known to be required for interaction with the pheromone receptors, create alleles that belong to this class. *GPA1* alleles of the second class confer a complex phenotype: mutant cells are apparently supersensitive to pheromone but also have an increased ability to recover from pheromone treatment. The existence of this phenotype has been invoked to support a model in which the activated form of Gpa1 stimulates downregulation of the pheromone response pathway, or what we have called hyperadaptivity. Others have argued that these alleles confer insensitivity to pheromone and not an augmented ability to recover from pheromone treatment (28). We sought to determine which of these two views is correct.

One method of assessing response to pheromone is the growth inhibition zone assay, or halo assay. In a halo assay, *MAT***a** cells are spread on the surface of a plate and challenged with a localized source of α-factor, the pheromone secreted by *MAT*α cells. After 24 to 48 h, a circular zone of growth inhibition where the concentration of α-factor is sufficient to prevent progression through the G_1 phase of the cell cycle can be seen. Halo size is thus a function of sensitivity to pheromone. Strains that are insensitive to pheromone, such as those expressing *GPA1* alleles of the first class, exhibit smaller than normal halos or no halos at all. In contrast, expression of a *GPA1* allele of the second class generates a heterogeneous cell population in which some cells respond to pheromone and form a halo, while others can divide and form colonies within the zone of growth inhibition (see Fig. 4). A priori, colonies growing within the halo could arise from cells that are unresponsive to pheromone or from cells that respond normally but recover more readily. Halo tests do not discriminate between insensitivity and hyperadaptivity. To distinguish these possibilities, a simple assay was devised to determine the kinetics and degree of response. Of the three *GPA1* alleles belonging to the second class, G50D, E355K, and E364K, the latter was chosen for detailed analysis.

The effect of pheromone treatment on the proliferation of strains either expressing the wild-type allele of *GPA1* or coexpressing wild-type *GPA1* along with the E364K allele is shown in Fig. 1. Wild-type cells transformed with either YCplac22/GPA1 or YCplac22/E364K were grown to mid-log phase in selective medium. At time zero, the cultures were treated with 0.5 μg of α-factor per ml, and changes in the proportion of budded cells and in cellular morphology were monitored by light microscopy. The proportion of budded cells serves as an index for pheromone responsiveness because pheromone causes cells to arrest in G_1, the only phase of the cell cycle in which yeast cells are unbudded. In addition, the pheromone-induced cellular enlargement and elongation provides a qualitative measure of the response. In the experiment represented in Fig. 1, for example, it is clear that the control cells are responding maximally to the administered dose of α-factor. Although the cells are 45% unbudded at zero time, typical of a mid-log-phase culture growing in this medium, the entire population arrests in G_1 of the first cell cycle following pheromone treatment, becoming 100% unbudded within one generation time. These cells continue to enlarge and elongate but remain blocked in G_1 for the duration of the experiment. Most of the wild-type cells send out multiple projections (Fig. 1B), a phenomenon diagnostic of terminal arrest (44a). For the first hour following pheromone treatment, the E364K strain appears to respond normally, accumulating the same proportion of unbudded cells as the wild-type culture. Between the 120- and 150-min time points, however, the percentage of unbudded E364K cells peaks at 80% and begins to decline. Eight hours after pheromone treatment, the wild-type culture is still 100% unbudded whereas the E364K culture is again growing logarithmically.

Is the ability of E364K cells to grow when exposed to an otherwise lethal dose of pheromone due to an adaptive mechanism or to the proliferation of a subpopulation of insensitive cells? Microscopic examination of the experimental culture reveals that both phenomena contribute to the pheromone resistance of the E364K strain (Fig. 1B). Two hours after pheromone treatment, the large majority of cells expressing E364K are unbudded and exhibit the pear-shaped (shmoo) morphology characteristic of the mating response. However, a small number of cells are budded. Some of the budded cells are of normal size and shape, apparently insensitive to pheromone. Others are enlarged and elongated, apparently having responded transiently to pheromone. At the 4- and 6-h time points, these morphological differences are more pronounced: the E364K cells can be easily recognized as either nonresponders (small, oval, budded cells), responders (very large and elongated cells, many with multiple projections), or transient responders (very large and elongated cells that are budding). The existence of the latter class of cells, the budded shmoos, demonstrates that the E364K allele of *GPA1* does not augment pheromone resistance in every case merely by conferring insensitivity. A substantial fraction of cells expressing E364K exhibit a response to pheromone followed by a rapid recovery. This observation is consistent with the hypothesis that Gpa1 stimulates an adaptive signal but does not explain the heterogeneous nature of the E364K culture.

A plausible hypothesis is that the pheromone responsiveness of individual E364K cells depends on their position in the cell cycle. Cells that have just passed Start (the point in late G_1 at which pheromone blocks cell cycle progression) when they are first exposed to α-factor may have time to fully induce an adaptive mechanism before once again reaching Start, their first opportunity to exhibit responsiveness (cell cycle arrest and projection formation). These cells, then, are insensitive to pheromone. Conversely, cells that are very close to but have not yet reached Start at the moment of pheromone addition may have insufficient time to adapt fully before reaching the arrest point. Such cells might be overwhelmed by the stimulus and hence become terminally arrested. Finally, cells that are midway through the cell cycle when challenged with pheromone might become partially adapted prior to reaching Start. Such cells would respond transiently and then recover.

A fixed temporal lag in the induction of the Gpa1-mediated adaptive mechanism could thus explain the heterogeneity of the E364K culture. If this hypothesis is correct, a synchronous population of E364K cells should exhibit a uniform response to α-factor. To test this idea, centrifugal elutriation was used to collect wild-type and E364K cells early in the G_1 phase of the cell cycle. Synchronized cultures were then divided into two parts, incubated with or without 0.5 μg of pheromone per ml, and scored for budding. As shown in Fig. 2, cells in the untreated cultures began to bud between 40 and 80 min after their release into fresh medium following elutriation. The variable delay prior to bud emergence can be attributed to differences in cell size in the elutriated populations and to variable

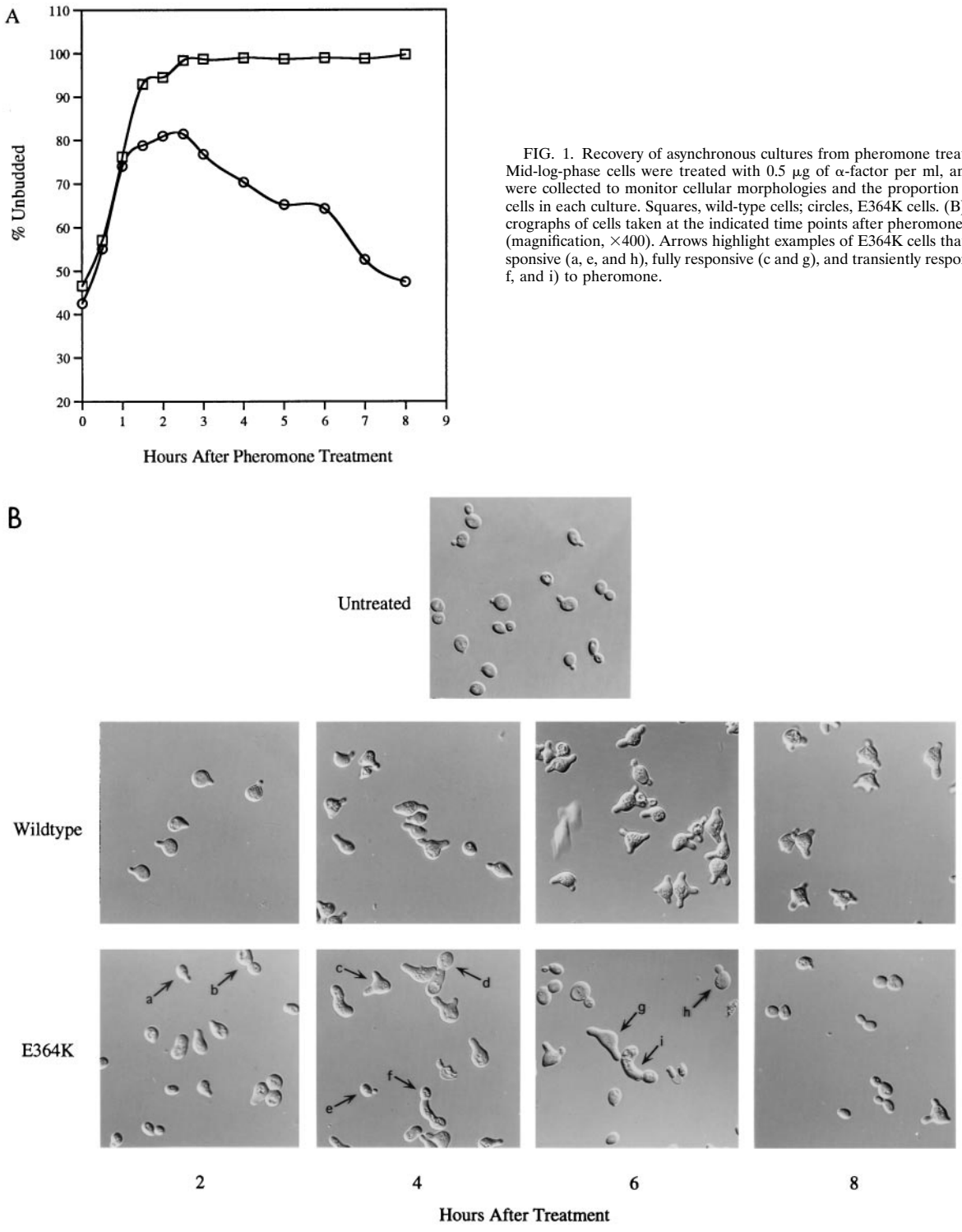

FIG. 1. Recovery of asynchronous cultures from pheromone treatment. (A) Mid-log-phase cells were treated with 0.5 μg of α-factor per ml, and samples were collected to monitor cellular morphologies and the proportion of budded cells in each culture. Squares, wild-type cells; circles, E364K cells. (B) Photomicrographs of cells taken at the indicated time points after pheromone treatment (magnification, ×400). Arrows highlight examples of E364K cells that are unresponsive (a, e, and h), fully responsive (c and g), and transiently responsive (b, d, f, and i) to pheromone.

periods of recovery from the elutriation procedure. The striking differences in the behavior of the three pheromone-treated cultures, on the other hand, can be attributed to functional differences in the three alleles of *GPA1*. In Fig. 2A, it is clear that the treated wild-type cells were unable to progress past Start. The treated E364K cells, although eventually capable of budding, showed a delay of 1 h (Fig. 2B). Thus, E364K does not block the pheromone response. Rather, this mutant form of Gpa1, in conjunction with pheromone, slowly induces a mechanism that overrides the mating signal. In a third elutriation experiment, we examined the pheromone responsiveness of early G_1-synchronized cells expressing the G322E allele of *GPA1*. G322E has previously been demonstrated to confer complete resistance to pheromone in a halo assay (46). As

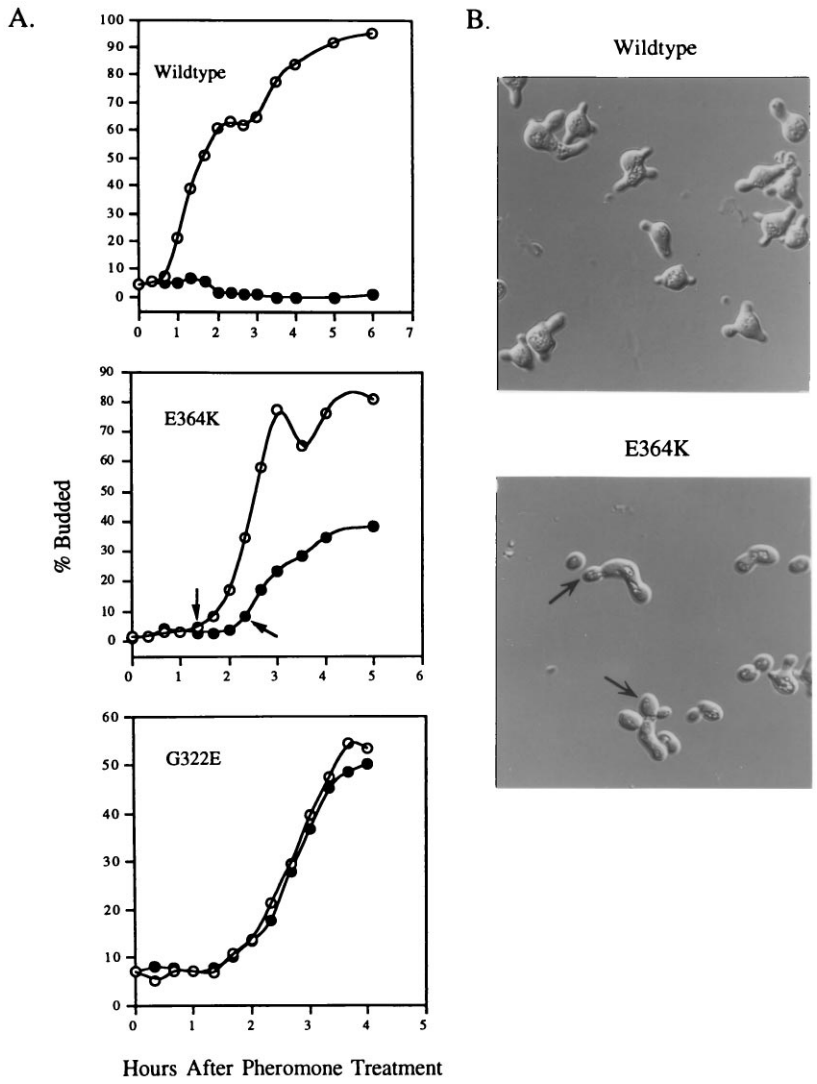

FIG. 2. Recovery of G_1-synchronized cells from treatment with pheromone. Cells in early G_1 were collected by centrifugal elutriation and resuspended in fresh medium lacking pheromone or containing 0.5 μg of α-factor per ml. Time points were taken to monitor the resumption of cell division and the morphological response to pheromone. (A) Graphs showing the proportion of budded cells in each culture plotted as a function of time after release into warm medium. Open symbols, untreated cultures; closed symbols, cells treated with α-factor. Arrows indicate the time points at which budded cells were first observed. (B) Photomicrographs showing wild-type and E364K cells at the 5-h time point (magnification, ×400). Budded shmoos can be seen in the E364K culture (arrows).

shown in Fig. 2C, α-factor has no effect on the proliferation of synchronous G322E cells, just as it has no effect on an asynchronous culture of this strain (data not shown). Since G322E is analogous to a form of $G_{\alpha s}$ that irreversibly binds to $G_{\beta\gamma}$ (31, 34), it is not surprising that this allele confers complete resistance to pheromone regardless of cell cycle position. Together, these experiments highlight a functional difference between G322E and E364K. G322E confers insensitivity—an inability to respond to pheromone. E364K confers a heightened ability to desensitize, or adapt, over time.

E364K is not overexpressed and does not confer pheromone resistance by sequestering G_β. The results shown in Fig. 1 and 2 suggest that E364K induces an adaptive mechanism that is delayed relative to the mating signal. Because the expression of *GPA1* is itself induced two- to threefold by pheromone (24), we wondered whether the augmented recovery was dependent on an increase in the level of E364K following pheromone treatment. To test this possibility, wild-type cells transformed with either YCplac22/GPA1 or YCplac22/E364K were again grown to mid-log phase in selective medium and treated with 0.5 μg of α-factor per ml. Aliquots of each culture were harvested at 2-h intervals during a 9-h time course to assess Gpa1 levels by immunoblot analysis. As shown in Fig. 3, the induction of Gpa1 in the experimental cells (lanes 6 to 10) was indistinguishable from that in the control cells (lanes 1 to 5). Moreover, cells transformed with the galactose-inducible construct $GAL1^{EG28}$-GPA1 and grown on medium containing galactose expressed significantly more Gpa1 than fully induced E364K cells (lane 16) but nevertheless showed no sign of hyperadaptivity in halo assays (Fig. 4). Cells in which the expression of *GPA1* is induced from the full-strength *GAL1* promoter form slightly smaller than normal halos, presumably because the great excess of inactive Gpa1 more effectively sequesters βγ, but develop no turbidity within the halos (9). These results clearly demonstrate that E364K does not stimulate adaptation by virtue of overexpression.

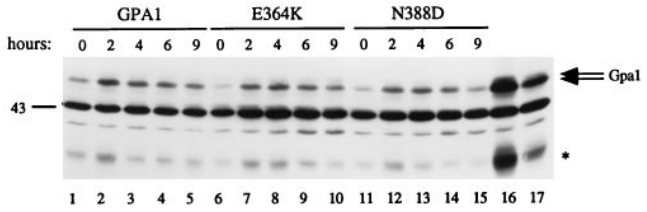

FIG. 3. Time course of Gpa1 induction in pheromone-treated cells. Mid-log-phase cells were treated with 0.5 μg of α-factor per ml, and portions of each culture were harvested at the indicated times. Crude lysates were prepared as described in Materials and Methods and electrophoresed on denaturing acrylamide gels. Proteins were then blotted to a PVDF-Plus membrane and probed with strip-purified antiserum raised against Gpa1. The arrows mark the full-length forms of Gpa1; the asterisk marks a Gpa1 degradation product. The indicated molecular mass is in kilodaltons. Lanes: 1 to 5, wild-type cells transformed with a centromeric plasmid containing wild-type *GPA1* (YCplac22/GPA1^{Wildtype}); 6 to 10, wild-type cells transformed with YCplac22/E364K; 11 to 15, wild-type cells transformed with YCplac22/N388D; 16, wild-type cells transformed with GAL1^{EG28}-GPA1, grown in the absence of pheromone; 17, wild-type cells transformed with GAL1^{EG43}-GPA1, grown in the absence of pheromone. The identity of the Gpa1 bands is known both from their overexpression in the *GAL1-GPA1* strains and from their absence in a *MAT***a**/α diploid strain (Fig. 8).

Although the supersensitive phenotype displayed by cells expressing E364K in a *gpa1*Δ background (46), as well as the results reported above, are inconsistent with the idea that Gpa1-mediated adaptation is dependent on direct binding of G_α to G_β, two additional experiments were performed to explore this possibility. First, the two-hybrid assay (5, 15) was used to quantitate the relative affinities of wild-type Gpa1, E364K, and G322E for G_β (Ste4). Clark et al. (7) and Whiteway et al. (49) have previously used this method to examine the interactions between Gpa1 and Ste4 and between Ste4 and Ste18. Their work demonstrated the utility of the two-hybrid assay in assessing G-protein subunit interaction. If adaptation involves sequestration of Ste4 by Gpa1, the hyperadaptive E364K form of Gpa1 would be expected to exhibit a greater affinity for Ste4 than the wild-type protein in this assay. Hybrid genes consisting of the DNA binding domain (codons 1 to 148) of *GAL4* fused in frame to each of the three alleles of *GPA1* (wild-type, E364K, and G322E) were constructed. These constructs were then used to transform the diploid yeast strain Y526 **a**/α, along with a chimera consisting of the *STE4* coding region fused in-frame to the transcriptional activation domain of *GAL4* (codons 768 to 881). A *MAT***a**/α diploid strain was used to prevent the Ste4 hybrid protein from adversely affecting cell growth by activating the pheromone response pathway. To derive the best possible estimations of G_α-G_β interaction, six independent cotransformants were assayed for each pair of chimeric proteins. For this assay, *GAL4*$^{1-147}$::*GPA1* and *GAL4*$^{768-881}$::*STE4* hybrid genes were cotransformed into strain Y526 **a**/α. The resulting strains were grown to mid-log phase in selective sucrose medium, and β-galactosidase activity was measured as described in Materials and Methods. β-Galactosidase activities were 55.0 ± 7.7, 46.9 ± 5.9, 84.0 ± 15.5, 0.32 ± 0.1, and 0 U for DNA binding domain fusions with *GPA1*, E364K, G322E, N388D, and vector, respectively (means and standard deviations of six independent cotransformants for each allele of *GPA1*). β-Galactosidase activity was not detectable in Y526 **a**/α cells singly transformed with any of the chimeric plasmids.

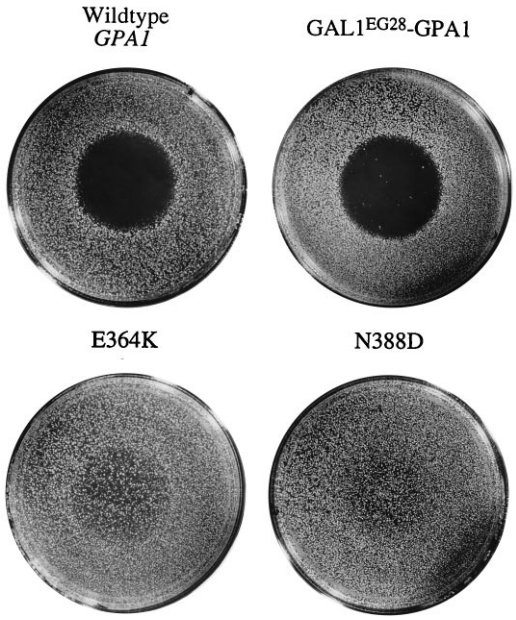

FIG. 4. N388D confers a dominant hyperadaptive phenotype. YCplac22 centromeric vectors containing either wild-type *GPA1*, N388D, or E364K were transformed into wild-type 15Dau cells. The *GPA1* overexpression strain was created by integrating a GAL1^{EG28}-GPA1 plasmid (YCplac128/GAL1^{EG28}-GPA1) at the *LEU2* locus of 15Dau. (The induced expression level of Gpa1 in these cells is shown in Fig. 3, lane 16.) Halo tests were performed on the resulting transformants in selective medium containing 4 μg of α-factor.

Although interaction with the Gal4$^{768-881}$::Ste4 protein was easily detectable for all three Gal4$^{1-148}$::Gpa1 hybrids, the β-galactosidase activity varied among transformants for a given pair of *GAL4* hybrids. Nevertheless, a clear pattern was apparent: the Gal4$^{1-147}$::E364K transformants exhibited about 15% less and the Gal4$^{1-147}$::G322E transformants exhibited about 50% more β-galactosidase activity than the wild-type control transformants. These differences are probably not due to variable expression of the Gal4$^{1-147}$::Gpa1 hybrid proteins: an immunoblot blot probed with a Gpa1 polyclonal antibody revealed similar levels of Gal4$^{1-147}$::Gpa1 in a subset of the transformants (data not shown). Rather, the results presented above most likely reflect differences in G_α-G_β affinity. Consistent with previous phenotypic analysis, these data suggest that E364K binds G_β with an affinity no greater than, and possibly less than, that of wild-type Gpa1, whereas G322E has an enhanced ability to interact with G_β.

A second experiment designed to assess G_β sequestration took advantage of the strict stoichiometric relationship between Gpa1 and Ste4 (9, 38, 50). Because as little as one extra copy of *STE4* is sufficient to activate the mating signal, the relative affinities of wild-type Gpa1 and E364K for G_β could potentially be measured in a strain in which excess Ste4 production is induced from a conditional promoter and pheromone pathway activity is monitored with a reporter construct. Such a strain was constructed by integrating two chimeric genes into 15Dau: *GAL1*^{EG43}-*STE4* allows modest induction of Ste4 when cells are grown on galactose-containing medium, and *FUS1-lacZ* (pSB231) (47) serves as an indicator of pathway activity, since β-galactosidase expression is driven by a pheromone-inducible promoter. This parent strain, 15Dau *GAL1*^{EG43}-*STE4 FUS1-lacZ*, was transformed with a centromeric plasmid, YCplac22, as well as YCplac22 recombinant constructs containing wild-type *GPA1* or E364K. The resulting strains were grown to log phase in selective sucrose-containing medium. Galactose was then added to 2% to induce Ste4, and aliquots of each culture were harvested over a 48-h period for determination of β-galactosidase activity.

Prior to induction of *GAL1*^{EG43}-*STE4*, the control strain

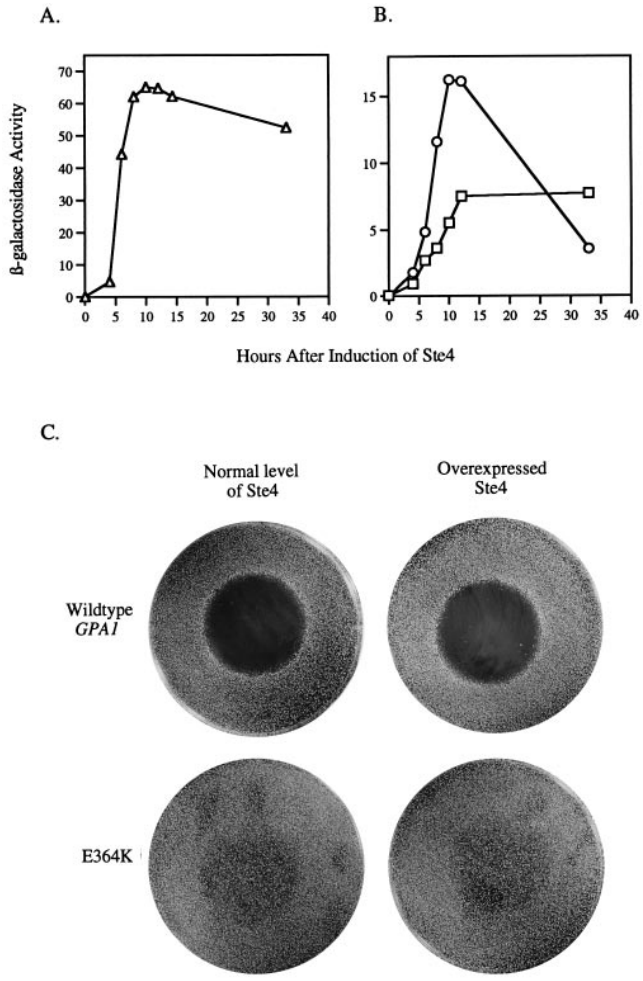

FIG. 5. Induction of pheromone pathway activity by excess Ste4. Cells containing *GAL1-STE4* and *FUS1-lacZ* transcriptional fusions were transformed with either YCplac22, YCPlac22/*GPA1*$^{wild\text{-}type}$, or YCplac22/E364K. Transformants were grown to mid-log phase in selective sucrose medium and then shifted to selective galactose medium. At the indicated time points, the cultures were sampled and β-galactosidase activity was measured as described in Materials and Methods. The results are representative of four similar experiments in which the mean ratio of β-galactosidase activity in E364K cells compared with wild-type cells 8 h after induction of Ste4 expression was 3.6 ± 1.8. (A) YCplac22 cells; (B) wild-type (squares) and E364K (circles) cells. (C) Halo tests performed 10 h after induction of the *GAL1-STE4* construct.

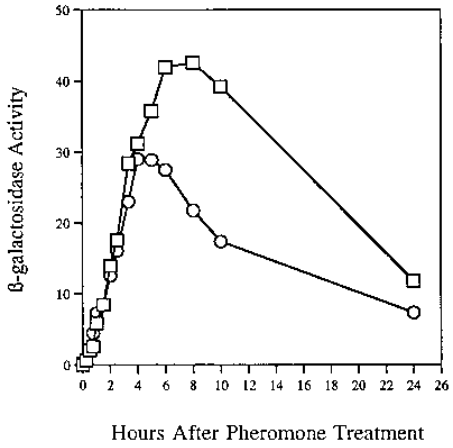

FIG. 6. N388D does not block the initial response to pheromone. Wild-type 15Dau cells containing the *FUS1-lacZ* transcriptional reporter were transformed with plasmids YCplac22/GPA1$^{wild\text{-}type}$ and YCplac22/N388D. Transformants were grown to mid-log phase in selective medium and treated with 0.5 μg of α-factor per ml. At the indicated time points, the cultures were sampled and β-galactosidase activity was measured as described in Materials and Methods. The results are representative of three similar experiments. Squares, wild-type cells; circles, N388D cells.

carrying the YCplac22 plasmid alone should express Gpa1 and Ste4 in the normal ratio, whereas cells transformed with either of the *GPA1*-containing centromeric vectors should produce roughly twice as much Gpa1 as usual. With the induction of *GAL1*^{EG43}*-STE4*, the initial change in pheromone pathway activity (prior to induction of Gpa1-mediated adaptation) should be a function of how well the excess Gpa1 can sequester the nascent Ste4. As expected, β-galactosidase activity rose to a high level in the control strain (Fig. 5A), indicative of pheromone pathway activation due to overproduction of Ste4. In contrast, the excess Gpa1 produced by cells transformed with the YCplac22/GPA1$^{wild\text{-}type}$ plasmid was able to sequester most of the nascent Ste4: the β-galactosidase activity rose to only 15% of the maximum level measured in the control cells (Fig. 5B). The results presented in Fig. 5B also indicate that E364K sequesters nascent Ste4 considerably less well than wild-type Gpa1. If Gpa1 and E364K have equal affinities for Ste4, we would expect equivalent induction of *FUS1-lacZ* in the two strains. In fact, the peak of β-galactosidase activity in the E364K strain was approximately threefold greater than that in the wild-type cells.

The relatively poor initial ability of E364K to sequester G_β in this assay led us to examine whether the E364K transformants would still exhibit pheromone resistance while overexpressing Ste4. The halo assays shown in Fig. 5C demonstrate that E364K confers resistance to pheromone whether excess Ste4 is produced or not. The halos formed by E364K cells plated 10 h after the induction of *GAL1*^{EG43}*-STE4* are as turbid as those formed by E364K cells expressing only the wild-type level of Ste4. Thus, E364K can terminate the pheromone response in cells expressing excess Ste4. These results, along with the results of the two-hybrid analysis, indicate that Gpa1 stimulates recovery from pheromone treatment by a mechanism other than G_β sequestration.

A mutation predicted to activate Gpa1 confers hyperadaptivity. In all well-characterized G-protein-mediated signaling pathways, G_α subunits interact with their effector proteins while in their GTP-bound, or activated, state. Because the biochemical nature of the E364K mutant protein is unclear, we created a novel mutation in *GPA1*, N388D, that is expected to activate G_α proteins. Mutations in the analogous position of other G proteins have been shown to decrease affinity for GDP and thus shift the protein's conformational equilibrium to the GTP-bound state (2, 11, 19, 37).

As shown in Fig. 4, the N388D allele of *GPA1* confers a dominant pheromone resistance phenotype at least as dramatic as that of E364K. When expressed in a wild-type background, N388D stimulates colony formation at doses of α-factor that are normally inhibitory to growth (Fig. 4), and it does so by a mechanism that is independent of Gpa1 overexpression (Fig. 3, lanes 11 to 15). Moreover, it is clear that N388D does not simply block the pheromone response. During the first 4 h following pheromone treatment, wild-type cells expressing N388D arrest in G_1 and form projections (data not shown) and induce the pheromone-specific *FUS1-lacZ* reporter (pSB231) (47) in a manner indistinguishable from that of control cells (Fig. 6).

TABLE 1. Adaptive and βγ sequestration activities of three forms of Gpa1

GPA1 allele	Halo appearance and diam (cm)[a]	Rescue of *gpa1*Δ[b]	Morphology of transformed cells (% normal)	Plasmid shuffle[c]
None (vector alone)	Clear, 3.8	No growth		0
GPA1	Clear, 3.8	45 h	90	20
E364K	Turbid, 3.5	45 h	50–70	20
N388D	Very turbid, 3.2	+/−[d]	0	0

[a] Halo tests were performed on wild-type cells carrying the indicated vectors.
[b] Ability to form colonies after *GAL1-GPA1* is repressed in a *gpa1*Δ strain.
[c] Percent colonies having lost the wild-type *GPA1* plasmid.
[d] N388D/*gpa1*Δ cells formed very small and aberrant colonies after 6 days.

Despite its ability to promote adaptation to pheromone, N388D cannot bind βγ: in experiments designed to compare the abilities of various forms of Gpa1 to sequester the Ste4/Ste18 dimer, N388D behaved like a null allele. A 15Dau-derived strain whose only means of expressing *GPA1* is from an attenuated *GAL1* promoter (15Dau *gpa1*::*ura3 GAL1*^{EG43}-*GPA1*) was transformed with the YCplac22 centromeric vector as well as various YCplac22 subclones containing the E364K, N388D, and wild-type alleles of *GPA1*. Portions of each transformation mixture were spread on both galactose-containing and glucose-containing media in order to assess transformation efficiency and cell viability in the absence of *GAL1*^{EG43}-*GPA1* expression. As expected, no colonies formed when cells were transformed with the YCplac22 vector and plated on glucose medium (Table 1), even though transformation efficiency, as judged by colony formation on galactose plates, was normal. Cells transformed with the YCplac22/N388D vector grew extremely slowly, taking 6 days to form very small and morphologically aberrant colonies, made up of severely elongated and misshapen cells (Table 1 and Fig. 7). In contrast, 15Dau *gpa1*::*ura3 GAL1*^{EG43}-*GPA1* cells transformed with YCplac22/GPA1 and YCplac22/E364K grew normally following repression of the genomic copy of *GPA1*, taking only 2 days to form large colonies. Microscopic examination of the YCplac22/GPA1-transformed cells showed that 90% were of normal morphology (Fig. 7). The remaining 10% exhibited signs of pheromone pathway activation, presumably due to loss of the YCplac22/GPA1 plasmid. Interestingly, the YCplac22/E364K transformants displayed a higher proportion of enlarged and elongated cells, suggesting either that the E364K mutant protein has a lesser affinity for βγ or that it is less stable than wild-type Gpa1 (data not shown).

In a second experiment designed to assess the ability of N388D to sequester βγ, a 15Dau *gpa1*Δ strain carrying a YCplac111/GPA1 vector was transformed with the YCplac22 vector and YCplac22 subclones containing E364K, N388D, and wild-type *GPA1*. After selection for colonies that contained both the resident and transforming plasmids, the resulting strains were grown for 10 generations on medium that allowed for loss of the YCplac111/GPA1 plasmid while maintaining the *GPA1* allele under analysis. Plasmid stability was then measured by replica plating single colonies grown on nonselective medium to the appropriate test medium. As shown in Table 1, 20% of the 15Dau *gpa1*Δ cells transformed with either YCplac22/GPA1 or YCplac22/E364K lost the original plasmid-borne copy of *GPA1* in the course of 10 generations, whereas none of the cells transformed with YCplac22/N388D could grow without a wild-type copy of *GPA1*. This experiment, as well as the one described above, clearly indicates that N388D, a mutant form of Gpa1 that stimulates dramatic resistance to pheromone when coexpressed with wild-type Gpa1, is unable to rescue a *gpa1*Δ strain and hence is likely to be severely defective in βγ binding. Consistent with this idea, a Gal4$^{1-147}$::N388D chimeric protein showed no interaction with the Gal4$^{768-881}$::Ste4 chimera in the two-hybrid assay (see above). Expression of the full length Gal4$^{1-147}$::N388D chimera was confirmed by immunoblot analysis (data not shown).

Careful consideration of the results represented in Fig. 4 and Table 1 raises an important question. When coexpressed with wild-type Gpa1, N388D can promote adaptation to pheromone. However, N388D is unable to inhibit the signaling activity of the βγ subunits released when the expression of wild-type Gpa1 is repressed. Does the N388D hyperadaptive phenotype require the presence of wild-type Gpa1 and its βγ binding activity, or does N388D hyperadaptivity depend on an activity that can only be induced by pheromone, not by free βγ? To address this question, 15Dau *gpa1*::*ura3 GAL1*^{EG43}-*GPA1* cells were again transformed with the YCplac22 subclones containing the E364K, N388D, and wild-type alleles of *GPA1*. As before, portions of each transformation mixture were spread on both galactose-containing and glucose-containing media in order to assess transformation efficiency and cell viability in the absence of *GAL1*^{EG43}-*GPA1* expression. In addition, aliquots of transformed cells were spread on glucose plates containing 10, 25, and 100 ng of α-factor per ml. The results of this experiment are summarized in Table 2. As expected, the E364K cells formed normal-size colonies after 2 days at 30°C at all doses of α-factor, whereas the cells expressing wild-type Gpa1 grew normally at the two lower doses of α-factor and were unable to overcome the highest dose. Remarkably, the growth rate and morphology of the N388D cells were indistinguishable from those of wild-type cells when α-factor was included in the growth medium, even though this strain formed extremely slowly growing and morphologically aberrant colonies when cultured in identical medium lacking α-factor (Table 2 and Fig. 7). Furthermore, when N388D cells that had been grown on α-factor plates were spread on plates lacking pheromone, they once again formed morphologically aberrant microcolonies composed of enlarged and misshapen cells (Fig. 7). Thus, α-factor is an essential growth supplement to *MAT***a** cells whose sole expressed form of Gpa1 is N388D. Taken together, these data suggest that the N388D mutant form of Gpa1 can block the mating signal by a mechanism that requires a pheromone-inducible factor but which is independent of βγ sequestration by G_α. It is noteworthy that the full rescue was observed at 10 ng of α-factor per ml, a dose at which little or no induction of N388D was seen (Fig. 8). This result argues against the possibility that the pheromone-inducible factor is N388D itself.

DISCUSSION

It is likely that all signaling pathways are modulated by antagonistic regulatory controls. Adaptive mechanisms prevent

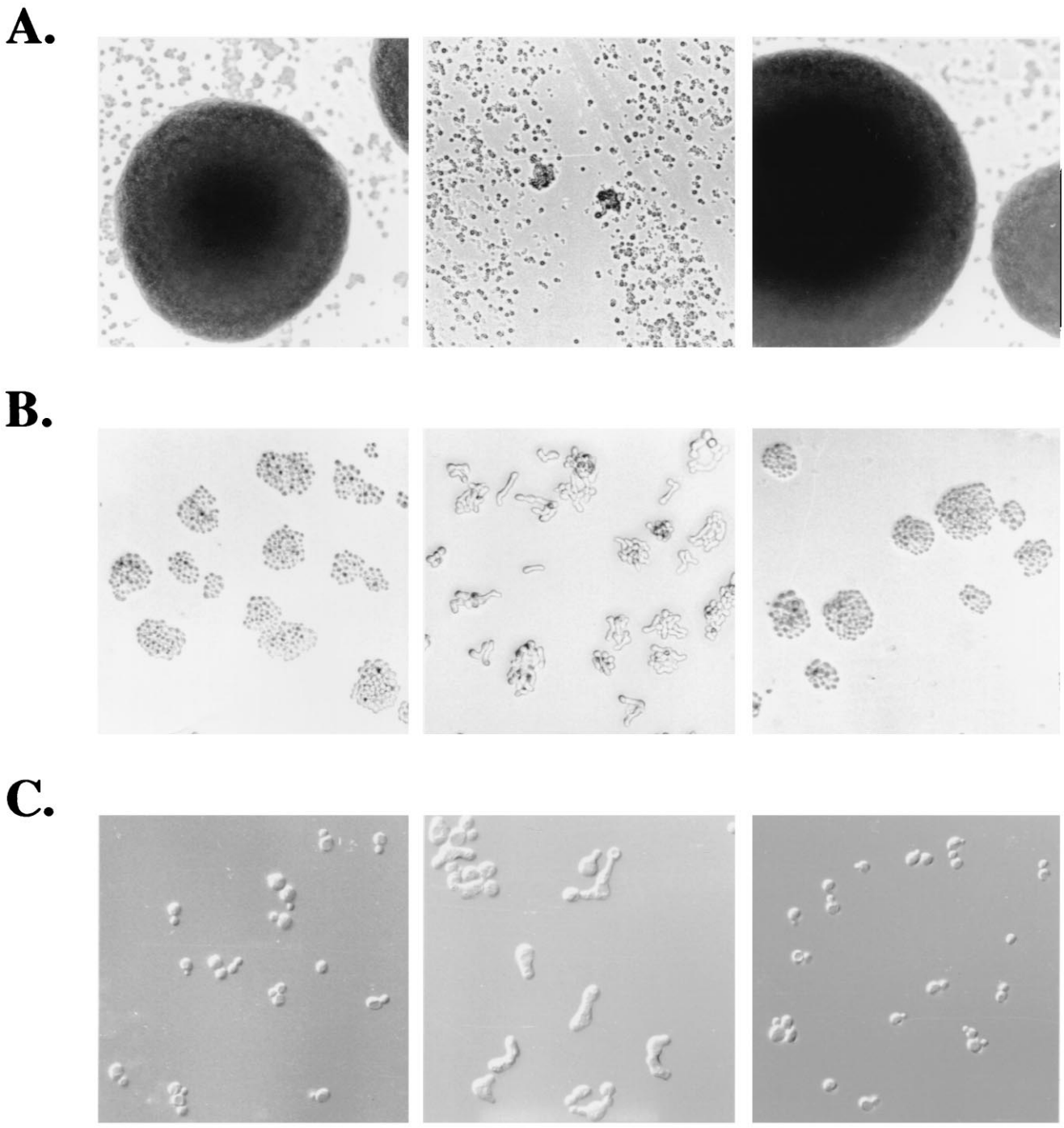

FIG. 7. Gpa1-mediated adaptation is potentiated by mating pheromone. (A) Photomicrographs of *GPA1*/*gpa1*Δ colonies (left) and N388D/*gpa1*Δ colonies (middle) growing on medium lacking α-factor and of N388D/*gpa1*Δ colonies growing on medium containing 10 ng of α-factor per ml (right). (B) Phase-contrast photomicrographs (magnification, ×100) of *GPA1*/*gpa1*Δ cells (left) and of N388D/*gpa1*Δ cells 14 h after being streaked onto plates lacking α-factor (middle) and onto medium supplemented with 10 ng of α-factor per ml (right). (C) Differential interference contrast (Nomarski) photomicrographs (magnification, ×400) of *GPA1*/*gpa1*Δ cells cultured without α-factor (left), N388D/*gpa1*Δ cells 14 h after being shifted to medium lacking α-factor (middle), and N388D/*gpa1*Δ cells growing in liquid medium containing 10 ng of α-factor per ml (right).

inappropriate induction of signals, allow for the fine-tuning of signaling activity, and are essential for the downregulation of signals when cells are chronically stimulated. The mating pheromone response of *S. cerevisiae* exemplifies all of these points. To mate efficiently, haploid yeast cells must sense one another's presence and polarize their growth such that contact is made between mating partners. This demands a constitutively expressed and highly sensitive signaling system. At the same time, the mating signal must be tightly controlled. In the absence of pheromone, a fortuitous rise in the basal activity of the mating signaling pathway could result in cell cycle arrest. During exposure to pheromone, negative regulatory mecha-

TABLE 2. Abilities of plasmid-borne *GPA1* alleles to stimulate colony formation in cells lacking wild-type Gpa1

GPA1 allele[a]	Colony formation[b]			
	0[c]	10	25	100
None (vector alone)	−	−	−	−
GPA1	++++	++++	++++	−
E364K	++++	++++	++++	++++
N388D	+/−	++++	++++	++++

[a] Test alleles were expressed in a *gpa1*Δ background as described in the text.
[b] ++++, normal-size colonies formed after 48 h at 30°C; +/−, microcolonies formed after 144 h at 30°C; −, no colonies formed.
[c] Dose of α-factor in nanograms per milliliter.

nisms presumably ensure that cellular responses such as projection formation (shmooing) and mating-specific gene expression will not be overinduced, so that cells which fail to mate can resume vegetative growth. The importance of such controls is reflected in the redundancy of means by which *S. cerevisiae* negatively regulates the pheromone response. Since desensitization of the yeast pheromone response was first observed (36), adaptive mechanisms have been identified throughout the pathway, operating on the ligand, the receptors, the G_β subunit, and one of the MAP kinases (Fus3). The *SST2* gene product and the recently discovered Mot2/Sig1 protein are thought to act at the level of the G protein (13, 30, 44).

In this work, we provide evidence that *S. cerevisiae* utilizes an adaptive mechanism that is stimulated by the pheromone-responsive G_α protein, Gpa1. Thus, Gpa1 plays at least two roles in regulating the pheromone response. In its inactive conformation, it sequesters the $G_{\beta\gamma}$ subunit, tethering it to the receptor so that it can be released at the appropriate time. During continuous stimulation, presumably when Gpa1 is in its active state, it downregulates the signal generated by $G_{\beta\gamma}$ via a mechanism other than sequestration. Our data also suggest that Gpa1-mediated adaptation is delayed relative to the mating signal and that it is induced by pheromone.

Gpa1 stimulates an adaptive signal that is induced by pheromone, is delayed relative to the mating signal, and does not depend on sequestration of βγ by α-factor. The possibility that Gpa1 stimulates adaptation was first raised by Miyajima et al., who examined the pheromone response of cells expressing

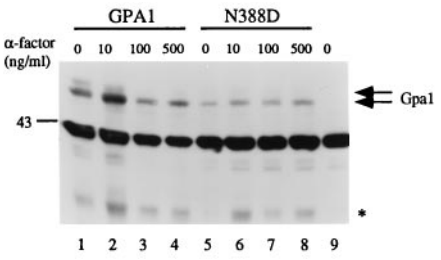

FIG. 8. Dose-response curve of Gpa1 induction in pheromone-treated cells. Cultures were grown to mid-logarithmic phase in selective glucose-containing medium, treated with the indicated doses of α-factor, and harvested after 5 h. Crude lysates were prepared and electrophoresed on denaturing acrylamide gels. Proteins were then blotted to a PVDF-Plus membrane and probed with strip-purified antiserum raised against Gpa1. The arrows mark the full-length forms of Gpa1; the asterisk marks a Gpa1 degradation product. The indicated molecular mass is in kilodaltons. Prior to initiation of the experiment, the cells expressing N388D were grown in medium containing 10 ng of α-factor per ml. To prepare the sample shown in lane 6, N388D cells were washed free of α-factor and cultured for 12 h prior to harvest. Lanes: 1 to 4, *GPA1*$^{\text{wild-type}}$/*gpa1*::*URA3* cells; 5 to 8, N388D/*gpa1*::*URA3* cells; 9, *MAT***a**/α diploid cells (congenic with strain 15Dau) grown in the absence of pheromone.

G50V, a mutant allele of *GPA1* that they created in vitro (35). When expressed in a *gpa1* null background, G50V confers both supersensitivity and resistance to pheromone. The halos are larger than normal and turbid. Cells expressing both G50V and wild-type *GPA1* exhibit normal-size halos that are also filled in. Thus, G50V confers recessive supersensitivity and dominant resistance. In a screen for additional pheromone-resistant alleles of *GPA1*, we identified three more mutations that confer similar halo phenotypes: G50D, E355K, and E364K (46). Because the G50V and G50D mutations of *GPA1* are analogous to activating mutations of mammalian Ras and were presumed to shift the conformational equilibrium of Gpa1 toward the active state, the unusual halo phenotypes were said to result from a decreased ability of the mutant form of Gpa1 to bind $G_{\beta\gamma}$, combined with an increased ability to stimulate adaptation. More recently, Kurjan et al. have called this inference into question (28). They reported that two additional mutations expected to activate Gpa1, N388K and D391A, confer a null phenotype. They also reevaluated the effect of G50V on the pheromone response while controlling for copy number of the mutant allele. When integrated as a single copy into the yeast genome, G50V was reported to confer poor growth due to a constitutively high but poorly inducible mating signal. In cells carrying a plasmid-borne copy of G50V, Kurjan et al. suggested that the high basal mating signal creates selective pressure for plasmid amplification and, ultimately, pheromone resistance. According to this view, G50V causes cells to become insensitive, presumably by sequestering $G_{\beta\gamma}$; it does not stimulate adaptation.

We sought to distinguish Gpa1-mediated adaptation from the more trivial possibility that pheromone-resistant alleles of *GPA1* simply confer insensitivity via βγ sequestration. To avoid amplification of the E364K centromeric plasmid, we performed all of our experiments in an E364K/*GPA1*$^{\text{wild-type}}$ background. Wild-type haploid cells transformed with the YCplac22/E364K centromeric plasmid grow normally and do not induce mating-specific genes (e.g., *FUS1*) in the absence of pheromone. Thus, there is no reason to believe that the E364K copy number is elevated or that the cells are constitutively desensitized. In fact, immunoblot analysis indicates that wild-type cells transformed with plasmid YCplac22/E364K do not accumulate greater steady-state or pheromone-induced levels of Gpa1 than cells transformed with plasmid YCplac22/GPA1 (Fig. 3). Moreover, overexpression of wild-type Gpa1 does not confer a hyperadaptive phenotype (reference 9 and Fig. 3 and 4). Nevertheless, when E364K/*GPA1*$^{\text{wild-type}}$ cells are challenged with α-factor in a plate assay, they exhibit normal-size halos that are filled with colonies. In other words, the culture appears to consist both of cells that display normal sensitivity and cells that are resistant to pheromone. By monitoring the morphology and proliferation of E364K/GPA1$^{\text{wild-type}}$ cells exposed to pheromone in liquid culture, we discovered that the treated E364K culture is actually composed of three subpopulations: cells that respond fully, those that respond transiently, and those that do not respond at all. That some cells expressing E364K are able to resume cell division despite exhibiting the same morphological changes as the permanently arrested wild-type control cells rules out any model in which E364K simply blocks the pheromone response (Fig. 1B). The heterogeneous nature of E364K cells responding to α-factor is understandable if one assumes that the Gpa1-mediated adaptive mechanism is induced by pheromone and that it develops slowly relative to the signal driven by $G_{\beta\gamma}$. We tested this possibility by repeating our liquid pheromone response assay with cultures that had been synchronized in early G_1. When E364K cells were given only a minimal cell cycle interval between being challenged

with α-factor and reaching Start, we were able to detect a full response (Fig. 2). The G_1-to-S phase transition of every cell in the culture was delayed by at least 60 min. From these results, we conclude that E364K does not confer an inability to respond to pheromone but rather confers a time-dependent ability to become resistant upon continual stimulation. We estimate that it takes at least two-thirds of a cell cycle (60 min in complete medium at 30°C) for E364K cells to overcome a large dose of α-factor. The results of this experiment also suggest that activation of the mating signal is necessary for full induction of Gpa1-mediated adaptation. If E364K alone were sufficient to fully induce adaptation, then all cells expressing this mutant form of Gpa1 would become constitutively desensitized and thus unresponsive to pheromone regardless of cell cycle position. This idea is strongly supported by the behavior of *MAT***a** cells whose only expressed allele of *GPA1* is N388D. Such cells are inviable when inoculated into standard growth media lacking α-factor, presumably because free βγ constitutively induces the mating signal. When grown in media containing α-factor, however, N388D/*gpa1*Δ cells show no sign of pathway activation. In conjunction with N388D, pheromone induces an adaptive mechanism that downregulates the mating signal.

Can the ability of E364K to confer pheromone resistance be due to $G_{\beta\gamma}$ sequestration? Because Gpa1 is thought to directly bind and inactivate Ste4, we performed two experiments to assess the ability of E364K to sequester $G_{\beta\gamma}$. In the first approach, we examined whether the two-hybrid assay could detect differences in the affinities of various forms of Gpa1 for Ste4. We presume that the G322E protein binds Ste4 with a greater than normal affinity since the glycine at position 322 of Gpa1 is analogous to the glycine at position 226 of $G_{\alpha s}$. Substantial evidence indicates that this residue is critical for the activation of G_α proteins. The G226A allele of $G_{\alpha s}$ encodes a protein that is effectively locked in its inactive conformation and that binds $G_{\beta\gamma}$ with a greater affinity than does the wild-type protein (31, 34). Given the high degree of both structural and functional conservation among G_α proteins, and the pheromone-unresponsive phenotype of cells expressing the G322E allele of *GPA1*, it is highly likely that the G322E mutant protein is unable to shift to the active conformation and release βγ. The results of our two-hybrid analysis support this idea. The $Gal4^{1\text{-}147}$::G322E/$Gal4^{768\text{-}881}$::Ste4 cotransformants averaged about 50% more β-galactosidase activity than did the wild-type control strains. Thus, despite being localized to the nucleus in a chimeric and unmyristoylated form, the behavior of G322E in this assay correlates well with its effect on the pheromone response in situ and with the biochemical characteristics of the G226A form of $G_{\alpha s}$ in vitro. In contrast, the $Gal4^{1\text{-}147}$::E364K hybrid protein did not show an unusually high affinity for $Gal4^{768\text{-}881}$::Ste4. Since the two-hybrid assay is sensitive to the effect of the G322E mutation on α-β interaction, our failure to observe a similar effect in the $Gal4^{1\text{-}147}$::E364K transformants can be taken as evidence that E364K inhibits the pheromone response by a mechanism that does not depend on enhanced $G_{\beta\gamma}$ sequestration.

In an effort to confirm the conclusions suggested by the two-hybrid analysis, we designed a means to test the relative abilities of wild-type Gpa1 and E364K to sequester $G_{\beta\gamma}$ in situ. We constructed a strain expressing the native copies of both *GPA1* and *STE4*, as well as one of the two test alleles carried on centromeric plasmids, in which we could conditionally express Ste4 from an attenuated *GAL1* promoter. It had previously been shown that a slight excess of Ste4 can activate the pheromone response, even without overexpression Ste18 (G_γ) (9, 38, 50). As expected, wild-type Gpa1 decreased induction of the pheromone response, as indicated by a *FUS1-lacZ* transcriptional reporter, presumably by binding the excess $G_{\beta\gamma}$. In comparison with wild-type Gpa1, the E364K protein was less capable of inhibiting activation of the pheromone response, as evidenced by a threefold-higher level of β-galactosidase activity 14 h after inducing Ste4 expression. This result is consistent with the view that E364K does not block the pheromone response by sequestering $G_{\beta\gamma}$ but is able to stimulate, along with unknown pheromone-specific factors, a delayed adaptive mechanism that downregulates the mating signal.

Our study of N388D, a novel allele of *GPA1*, provides strong support for each of these inferences. Although stably expressed, the N388D mutant protein cannot rescue a *gpa1*Δ strain. Therefore, the N388D form of Gpa1 must not be able to bind βγ well enough to prevent high constitutive induction of the mating signal. This idea is supported by our failure to observe interaction between N388D and Ste4 in the two-hybrid assay (see Results) and the inability of N388D to bind to a Ste4-Ste18 ($G_{\beta\gamma}$) affinity column (5a). Despite its defect in βγ binding, however, N388D is able to downregulate the pheromone response and confer viability in a *gpa1*Δ background when cells are exposed to pheromone. This finding suggests the existence of a pheromone-inducible activity which, in conjunction with Gpa1, promotes adaptation. The surprising observation that the N388D-dependent desensitization mechanism is activated by the occupied pheromone receptor, but not by free βγ, suggests the involvement of the receptor itself. Although the α-factor receptor (Ste2) is thought to stimulate recovery in combination with a pheromone-inducible gene product called Afr1 (25), the hyperadaptive activities of both E364K and N388D are completely uncompromised in a strain expressing *ste2*T-326 (26), an adaptive activity-deficient C-terminal truncation allele of *STE2* (46a). Similarly, the *SST2* gene product is highly induced by pheromone and has been shown to downregulate the mating signal at a point prior to βγ (13), but E364K and N388D both stimulate recovery from high doses of pheromone in an *sst2*Δ background (46b). Moreover, the inducible desensitization mechanism is not likely to depend on an increase in Gpa1 expression since cells expressing N388D in a *gpa1*Δ background grow normally in medium containing 10 ng of α-factor per ml (Table 2 and Fig. 7), a dose that does not significantly increase the steady-state level of the mutant G_α protein (Fig. 8). In light of these data, it is likely that the pheromone-inducible, G_α-dependent adaptation effector has yet to be discovered. Recently, we have identified a candidate for this inducible effector—a protein which is moderately and slowly induced by α-factor, peaking between 2 and 4 h after pheromone treatment (45a). A slow increase in expression in response to stimulus might be expected of a protein involved in the inhibition, rather than the transmission, of the mating signal.

For years following the discovery of heterotrimeric G proteins, it was commonly believed that only the G_α subunits possessed signaling activity. The βγ subunits were thought to play an ancillary role, inhibiting the activity of the α subunits. It is now clear that both the α and βγ subunits interact with effector molecules and transduce signals (for reviews, see references 3, 6, and 20). *S. cerevisiae* has provided an example of a G protein with dual signaling activities. Considered as a whole, our data suggest a picture in which the pheromone-responsive heterotrimeric G protein in budding yeast cells has a self-regulatory signaling function. Upon activation, the heterotrimer separates into its two subunits, one of which stimulates the pheromone response, while the other slowly induces a negative regulatory mechanism that ultimately shuts off the mating signal downstream of the receptor. By means that are

not yet clear, the positive and negative signaling activities are balanced to allow for response and recovery, as well as to prevent inappropriate induction while preserving sensitivity. Antagonistic regulation of a MAP kinase cascade by the βγ and α subunits of a G protein has also been observed in COS-7 cells (10).

The nature of the hyperadaptive alleles of *GPA1*. One can imagine two ways by which a mutation such as E364K could create a hyperadaptive allele of *GPA1*. The substitution might affect the conformational equilibrium of Gpa1 such that the activated state (the GTP-bound form) is favored, or the alteration might augment the interaction of Gpa1 with the adaptation effector. A number of observations bear on this question. First, because there is an impressive precedent for mutations that activate GTP-binding proteins and thereby increase signaling activity, it would not be surprising to discover similar mutational effects on Gpa1. Second, the three-dimensional crystal structures of the active and inactive forms of transducin-alpha (T_α-GTP and T_α-GDP) implicate positions 232 and 241, analogous to positions 355 and 364 of Gpa1, as participants in salt bridges that affect the conformation of G_α proteins (29, 37). As reported by Stone and Reed (46), the E355K allele of *GPA1* confers a halo phenotype very similar to that of E364K. Third, position 388 of Gpa1 is analogous to a position that contacts the guanine nucleotide ring in both small GTP-binding proteins, such as Ras and EF-Tu, and G_α subunits (2, 11, 19, 37). Mutations at this position have been found to increase the dissociation rate of GDP and thus shift the conformational equilibrium of G proteins to the GTP-bound state, as predicted by the three-dimensional crystal structures of α-GTP and α-GDP (17a, 29, 37). The effect that the N388D form of Gpa1 has on yeast cells is very informative. N388D cannot rescue cells lacking wild-type Gpa1 (Tables 1 and 2) yet acts as a potent stimulator of adaptation in cells treated with pheromone (Table 2 and Fig. 7). Presumably, the βγ dimer is free to signal in N388D/*gpa1*Δ cells because most of the G_α is in its active conformation.

It has been reported that other mutations predicted to activate Gpa1, such as N388K and D391A, create null alleles instead of hyperadaptive alleles of *GPA1* (28). Because the functions of these alleles were assessed by methods that differed from ours, as well as in a different genetic background, the results are not directly comparable. It is also possible that the proteins encoded by these alleles are unstable or are not localized properly. As shown in Fig. 3, the steady-state and pheromone-induced levels of the E364K and N388D proteins are similar to those of wild-type Gpa1.

In addition to their presumed role in maintaining three-dimensional structure, the residues E355 and E364 of Gpa1 are located in a domain thought to be involved in the interaction between T_α and its effector, cyclic cGMP phosphodiesterase (29, 37), between $G_{\alpha s}$ and its effector, adenylate cyclase (29, 37), and between $G_{\alpha q}$ and its effector, phospholipase C (48). This observation suggests an alternative to the idea that the E364K substitution affects the conformation of Gpa1. The E355K and E364K mutations of *GPA1* may augment adaptive function by increasing the affinity of Gpa1 for the adaptation effector or by otherwise increasing the efficacy of the Gpa1-effector interaction. We are currently attempting to purify Gpa1 in order to assess the effects of various mutations on the conformation of the protein in vitro. We are also conducting a genetic screen in an effort to identify the G_α-driven adaptation effector. Thus far, we have found two complementation groups whose functions are required for Gpa1-mediated adaptation.

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