THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 34, Issue of August 20, pp. 35287–35297, 2004
Printed in U.S.A.

Dominant-negative Inhibition of Pheromone Receptor Signaling by a Single Point Mutation in the G Protein α Subunit*

Received for publication, May 3, 2004, and in revised form, June 9, 2004
Published, JBC Papers in Press, June 14, 2004, DOI 10.1074/jbc.M404896200

Yuh-Lin Wu‡, Shelley B. Hooks§, T. Kendall Harden§, and Henrik G. Dohlman‡§¶

From the ‡Department of Biochemistry and Biophysics and the §Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599-7260

In yeast, two different constitutive mutants of the G protein α subunit have been reported. Gpa1^{Q323L} cannot hydrolyze GTP and permanently activates the pheromone response pathway. Gpa1^{N388D} was also proposed to lack GTPase activity, yet it has an inhibitory effect on pheromone responsiveness. We have characterized this inhibitory mutant (designated Gα^{ND}) and found that it binds GTP, interacts with G protein βγ subunits, and exhibits full GTPase activity *in vitro*. Although pheromone leads to dissociation of the receptor from wild-type G protein, the same treatment promotes stable association of the receptor with Gα^{ND}. We conclude that agonist binding to the receptor promotes the formation of a nondissociable complex with Gα^{ND}, and in this manner prevents activation of the endogenous wild-type G protein. Dominant-negative mutants may be useful in matching specific receptors and their cognate G proteins and in determining mechanisms of G protein signaling specificity.

Mammalian G protein-coupled receptors (GPCRs)[1] respond to a variety of signaling molecules, including hormones, neurotransmitters, odors, and light. These signals regulate diverse cellular processes, such as the control of blood pressure and heart rate, perception of pain, cell proliferation, inflammation, and platelet aggregation (1–3). Upon binding of an agonist to its cognate receptor, the G protein α subunit transits from a GDP-bound to a GTP-bound state and liberates the G protein βγ subunit complex. The dissociated α (in the GTP-bound state) and βγ subunits then activate a variety of downstream effectors. Regulators of G protein signaling reverse this process by binding to Gα-GTP and promoting GTP hydrolysis, after which the subunits reassociate and signaling terminates (4).

A prominent feature of G protein signaling is the tremendous diversity of the component proteins. Human genome analysis has revealed genes that encode several hundred candidate GPCRs, 16 α subunits, 5 β subunits, and 12 γ subunits (5). Further diversity results from alternative mRNA splicing and the potential of a given receptor to transduce signals to multiple G protein subunit subtypes and effectors. These signaling components do not assemble randomly; rather, one receptor typically activates only a subset of G protein heterotrimers, and the dissociated subunits activate only a subset of downstream effectors (5).

Clearly, a major challenge is to define the coupling specificity of specific receptors, G proteins, and effectors. Bacterial toxins have long been used to perturb signaling mediated by susceptible G proteins. Cholera toxin catalyzes the ADP-ribosylation of $G\alpha_s$ resulting in inactivation of its GTPase activity, thus maintaining $G\alpha_s$ in the active GTP-bound state. Pertussis toxin catalyzes ADP-ribosylation of $G\alpha_i$, and this modification blocks G protein coupling to GPCRs (6). However, pertussis toxin does not modify all members of the $G\alpha_i$ subfamily, and no toxins have been identified that modify members of the $G\alpha_q$ and $G\alpha_{12}$ subfamilies. Thus, more general approaches are needed to analyze receptor and G protein coupling specificity.

An alternative approach to studying G protein signal specificity has been to mutate residues in Gα critical for GTPase activity. One early example of an activated G protein allele was described by Landis *et al.* (7), who showed that certain types of human pituitary tumors are associated with GTPase-deficient mutants of $G\alpha_s$. Another GTPase-deficient mutant replaces Gln-204 in $G\alpha_i$ (Gln-277 in $G\alpha_s$ and Gln-323 in Gpa1) (8–10). A crystal structure of $G\alpha_i^{Q204L}$ and additional biochemical analysis suggest that the catalytic Gln acts by stabilizing the trigonal-bipyramidal transition state and by helping to orient the hydrolytic water molecule (11, 12). This mutation is widely used to identify signaling pathways activated by Gα subunits, and we recently used this approach to show that the yeast Gα subunit Gpa1 directly activates the mating response pathway, in conjunction with Gβγ (13).

Another approach is to inactivate G protein function by using mutants that confer a dominant-negative effect on signaling (14). Dominant-negative mutants are proteins that disrupt the function of the endogenous wild-type protein when overexpressed. Thus, highly specific dominant-negative Gα proteins have tremendous potential for ascertaining the signaling specificity of diverse G proteins in complex systems. Although such mutants have been reported (15–21) for various Gα subunits, they have not yet been proved to be generally applicable to studying G protein signaling.

In contrast to the large number and variety of mammalian GPCRs, only two distinct G protein signaling systems exist in yeast. The first regulates the response to mating pheromones, which are secreted by haploid **a** and α cell types in preparation for mating. Pheromone binding to receptors (*e.g.* Ste2 in **a** cells) triggers dissociation of Gα (Gpa1) from the Gβγ heterodimer (Ste4/Ste18). The dissociated subunits proceed to activate a mitogen-activated protein kinase cascade, resulting in new gene transcription, cell cycle arrest, and eventually cell fusion to form the **a**/α diploid (22). The second signaling pathway

* This work was supported by National Institutes of Health Grants P01-GM65533 (to T. K. H. and H. G. D.) and F32-GM66561 (to S. B. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, University of North Carolina, CB 7260, Chapel Hill, NC 27599-7260. Tel.: 919-843-6894; Fax: 919-966-2852; E-mail: hdohlman@med.unc.edu.

[1] The abbreviations used are: GPCRs, G protein-coupled receptors; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; Ni-NTA, nickel-nitrilotriacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione *S*-transferase.

mediates the cellular response to glucose and environmental stressors such as high osmolarity and heat shock. Components of this pathway include the Gα protein Gpa2 working in conjunction with the putative glucose receptor Gpr1 (23, 24). Recent studies of Gpr1 signaling have identified two candidate Gβ subunits, Gpb1 and Gpb2, and a candidate Gγ, Gpg1 (25, 26). The Gpb1/2 proteins lack the seven WD40 repeats found in classical Gβ proteins, but instead contain seven kelch repeats implicated in protein-protein interaction (26). The effector for this G protein has not been positively identified, and generally speaking much less is known about this pathway.

Two different constitutive mutants in the yeast Gα subunit have been described. Gpa1^{Q323L} binds but does not hydrolyze GTP (13, 27). Gpa1^{N388D} was proposed to lack GTPase activity but paradoxically has an opposing or *inhibitory* effect on the pathway (28–30). We previously characterized the biochemical and physiological function of Gpa1^{Q323L} (13, 27), and here we characterize the inhibitory Gα^{ND} mutant. We report that Gα^{ND} binds and hydrolyzes GTP but is unable to dissociate effectively from receptors and therefore acts as a potent dominant-negative inhibitor of receptor signaling.

MATERIALS AND METHODS

Strains, Media, and Plasmids—Established methods were used for the growth and genetic manipulation of bacteria and yeast (31). *Escherichia coli* strain DH 5α was used for plasmid maintenance and amplification. The strains of yeast *Saccharomyces cerevisiae* used in this study are as follows: YPH499 (*MAT***a** *leu2*-Δ1 *his3*-Δ200 trp1-Δ63 *ade2*-101^{oc} *lys2*-801^{am} *ura3*-52), YPH501 (YPH499 *MAT***a**/α) (32), YGS5 (YPH499 *gpa1*::*hisG ste11*^{ts}) (33), BY4741 (*MAT***a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; from Research Genetics, Huntsville, AL), and a BY4741-derived *gpa1 ste7* mutant strain (*MAT***a** *ste7*::KanMX *gpa1*::*hisG*, provided by Paul Flanary, University of North Carolina).

Yeast cells were grown in synthetic medium supplemented with adenine, amino acids, 2% glucose (SCD), or 2% galactose plus 0.2% sucrose (SCG) to express *GAL1/10*-inducible genes. Leucine, uracil, tryptophan, or histidine was omitted to maintain selection of plasmids as needed. Yeast cells were grown at 30 °C unless otherwise stated. The absence of *GPA1* in strain YGS5 normally results in constitutive G$\beta\gamma$ signaling and growth arrest; however, these cells can be maintained at 34 °C due to inactivation of the temperature-sensitive *ste11* mutant.

Several expression plasmids used in this study have been described previously: pRS315 (*CEN*, *LEU2*), pRS423 (2 μm, *HIS3*), pRS424 (2 μm, *TRP1*) (32), pAD4M (2 μm, *LEU2, ADH1* promoter and terminator), and pAD4M-GST and pAD4M-GPA1-GST (34). p*GAL*^{H} (2 μm, *LEU2*) and p*GAL*^{L} (2 μm, *LEU2*) contain partially active *GAL1/10* promoter sequences having 18–20 (p*GAL*^{H}) or 1% (p*GAL*^{H}) full activity (35) (provided by Ming Guo, Yale University). pRS315-GPA1 contains the *GPA1* gene under the control of the native promoter.

Wild-type human Gα_{i2} in pcDNA3.1 mammalian expression vector was obtained from the Guthrie cDNA Resource Center (Sayre, PA). The N270D mutation was introduced using a QuikChange mutagenesis kit (Stratagene, Alameda, CA). The mutant was amplified by PCR and ligated into pFastBacHta (Invitrogen) digested with BamHI and XhoI, which introduced an in-frame hexahistadine tag at the N terminus (His$_6$-Gα_{i2}^{N270D}-pFastBacHta). Other Sf9 expression plasmids were described previously (36, 37).

GPA1^{Q323L}, *GPA1*^{N388D}, *GPA2*^{N365D}, and Gα_{i2}^{N270D} mutations were introduced using QuikChange. The primer sequences are as follows: 5′-TCGACGCTGGAGGCCTGCGTTCTGAACG-3′ and 5′-CGTTCAGAACGCAGGCCTCCAGCGTCGA-3′ for *GPA1*^{Q323L}; 5′-CGTTTATTTTGTTTTTAGATAAAATTGATTTGTTC-3′ and 5′-GAACAAATCAATTTTATCTAAAAACAAAATAAACG-3′ for *GPA1*^{N388D}; 5′-TCTGTCGTACTCTTTCTGGATAAAATCGACCTTTT TG-3′ and 5′-CAAAAAGGTCGATTTTTATCCAGAAAGAGTACGACAGA-3′ for *GPA2*^{N365D}; 5′-TCCATCATCCTCTTCCTCGACAAGAAGGACCTGTTTG-3′ and 5′-CAAACAGGTCCTTCTTGTCGAGGAAGAGGATGATGGA-3′ for Gα_{i2}^{N270D}. Each mutation was confirmed by sequencing analysis.

Expression of G$\beta\gamma$ (Ste4/Ste18) was under the control of the bidirectional *GAL1/10* promoter in pRS424-GAL-STE4/STE18. This plasmid was constructed by combining a SalI-EcoRI digestion product containing the *GAL1/10* promoter and *STE4* (from pL19, provided by Malcolm Whiteway, University of Montreal) (38) with an EcoRI-SacI digestion product of *STE18* amplified by PCR using the primers 5′-GGGAATTCTAGGATAGTAGCAATCGCA-3′ and 5′-GAGGCTCTACGTAGCAAG-3′ and a SalI-SacI digestion product of plasmid pRS424. Expression of *STE2* or *STE7* was achieved by PCR amplification and subcloning into the pYES2.1/V5-His-TOPO (2 μm, *URA3*, *GAL1/10* promoter, *CYC1* terminator; Invitrogen). Amplification primers used were 5′-CCCAAGCTTCCAGAATGTCTGATGCGGCTCCTTC-3′ and 5′-CCCAAGCTTTAAATTATTATTATCTTCAGTC-3′ for *STE2*; 5′-GCATCGGATCATATCTGTTT-3′ and 5′-GCTGGAAAAAGAAGAGACTA-3′ for *STE7*. A stretch of double-stranded DNA encoding three tandem repeats of the FLAG tag (sense, 5′-GATTATAAAGATGACGATGACAAGGATTATAAAGATGACGATGACAAGGATTATAAAGATGACGATGACAAG-3′) in pYES2.1/V5-His-TOPO was digested with HindIII and ligated to HindIII-digested *STE2* that had been PCR-amplified using the primers 5′-CCCAAGCTTCCAGAATGTCTGATGCGGCTCCTTC-3′ and 5′-CCCAAGCTTTAAATTATTATTATCTTCAGTC-3′, yielding pYES-STE2-FLAG.

Expression of Gα_{i2}^{ND} in Insect Cells—A baculovirus encoding human Gα_{i2}^{N270D} with an N-terminal hexahistidine tag was prepared and amplified using plasmid his$_6$-Gα_{i2}^{N270D}-pFastBacHta according to the manufacturer's instructions (Invitrogen). Four liters of Sf9 cells at a density of 1.5×10^6 cells/ml were infected with the virus at a multiplicity of infection of 2 and harvested 48 h after infection by centrifugation at $1,000 \times g$ for 15 min at 4 °C. All subsequent steps were carried out at 4 °C. Cells were resuspended in 400 ml of ice-cold cell lysis buffer (20 mM HEPES, pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 9.8 mM 2-mercaptoethanol, 0.01 mM GDP, 500 nM aprotinin, 10 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, 1 nM pepstatin, 10 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone) and lysed by passage through a pressurized Emulsiflux (Avestin, Ottawa, Canada). The lysate was centrifuged at $500 \times g$ for 15 min to remove intact cells and nuclei. The cleared lysate was further centrifuged at $150,000 \times g$ for 35 min in an ultracentrifuge (Beckman). The resulting supernatant fraction was passed over an equilibrated 2.5-ml nickel-nitrilotriacetic acid (Ni-NTA)-agarose resin (Qiagen) column at a flow rate of ~2 ml/min, and the flow-through was reapplied to the column. The column was washed with 10 ml of high salt wash (cell lysis buffer + 300 mM NaCl), and 10 ml of 10 mM imidizole in cell lysis buffer. His-Gα_{i2}^{N270D} was eluted with 10 ml of 150 mM imidazole in cell lysis buffer, diluted 1:4 in Buffer A (20 mM HEPES, pH 8, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 0.01 mM GDP, 500 nM aprotinin, 10 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, 1 nM pepstatin, 10 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone), and loaded onto an equilibrated 1-ml HiTrap Q-Sepharose FPLC column (Amersham Biosciences) according to the manufacturer's recommendations. A linear gradient from 0 to 500 mM NaCl (30 ml) was used to elute proteins bound to the column. Five hundred microliter fractions were collected and immediately assayed for [^{35}S]GTPγS binding and GTPase activity.

[^{35}S]GTPγS Binding Assay—[^{35}S]GTPγS binding assays were performed in triplicate in conical polypropylene tubes (Sarstedt) in a 100-μl reaction volume. Fifty microliters of each sample was added to 50 μl of 2× GTPγS binding buffer (50 mM HEPES, pH 8, 4 mM $MgCl_2$, 1 mM EDTA, 5 μM GTPγS, 500,000 cpm/reaction [^{35}S]GTPγS) and incubated in a 30 °C water bath for 60 min. Reactions were then transferred to ice and diluted with 5 ml of ice-cold stop buffer (20 mM Tris-HCl, pH 8, 25 mM $MgCl_2$, 120 mM NaCl). The diluted reactions were filtered over pre-wetted 0.45-μm type HA nitrocellulose filters (Millipore) using a vacuum manifold and washed twice with 5 ml of stop buffer. The filters were then added to scintillation vials with scintillant, and radioactivity was determined using a scintillation counter.

GTPase Activity—Steady state GTPase assays were performed in duplicate in conical polypropylene tubes in a 50-μl reaction volume. Twenty five microliters of each sample was added to 25 μl of 2× GTPase buffer (40 mM HEPES, pH 8, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM EDTA, 4 μM GTP, 625,000 cpm/reaction [γ-^{32}P]GTP) and incubated in a 30 °C water bath for 30 min. To terminate the reaction, the tubes were moved to an ice bath, and 950 μl of suspended 5% activated charcoal (Sigma) in 20 mM NaH_2PO_4 was added to each tube. The reaction tubes were subjected to centrifugation at $3,000 \times g$ in a swinging bucket centrifuge for 10 min to collect the charcoal pellet. Six hundred microliters of the cleared supernatant were transferred to scintillation vials with scintillation fluid for quantification of radioactivity.

Pheromone Signaling Assays—Two outcomes of pheromone signaling were measured. The first was pheromone-dependent growth inhibition (halo assay) (39). Briefly, 100 μl of a saturated cell culture was mixed with 2 ml of water and 2 ml of 1% (w/v) dissolved agar (55 °C) and poured onto selective SCD or SCG agar plates. Synthetic α-factor was spotted onto sterile paper disks and placed on the nascent lawn. The resulting zone of growth inhibition was recorded after 48 h.

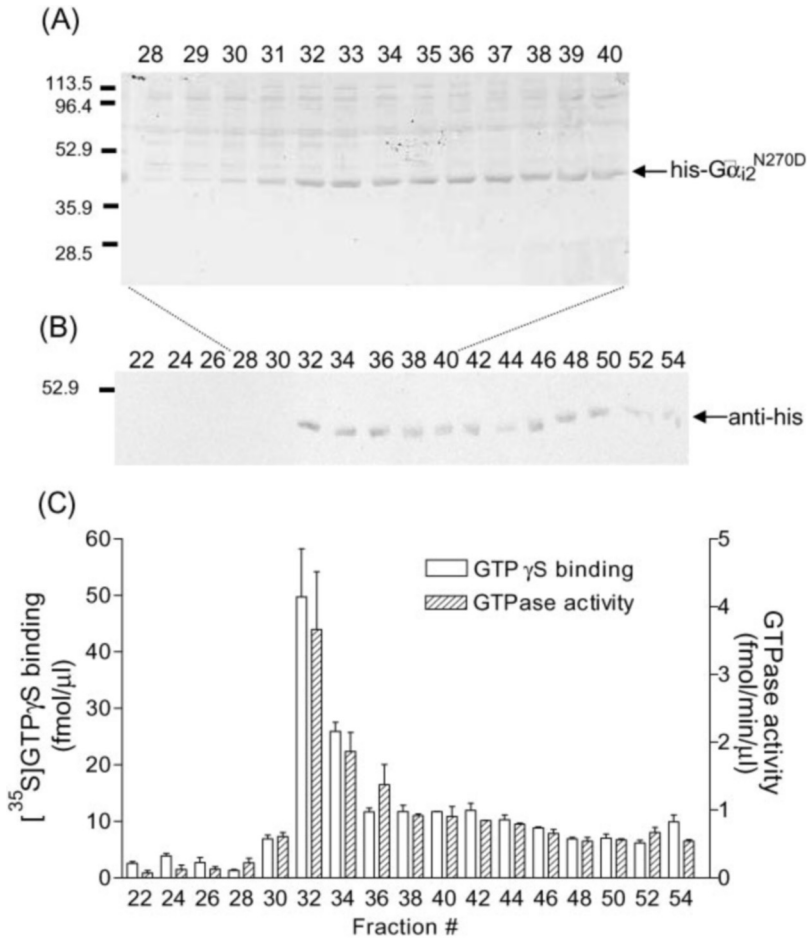

FIG. 1. **Nucleotide binding and GTPase activity of purified $G\alpha_{i2}^{N270D}$.** Recombinant his_6-$G\alpha_{i2}^{N270D}$ was purified from Sf9 cell lysates by Ni-NTA affinity chromatography followed by ion exchange chromatography, as described under "Experimental Procedures." *A*, His_6-$G\alpha_{i2}^{N270D}$ was eluted from an ion exchange column using a 0–500 mM NaCl linear gradient. Purity was assessed by Coomassie Blue staining of 12.5% acrylamide SDS-PAGE gels. Fraction numbers are provided *above* each lane, and the position of molecular weight standards (kDa) are indicated on the *left*. *B*, elution fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-pentahistidine antibody to detect the presence of his_6-$G\alpha_{i2}^{N270D}$. Fraction numbers are provided *above* each lane. *C*, elution fractions were assayed for [^{35}S]-GTPγS binding and GTPase activities. *Open bars*, [^{35}S]GTPγS binding. Samples (in triplicate) of each fraction were incubated with 2.5 μM [^{35}S]GTPγS for 60 min at 30 °C. The reactions were filtered over nitrocellulose, and the retained radioactivity was quantitated by scintillation counting to determine GTPγS binding activity. *Hatched bars*, GTPase activity. Samples (in duplicate) of each eluate fraction were incubated with 2 μM [γ^{32}P]GTP for 30 min at 30 °C. Activated charcoal was added to each reaction and collected by centrifugation, and radioactivity in the supernatant was quantitated by scintillation counting to determine free phosphate. Fraction numbers are indicated *below* each bar. *Error bars*, mean ± S.E. Note that a wider range of fractions are included in *B* and *C* than in *A*.

The second assay was pheromone-dependent reporter transcription activity (39). In this method a pheromone-inducible promoter (*FUS1*) drives expression of a reporter enzyme (β-galactosidase) (39). A saturated cell culture in selective SCD medium was diluted 1:200 in fresh SCD medium, allowed to grow overnight, washed, and resuspended in SCG medium to $A_{600\ nm}$ ~0.6. After 4 h the cells were aliquoted (90 μl, in triplicate) to 96-well plates containing 10 μl of α-factor and incubated for 90 min at 30 °C. β-Galactosidase activity was measured by adding 20 μl of a freshly prepared solution of 83 μM fluorescein di-β-D-galactopyranoside (Marker Gene Technologies Inc.), 137.5 mM PIPES, pH 7.2, 2.5% Triton X-100 and incubating at 37 °C until a bright yellow color appeared. The reaction was stopped by the addition of 20 μl of 1 M Na_2CO_3, and the fluorescence activity was monitored using an excitation of 485 nm and an emission of 530 nm.

Heat Shock Assay—A saturated cell culture in selective SCD medium was diluted 1:20 into fresh SCD medium and incubated for 48 h. Cell cultures were then transferred to glass tubes and placed in a 50 °C water bath for 45 min. Heat-shocked (50 °C) and nonheat-shocked (30 °C) cells were diluted and plated on selective SCG agar plates. Surviving cell colonies were counted after 2–3 days (23).

Gpa1 and $Gpa1^{N388D}$ Expression Assay—To compare expression of Gpa1 *versus* $Gpa1^{N388D}$, protein concentration was examined in the *gpa1*Δ deletion YGS5 strain and in the diploid YPH501 strain, which normally does not express the receptor or G protein. To assess regulation by pheromone, α-factor (2.5 μM) was added at $A_{600\ nm}$ ~0.6 and incubation continued for an additional 2 h. Cells were grown to mid-log phase in SCG ($A_{600\ nm}$ ~1.0), and cell growth was stopped by addition of 10 mM (final concentration) NaN_3. Cells were harvested by centrifugation at 1,000 × *g* for 10 min. Cells were washed once with 10 mM NaN_3 and resuspended in phosphate-buffered saline, pH 7.3. Cells were then lysed by vortexing with glass beads four times for 1 min each and then centrifuged at 10,000 × *g* for 30 s. The resulting supernatant was collected and mixed with SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 14.4 mM 2-mercaptoethanol, 10 μg/ml bromphenol blue, 4% SDS) and heated at 100 °C for 10 min. The samples were allowed to cool and subjected to immunoblotting analysis (see below). To compare stability of Gpa1 and $Gpa1^{N388D}$, cycloheximide was added (10 μg/ml final concentration) for various times prior to harvesting the cells.

Co-purification of Gpa1 with Gβγ—YPH501 cells expressing receptor (Ste2), Gβγ (Ste4/Ste18), and either Gα (Gpa1) or $G\alpha^{ND}$ ($Gpa1^{N388D}$) fused to glutathione *S*-transferase (GST) or GST alone were grown in selective SCG medium. All the following procedures were carried out at 4 °C. After termination of cell growth, 50 $A_{600\ nm}$ units of cells were resuspended in purification lysis buffer (40 mM triethanolamine, pH 7.2, 2 mM EDTA, 150 mM NaCl, 2 mM dithiothreitol, 0.2 mM 4-[2-aminoethyl]benzenesulfonyl fluoride HCl, 15 μg/ml leupeptin, 20 μg/ml pepstatin, 1 mM benzamidine, 10 μg/ml aprotinin, 100 μM glycerol 2-phosphate, 0.5 mM sodium orthovanadate). Cells were split into two equal portions, and each portion was resuspended in 1 ml of lysis buffer containing 3 mM $MgCl_2$ and 10 μM GDP (condition 1, "$-AlF_4^-$") or 3 mM $MgCl_2$, 10 μM GDP, 30 μM $AlCl_3$, and 10 mM NaF (condition 2, "$+AlF_4^-$"). Cells were lysed by vortexing with glass beads four times for 1 min each. The resultant lysates were harvested by centrifugation at 1,000 × *g* for 10 min and solubilized by addition of Triton X-100 (1% final concentration) and rocking for 1 h. The samples were then centrifuged at 1,000 × *g* for 10 min, and the resulting supernatant was mixed with 100 μl of a 30% slurry of glutathione-Sepharose 4B (Amersham Biosciences) in the appropriate lysis buffer (condition 1 or condition 2) and incubated for 2 h. The glutathione-Sepharose 4B was centrifuged at 10,000 × *g* for 10 min and washed three times with 1 ml of phosphate-buffered saline. The bound proteins were eluted by heating at 100 °C in SDS-PAGE sample buffer.

Co-immunoprecipitation of Receptor (Ste2) and Gpa1—YPH501 cells expressing Gβγ (Ste4/Ste18), Gpa1, or $Gpa1^{N388D}$ and receptor (Ste2) tagged or nontagged with the FLAG epitope were grown in selective SCG medium to $A_{600\ nm}$ ~1.0. All the following procedures were conducted at 4 °C. Twenty five $A_{600\ nm}$ units of cells were resuspended in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 10% glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1× of protease inhibitor mixture (catalog number 1873580; Roche Applied Science)), followed by vortexing with glass beads four times for 1 min each. Cell lysates were harvested by centrifugation at 1,000 × *g* for 10 min, and the supernatant was subjected to

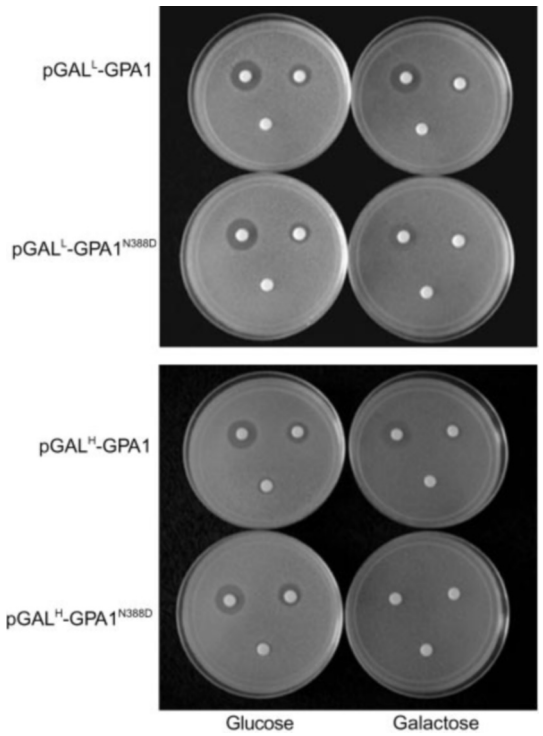

FIG. 2. **Gpa1^{N388D} inhibits pheromone-dependent growth arrest.** To measure pheromone-dependent growth arrest, wild-type cells (strain BY4741) were transformed with wild-type *GPA1* or *GPA1^{N388D}* expressed from an attenuated *GAL1/10* promoter having 1 (*GAL^{L}*) or 20% (*GAL^{H}*) of wild-type activity. Cells were plated onto medium containing either galactose (to induce expression) or glucose (to repress expression) and exposed to filter disks spotted with α-factor pheromone (*clockwise from top left*, 75, 25, and 8 μg). The resulting zone of growth inhibition was documented after 2–3 days.

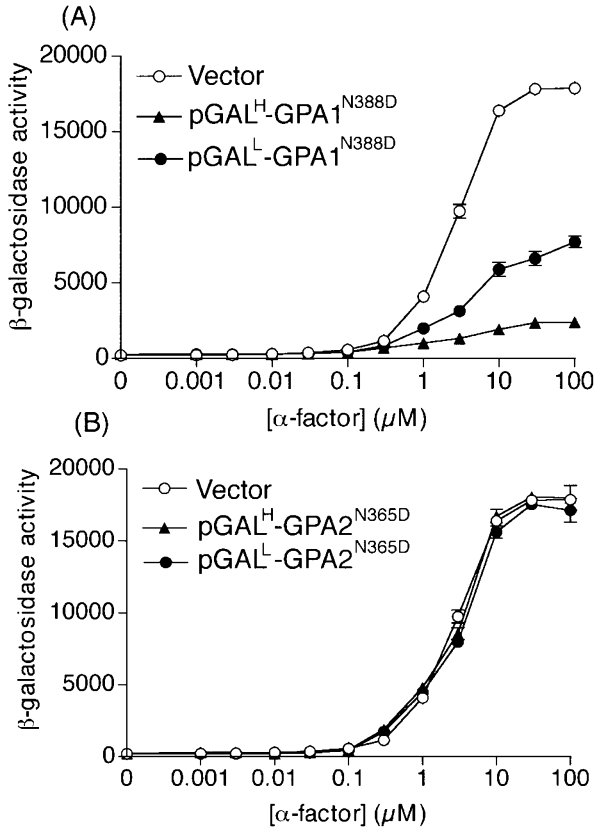

FIG. 3. **Gpa1^{N388D} inhibits pheromone-induced transcription.** To measure pheromone-dependent transcription, wild-type cells (strain BY4741) were co-transformed with a reporter plasmid containing the pheromone-responsive *FUS1* promoter fused to *lacZ* and a second plasmid containing *GPA1^{N388D}* (*A*), *GPA2^{N365D}* (*B*), or no insert (*Vector*, *A* and *B*) expressed from either the *GAL^{L}* or *GAL^{H}* promoter. Cells were grown in the presence of galactose and analyzed for β-galactosidase activity following treatment with the indicated concentrations of α-factor. β-Galactosidase activity units are arbitrary. Each point is an average of three measurements, and the data shown are representative of three independent experiments. *Error bars*, S.E.

1 h of rocking to liberate membrane-bound proteins. The samples were then centrifuged at 10,000 $\times$ *g* for 10 min, and the resulting supernatant was incubated with 40 μl of a 50% slurry of EZview^{TM} Red anti-FLAG M2 affinity gel (Sigma). After 2 h of gentle agitation, the gel was centrifuged at 10,000 $\times$ *g* for 30 s and washed three times with IP lysis buffer. Elution of FLAG-tagged protein was achieved by incubating the gel with 15 μg of 3$\times$ FLAG peptide in 50 μl of elution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) with gentle shaking for 30 min. Supernatant was harvested by centrifugation at 8,000 $\times$ *g* for 30 s and subjected to immunoblotting assay.

Immunoblot Detection—Protein samples in SDS-PAGE sample buffer were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies against Gpa1 (1:1,000 dilution) (40), FLAG (1:3,000; Sigma), GST (1:1,500; from Joan Steitz, Yale University), Ste4 (1:2,000; from Duane Jenness, University of Massachusetts), or Gα_{i2} (1:2,500; Qiagen). Antibodies were detected using secondary antibodies such as horseradish peroxidase-conjugate goat anti-mouse IgG or anti-rabbit IgG (Bio-Rad). The signal was detected by the ECL system (Amersham Biosciences) according to the manufacturer's instructions.

RESULTS

Gα^{ND} Can Bind and Hydrolyze GTP—Gpa1^{N388D} is a potent inhibitor of pheromone signaling. Other investigators have suggested that this mutation impairs GTPase function and proposed that the inhibition of signaling might occur through activation of a "desensitization effector" (28–30, 41). However, the GTPase activity of the ND mutant has not been measured in any system. A previous attempt to purify recombinant Gpa1^{N388D} from bacteria yielded a product unable to hydrolyze GTP, but which was also unable to bind to GTP or G$\beta\gamma$ (28). These findings suggest that the mutant protein is unstable and loses activity during purification. As an alternative strategy we attempted to purify the analogous mutant form of Gα_{i2} expressed in insect cells. Gα_{i} is the closest mammalian homologue to Gpa1; both proteins have nearly identical guanine nucleotide binding pockets, and a direct comparison revealed that they have very similar kinetic properties *in vitro* (27).

As shown in Fig. 1, we were able to purify small quantities of the mutant protein from insect cells. We generated a baculovirus encoding human Gα_{i2}^{N270D} fused at the N terminus to a hexahistidine affinity tag (his$_6$-Gα_{i2}^{N270D}). Sf9 cell infection with the virus resulted in heterologous expression of the mutant protein, although at significantly lower levels than that observed following infection with a similar virus encoding wild-type Gα_{i2} (data not shown). Lysates were separated by high speed centrifugation into particulate and soluble fractions, and the soluble fraction was passed over an Ni-NTA affinity column. After extensive washing the his$_6$-Gα_{i2}^{N270D} was eluted with 150 mM imidazole. Fractions were then resolved by SDS-PAGE and visualized by staining with Coomassie Blue as well as by immunoblotting and detection with anti-pentahistidine antibodies. Both detection methods revealed a single prominent band migrating at 40.5 kDa, which is the predicted molecular mass of his$_6$-Gα_{i2}^{N270D} (data not shown).

To further purify his$_6$-Gα_{i2}^{N270D}, the 150 mM imidazole eluate was diluted and passed over an anion exchange resin (HiTrap Q-Sepharose). The column was washed, and bound proteins were eluted with a linear salt gradient. Elution fractions were again analyzed by protein staining and immunoblotting as described above. As shown in Fig. 1*A*, his$_6$-Gα_{i2}^{N270D} represented greater than 50% of the total protein in fractions with significant [^{35}S]GTPγS binding activity (see below). his$_6$-

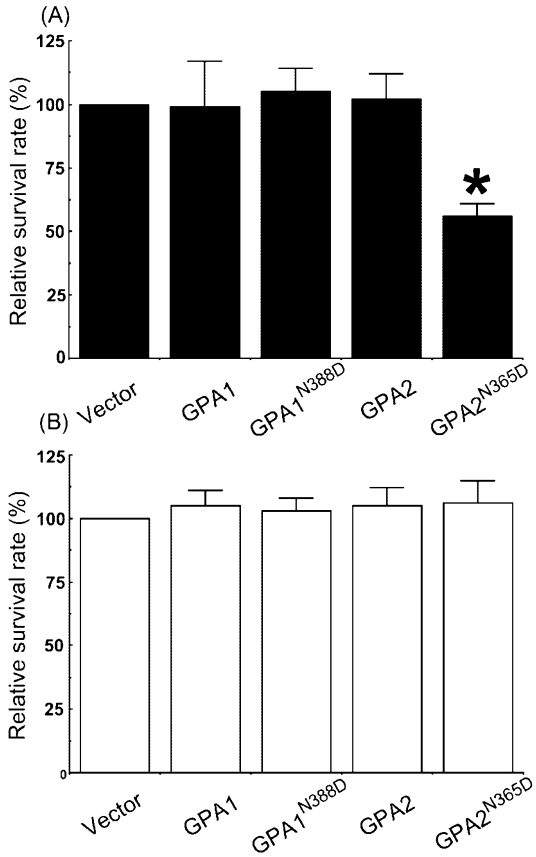

FIG. 4. **Gpa2^{N365D} inhibits recovery from heat shock.** Wild-type cells (strain YPH499) were transformed with a plasmid containing no insert (*Vector*), wild-type *GPA1*, wild-type *GPA2*, *GPA1^{N388D}*, or *GPA2^{N365D}* under the control of the *GAL^{H}* promoter. Cells in liquid medium were placed in either a 50 °C water bath for 45 min (*A*) or maintained at the normal growth temperature of 30 °C (*B*) and then plated onto solid medium. Colonies were counted after 2–3 days. Heat shock recovery was expressed as the relative survival rate of each transformed strain and expressed relative to the vector control group (defined as 100%). Each value is an average of three measurements, and the data shown are representative of four independent experiments. *Error bars*, S.E.

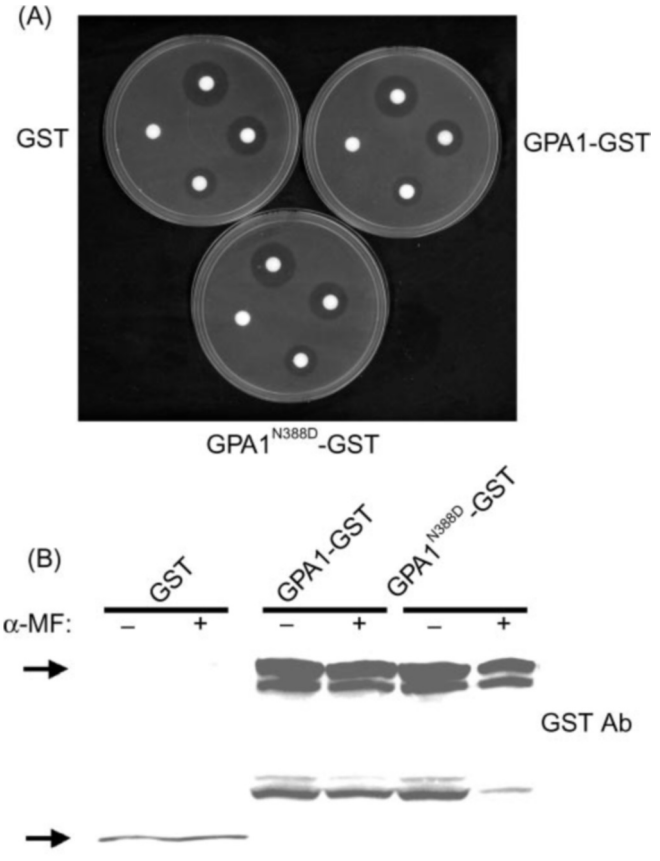

FIG. 5. **Signal inhibition by Gpa1^{N388D} requires receptor coupling activity.** To determine whether coupling to receptors is required for Gpa1^{N388D} activity, wild-type cells (strain BY4741) were transformed with plasmid pAD4M containing *GPA1^{N388D}*-GST, *GPA1*-GST, or GST alone. Fusion to GST preserves G$\beta\gamma$ binding but blocks activation by receptors, as discussed above. *A*, cells were plated onto selective medium and exposed to disks spotted with α-factor pheromone (*clockwise from top*, 75, 25, 8, and 0 μg). The resulting zone of growth inhibition was documented after 2–3 days. *B*, total lysates were prepared from the cells used in *A*, resolved by 7.5% SDS-PAGE, and subjected to immunoblotting with anti-GST antibodies (*Ab*). *Arrows* indicate full-length protein specifically detected by the antibody. Other bands are presumed to be degradation products.

Gα_{i2}^{N270D} immunoreactivity did not elute as a discrete species but rather in two broad peaks over a wide range of NaCl concentrations (Fig. 1*B*). These results suggest that the protein was not monodisperse, consistent with our observations that protein solubility and GTPγS binding activity of his$_6$-Gα_{i2}^{N270D} decline rapidly (see below).

The Q-Sepharose eluate was assayed for [^{35}S]GTPγS binding and GTPase activities. As shown in Fig. 1*C,* substantially more [^{35}S]GTPγS binding and GTPase activity was observed in the first peak (fractions 32–36) than in the second peak, suggesting that the his$_6$-Gα_{i2}^{N270D} present in the later fractions was inactive. Even in this early peak the active form of the protein (determined by GTPγS binding) was ~15% of the estimated total concentration of Gα_{i2}^{N270D} (determined by protein staining). Wild-type his-Gα_{i2} purified under the same conditions eluted as a single discrete peak at ~200 mM NaCl, and these fractions exhibited nearly stoichiometric [^{35}S]GTPγS binding (data not shown).

Significant loss of [^{35}S]GTPγS binding activity of Gα_{i2}^{N270D} occurred as a function of time and temperature. For this reason all purification steps were carried out at 4 °C, and all assays of [^{35}S]GTPγS binding and GTPase activities were performed within 10 h of cell lysis. Despite these precautions, it appeared that much of the protein was inactive, further suggesting that the protein is unstable (data not shown).

Finally, we observed that co-expression with Gβ_1 and Gγ_2 increased the proportion of Gα_{i2}^{N270D} associated with the membrane fraction, suggesting that the mutant protein interacts with G$\beta\gamma$ dimers and is recruited to the membrane by this interaction (data not shown). We have also co-expressed untagged Gα_{i2}^{N270D} and pentahistidine-tagged Gγ_2 with Gβ_1 and purified the heterotrimeric complex by Ni-NTA and ion exchange chromatography. We found that Gα_{i2}^{N270D} co-eluted with his$_6$-G$\beta\gamma$ in this procedure, further suggesting that the mutant associates with G$\beta\gamma$ (data not shown).

Gpa1^{N388D} Is a Receptor- and Gα Subtype-selective Dominant-negative Mutant—The data presented in Fig. 1 indicate that Gα^{ND} binds and hydrolyzes GTP normally. This makes it unlikely that Gpa1^{N388D} functions by activating any effector protein, because effectors recognize only the GTP-bound form of Gα. We therefore considered whether the mutant functions as a dominant negative. Dominant-negative mutants will, when overexpressed, inhibit the function of the endogenous wild-type protein (14). In this scenario, the Gpa1^{N388D} phenotype could result from interference with receptor-G protein coupling, from inhibition of G protein subunit dissociation, or from both.

As an initial test of the model we asked whether Gpa1^{N388D} specifically inhibits the signaling activity of the pheromone

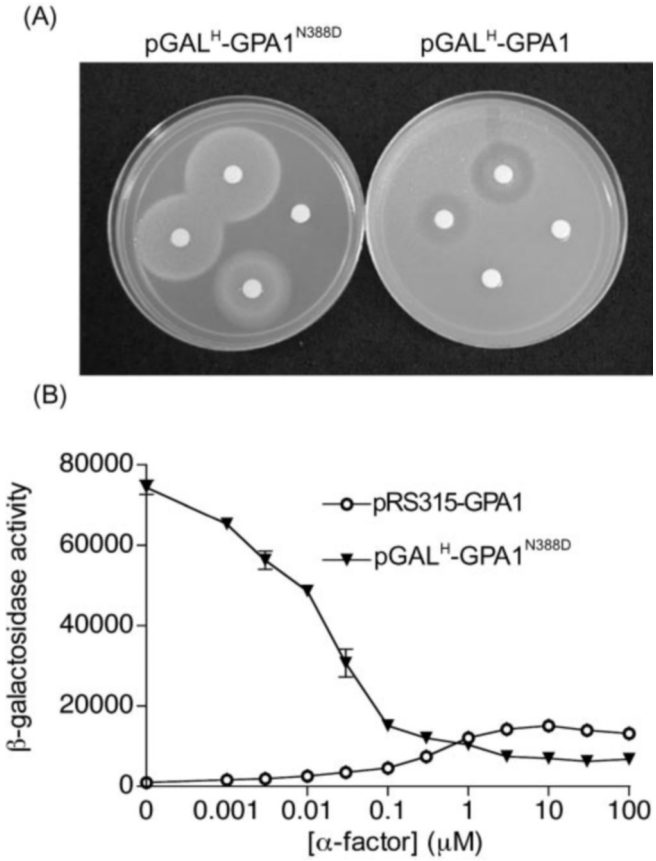

FIG. 6. **Pheromone-dependent inhibition of constitutive signaling by Gpa1^{N388D} in a *gpa1*Δ mutant strain.** *A*, *gpa1*Δ *ste7*Δ mutant strain was transformed with plasmids containing *STE7* under the control of the *GAL1/10* promoter and either wild-type *GPA1* or *GPA1^{N388D}* under the control of the *GAL^{H}* promoter. Cells were plated onto galactose-containing medium and exposed to α-factor pheromone (*counter-clockwise from top*, 75, 25, 8, and 0 μg). The resulting zone of pheromone-dependent cell growth was recorded after 2 days. *B*, the same strain was transformed with plasmids containing *STE7* (*GAL1/10* promoter), the *FUS1*-lacZ reporter, and either *GPA1^{N388D}* (*GAL^{H}* promoter) or wild-type *GPA1* regulated by its native promoter (pRS315-GPA1). Each point is an average of three measurements, and the data shown are representative of three independent experiments. *Error bars*, S.E.

receptor Ste2. Two distinct GPCR signaling pathways exist in yeast. In haploid cells, the primary function of Ste2 is to modulate the activity of Gpa1 (Gα) and the Ste4/Ste18 dimer (Gβγ) (22). Activation of this pathway leads to growth arrest and mating. Although less well characterized, a putative glucose receptor Gpr1 modulates the activity of Gpa2 (Gα), Gpb1 or Gpb2 (Gβ-like proteins), and Gpg1 (Gγ). Activity of this pathway allows the cell to adapt to cell stress (42).

We first compared pheromone signaling in wild-type cells that express Gpa1^{N388D} or the analogous mutant form of Gpa2, Gpa2^{N365D}. Both Gα mutants were expressed using the attenuated galactose-inducible promoters, *GAL^{H}* (20% of wild-type activity) and *GAL^{L}* (1% of wild-type activity). Two outcomes of pheromone signaling were measured. The first was pheromone-mediated cell growth arrest (halo assay). Pheromone spotted onto a filter disk produces a zone of growth arrest the size of which correlates with pheromone sensitivity. As shown in Fig. 2, modest overexpression of Gpa1^{N388D} inhibited the response to pheromone, as described previously (30). Inhibition was dependent on the expression level of Gpa1^{N388D} as the *GAL^{H}* promoter conferred more effective inhibition (Fig. 2, *bottom panel*) than *GAL^{L}* (*top panel*). Inhibition was absent when expression was repressed by growth in glucose. Expression of wild-type Gpa1, wild-type Gpa2, or Gpa2^{N365D} also did not affect the halo response (Fig. 2 and data not shown).

We then examined the effects of the ND mutation by using a pheromone-dependent gene transcription assay. In this method, induction of a pheromone-inducible promoter (from *FUS1*) leads to increased expression of a reporter enzyme (β-galactosidase). As shown in Fig. 3*A*, expression of Gpa1^{N388D} caused a significant reduction in β-galactosidase activity, and the effect was again dependent on the level of mutant expression (*GAL^{H}* was more effective than *GAL^{L}*). Inhibition was observed only when expression of the mutant was induced by galactose (data not shown). Expression of wild-type Gpa1, wild-type Gpa2, or Gpa2^{N365D} had no effect on signaling (Fig. 3*B*, and data not shown). Thus, the two functional assays are in agreement and together show that pheromone signaling is diminished upon overexpression of Gpa1^{N388D} but not Gpa2^{N365D}.

We then characterized the effect of the ND mutation on signaling by Gpa2. No reporter-transcription assay is available that is selective for signaling by Gpa2 (the widely used *FLO11* reporter is also induced by pheromone, data not shown). However, Gpa2 was shown previously (23, 25) to regulate survival after heat shock. Thus, we investigated whether Gpa2^{N365D} would likewise enhance heat shock sensitivity. As shown in Fig. 4, Gpa2^{N365D} significantly reduced the survival rate of cells grown at 50 °C for 45 min (Fig. 4*A*). In contrast, wild-type Gpa2, Gpa1, or Gpa1^{N388D} exhibited no effect on heat shock sensitivity. Likewise, no difference was observed among any of the tested strains maintained at 30 °C (Fig. 4*B*). Taken together, these data indicate that Gpa2^{N365D} (but not Gpa1^{N388D}) acts in the Gpr1-Gpa2 pathway, whereas Gpa1^{N388D} (but not Gpa2^{N365D}) diminishes Ste2-Gpa1-mediated pheromone signaling. Stated differently, GαND functions as a receptor-selective dominant-negative mutant.

Receptor Coupling Is Required for Gpa1^{N388D} Dominant-negative Activity—The data above indicate that Gpa1^{N388D} specifically regulates Ste2 activity. This inhibitory effect could be due to direct binding to the receptor, binding to Gβγ, or both. To determine whether receptor coupling is required, we tested the activity of Gpa1^{N388D} fused at its C terminus to glutathione *S*-transferase. The C-terminal region of Gα is required for coupling to receptor but not for binding to Gβγ. We have shown previously (34) that Gpa1-GST blocks receptor coupling but preserves binding to guanine nucleotides and to Gβγ. As shown in Fig. 5, the Gpa1^{N388D}-GST fusion had no effect on the growth arrest response. Expression of GST alone or wild-type Gpa1-GST was also without effect in this assay (Fig. 5*A*). Equal expression of each protein was confirmed by immunoblotting (Fig. 5*B*). These data indicate that the dominant-negative activity of Gpa1^{N388D} requires direct coupling to its receptor.

Dominant-negative Activity of Gpa1^{N388D} Depends on Receptor Activation—Gpa1^{N388D} evidently couples to its receptor (Ste2), yet appears unable to undergo receptor activation. If Gpa1^{N388D} and the receptor indeed form an unproductive complex, it remains unclear why any response to pheromone occurs at least initially. One possibility is that Gpa1^{N388D} is not normally associated with the receptor but is recruited in response to prolonged pheromone stimulation. In contrast, any wild-type Gpa1 normally associated with the receptor would be displaced upon pheromone stimulation, allowing Gpa1^{N388D} to bind and thereby prevent further activation of wild-type Gpa1.

To test this aspect of the model, Gpa1^{N388D} was expressed in the absence of wild-type Gpa1 (*i.e.* a *gpa1*Δ mutant strain). Cells lacking *GPA1* are normally not viable due to constitutive release of Gβγ leading to cell division arrest (43, 44). However,

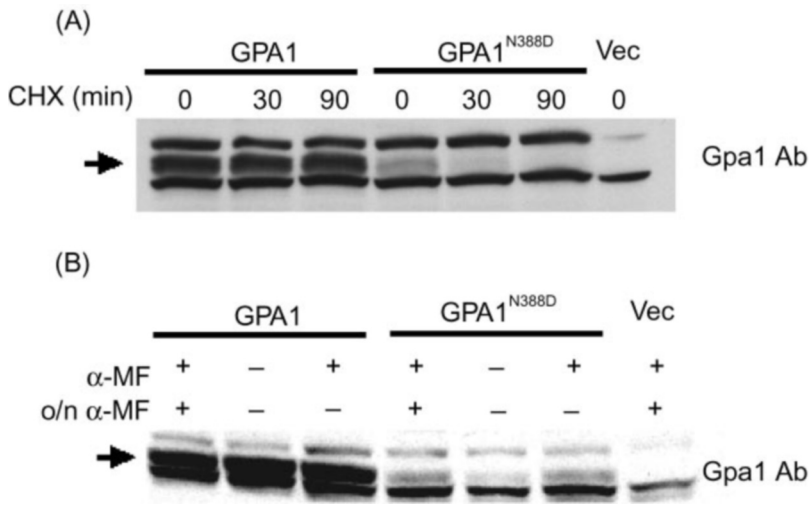

FIG. 7. **Pheromone-dependent expression of Gpa1^{N388D}.** *A*, to measure Gpa1^{N388D} expression, a *gpa1* mutant (strain YGS5) was transformed with a plasmid containing either no insert (*Vec*), wild-type *GPA1*, or *GPA1^{N388D}* under the control of the *GAL^{H}* promoter. Cells were grown to mid-log phase in galactose medium and then treated with the protein synthesis inhibitor cycloheximide (*CHX*) for the indicated times. Whole cell lysates were resolved by 7.5% SDS-PAGE and subjected to immunoblotting with anti-Gpa1 antibodies. *B*, the same cells as in *A* were grown for 18 h in the absence or presence of 2.5 μM α-factor, as indicated (*o/n α-MF*). Another addition of α-factor was made 2 h prior to collecting the cells, as indicated (*α-MF*). Whole cell lysates were resolved by 7.5% SDS-PAGE and subjected to immunoblotting with anti-Gpa1 antibodies (*Ab*). *Arrows* indicate protein specifically detected by the antibody (*Ab*). Data are representative of four experiments.

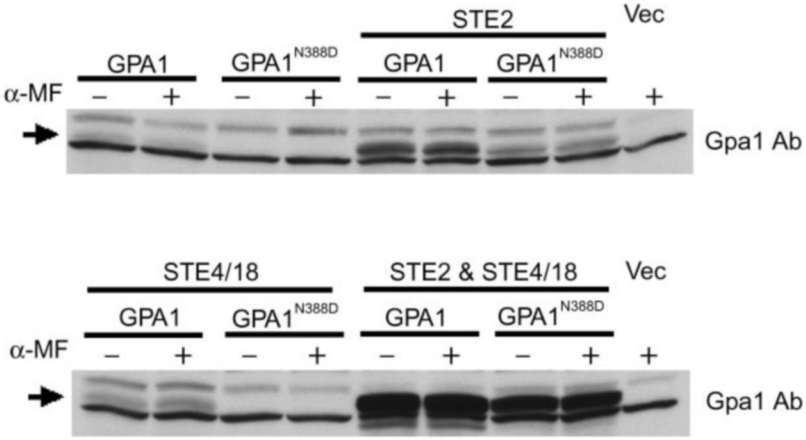

FIG. 8. **Receptor- and G$\beta\gamma$-dependent expression of Gpa1^{N388D}.** To determine the relative contribution of receptor and G$\beta\gamma$ to stable Gpa1^{N388D} expression, wild-type diploid cells (strain YPH501) were transformed with plasmids containing no insert (*Vec*), wild-type *GPA1*, or *GPA1^{N388D}*, alone or in combination with plasmids that express receptor (*STE2*), G$\beta\gamma$ (*STE4/18*), or both, as indicated. Whole cell lysates were resolved by 7.5% SDS-PAGE and subjected to immunoblotting with anti-Gpa1 antibodies (*Ab*). The *arrows* indicate the protein specifically detected by the Gpa1 antibody. Data are representative of four experiments.

the cells used here also do not express the downstream kinase gene *STE7* and are viable. Thus, signaling can be monitored by growth arrest and reporter transcription assays following induction of *STE7* expression from a plasmid.

As predicted by the model, Gpa1^{N388D} blocked cell division arrest in pheromone-treated cells (those nearest the source of pheromone) (Fig. 6*A*) (45). Cells grew poorly at the perimeter of the halo, where pheromone concentrations are reduced (Fig. 6*A*). Cells expressing wild-type Gpa1 exhibited a more typical growth arrest phenotype. Cells closest to pheromone underwent cell division arrest, whereas those further away continued to grow. This pattern of signaling supports the model that Gpa1^{N388D} can inhibit signaling, but only upon receptor activation. This is in contrast to wild-type Gpa1, which promotes signaling upon receptor activation.

The growth arrest data suggest that Gpa1^{N388D} can form a stable complex with pheromone-occupied receptor and G$\beta\gamma$. Alternatively, Gpa1^{N388D} could subserve a role in cell division cycling independent of its ability to bind G$\beta\gamma$. To rule out this possibility we measured the transcription induction response in the same cells. Transcription induction coincides with, but does not require, cell division arrest (46). Whereas wild-type Gpa1 conferred dose-dependent activation of the transcription reporter (Fig. 3), Gpa1^{N388D} (in the absence of wild-type protein) produced a high basal transcription activity and a dose-dependent *inhibition* of the response (Fig. 6*B*). These data mirror that seen in the growth arrest assay, and further suggest that Gpa1^{N388D} binds to pheromone-activated receptor and G$\beta\gamma$. However, rather than leading to activation, the mutant appears to remain stably bound to receptor and G$\beta\gamma$.

Pheromone Stimulation Increases Gpa1^{N388D} Stability and Expression—The above results suggest that pheromone triggers the association of Gpa1^{N388D} with G$\beta\gamma$ and receptor. Thus, we investigated the mechanism by which pheromone treatment can unmask the apparent ability of Gpa1^{N388D} to sequester G$\beta\gamma$. One possibility is that pheromone is required for stable *expression* of Gpa1^{N388D} (47). Indeed, purification of G$\alpha_{i2}{}^{N270D}$ or Gpa1^{N388D} yielded protein that was unstable *in vitro* (28). Thus we considered whether Gpa1^{N388D} is also unstable *in vivo*.

To determine whether Gpa1^{N388D} is unstable, we monitored its rate of loss in cells following treatment with the protein synthesis inhibitor cycloheximide. As shown in Fig. 7*A*, overexpressed wild-type Gpa1 protein was quite stable, with almost no change after a 90-min treatment with cycloheximide. In contrast, Gpa1^{N388D} was expressed at much lower levels, and expression was undetectable after only 30 min of translation inhibition (Fig. 7*A*). These data indicate that the mutant is unstable.

To determine whether stability is influenced by receptor activation, the same cells were treated with pheromone, and Gpa1^{N388D} expression was again measured by immunoblotting. As shown in Fig. 7*B*, addition of pheromone resulted in elevated expression of Gpa1^{N388D} but had no effect on the already high levels of wild-type Gpa1. These data suggest that pheromone promotes more stable expression of Gpa1^{N388D}. The elevated expression might allow the mutant protein to associate with G$\beta\gamma$ and thereby inactivate the signal.

In Vivo Reconstitution of Receptor, Gβγ, and Gpa1^{N388D}—The data presented above indicate that pheromone-occupied receptor slows the degradation of Gpa1^{N388D}. Therefore, we asked whether binding to Gβγ also contributes to stable expression of the mutant protein. These experiments were conducted in diploid cells, which normally lack the receptor and G protein subunits. Through heterologous expression of each component, alone or in combination, we determined the relative contribution of each to stabilized expression of Gpa1^{N388D}.

As shown in Fig. 8 (*top panel*), Gpa1 and Gpa1^{N388D} proteins were barely detectable when expressed alone. The abundance of both the mutant and wild-type protein was enhanced by co-expression of the receptor and was further enhanced by co-expression of both the receptor *and* Gβγ (*bottom panel*). Most surprising, pheromone treatment in this case did not appear to affect the expression of either the wild-type or mutant protein. This could be due to overexpression of the signaling proteins, which might dampen signaling efficiency or reflect the absence in diploids of another required signaling component such as the haploid-specific proteins Ste5, Far1, Sst2, or Fus3. Nevertheless, these results support our hypothesis that receptor helps to stabilize the expression of Gpa1^{N388D}. These data also reveal a contribution of Gβγ to Gα stability.

GTPase Activity Is Not Required for Gpa1^{N388D} Dominant-negative Activity—The intrinsic GTPase activity of the Gα subunit is normally required for G protein subunits to reassociate and for signaling to cease (48). Agonist-occupied receptors function by stabilizing the guanine nucleotide-free state, so stable formation of a receptor-G protein complex should not require the ability to catalyze GTP hydrolysis. To test this aspect of the model, we introduced a second mutation (Q323L) that is incompatible with GTPase activity (27). The effect of the Q323L/N388D double mutant was compared with N388D alone, using both the cell growth inhibition assay and the transcription activation assay. As shown in Fig. 9, Gpa1$^{Q323L/N388D}$ and Gpa1^{N388D} produced a similar response in both assays. These data demonstrate that GTP hydrolysis is not required for Gpa1^{N388D} to inhibit pheromone signaling, in agreement with our model.

Gpa1^{N388D} Binds Gβγ (Ste4/18)—Typically, Gα subunits bind to Gβγ in the presence of GDP but not GTP (48). Our model predicts that Gpa1^{N388D} binds the receptor, Gβγ and guanine nucleotides but fails to dissociate from Gβγ following receptor activation. One possibility is that Gpa1^{N388D} is locked in the inactive conformation and therefore does not undergo the conformational changes needed to liberate Gβγ. Alternatively, Gpa1^{N388D} might couple to the receptor but is unable to undergo receptor-dependent guanine nucleotide exchange required for subunit dissociation. To rule out the first of these two possibilities, we investigated whether Gpa1^{N388D} undergoes the conformational change necessary for Gβγ dissociation. Gpa1^{N388D} and Gpa1 were fused to GST, expressed, and purified by glutathione-Sepharose affinity chromatography. As shown in Fig. 10, Ste4/Ste18 (Gβγ) bound to either Gpa1-GST or Gpa1^{N388D}-GST, when purified in the presence of GDP. Addition of AlF_4^- converts Gα to the active conformation, and this treatment led to dissociation of Ste4/Ste18 from either protein; indeed, the binding properties of Gpa1^{N388D}-GST were almost identical to that of wild-type Gpa1-GST (Fig. 10). These data indicate that Gpa1^{N388D} retains the ability to undergo a conformational change leading to Gβγ release. These data also support our model that Gpa1^{N388D} forms a stable complex with Gβγ as well as receptor but does not liberate Gβγ from receptor after pheromone stimulation, and as a consequence inhibits pheromone signaling.

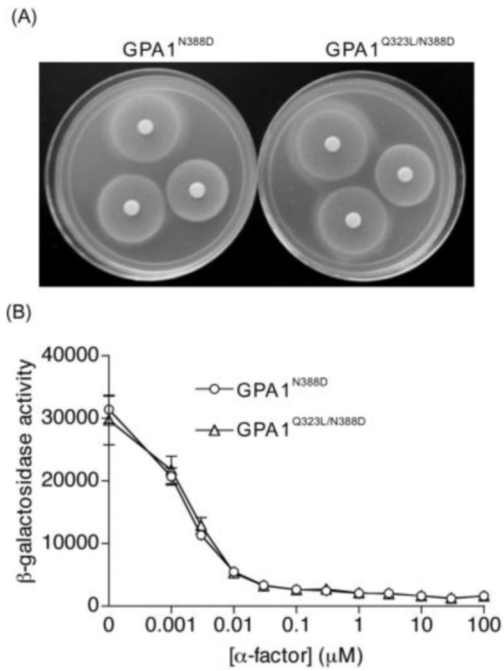

FIG. 9. **GTPase activity is not required for Gpa1^{N388D} function.** To determine whether GTP hydrolase activity is required for Gpa1^{N388D} function, a second mutation (Q323L) was introduced into *GPA1^{N388D}*. The *gpa1*Δ *ste7*Δ mutant strain was transformed with a plasmid containing either *GPA1^{N388D}* or *GPA1$^{N388D/Q323L}$* under the control of the *GAL^{H}* promoter and analyzed by using the growth inhibition assay (α-factor pheromone, *counter-clockwise from top*, 75, 25, and 8 μg) (*A*) and the transcription activation assay (*B*) as described above. Each point is an average of three measurements, and the data shown are representative of three independent experiments. *Error bars*, S.E.

Pheromone Promotes Stable Coupling of Gpa1^{N388D} to Receptor—The previous experiments demonstrated that pheromone promotes interaction of the receptor with Gpa1^{N388D} and Gβγ. G protein subunit binding is reversible but does not occur even with pheromone stimulation. These results suggest that pheromone promotes coupling but not activation of Gpa1^{N388D}. As a final test of this hypothesis, we investigated whether Gpa1^{N388D} is recruited to the receptor in response to pheromone binding. Our approach was to immunopurify receptor and track the interaction of Gpa1^{N388D} before and after pheromone stimulation. Diploids were used because (in contrast to haploid cells) they express similar levels of Gpa1 and Gpa1^{N388D}, thereby allowing a more valid comparison of binding of wild-type and mutant proteins.

The receptor was fused to a triple-FLAG epitope tag (Ste2-FLAG) and co-expressed with either wild-type Gpa1 or Gpa1^{N388D} as well as Ste4/18. The receptor and any associated G protein were then immunoprecipitated and resolved by gel electrophoresis and immunoblotting. As shown in Fig. 11, treatment with pheromone caused diminished binding of receptor to wild-type Gpa1. In contrast, pheromone treatment *enhanced* the interaction of receptor with Gpa1^{N388D}. Even without pheromone treatment, the receptor appeared to have a higher affinity for Gpa1^{N388D} than wild-type Gpa1 (Fig. 11). These data indicate that receptor activation by pheromone not only stabilizes Gpa1^{N388D} expression but also actively promotes assembly of a receptor-Gpa1^{N388D}-Gβγ complex. This complex would preclude access of endogenous wild-type Gpa1 and therefore inhibit pheromone signal propagation in a dominant-negative manner.

DISCUSSION

We have characterized a novel mutant (designated GαND) and found that it acts as a potent dominant-negative inhibitor

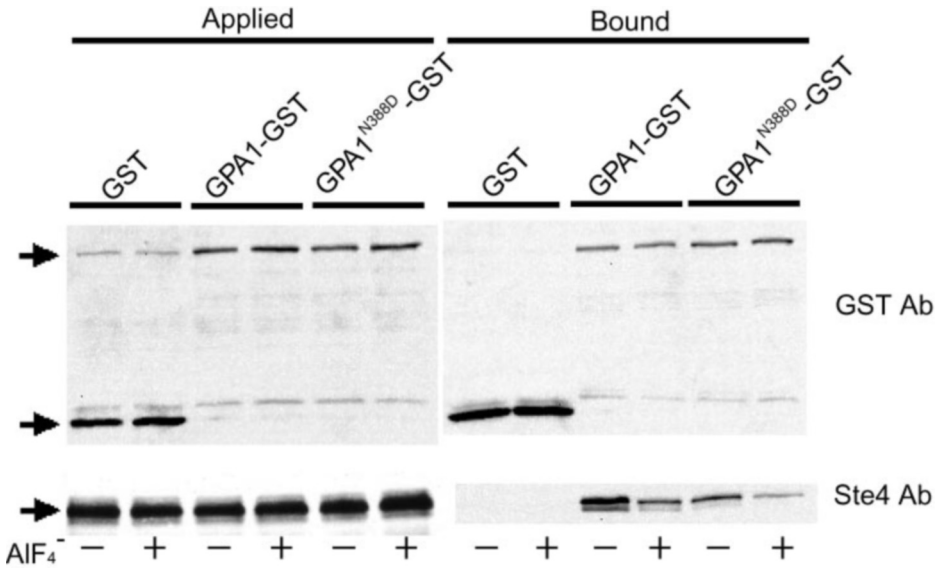

FIG. 10. **Gβγ binds preferentially to Gpa1^{N388D} in the GDP-bound conformation.** To determine whether Gpa1^{N388D} binds to Gβγ in a guanine nucleotide-dependent manner, diploid cells (strain YPH501) were transformed with plasmids containing *STE2* (receptor), *STE4/18* (Gβγ), and either wild-type *GPA1*-GST, *GPA1^{N388D}*-GST, or GST alone. The cells were disrupted in the presence of GDP (−) or GDP and AlF_4^- (+), as indicated. Detergent-solubilized lysates were then immobilized on glutathione-Sepharose, washed, and eluted with SDS-PAGE sample buffer. Samples of total cell lysates (*Applied*) and retained proteins (*Bound*) were resolved by 10% SDS-PAGE and subjected to immunoblotting with antibodies (*Ab*) against GST or Ste4, as indicated. Data shown are representative of three independent experiments. *Arrows* indicate proteins specifically detected by the indicated antibodies.

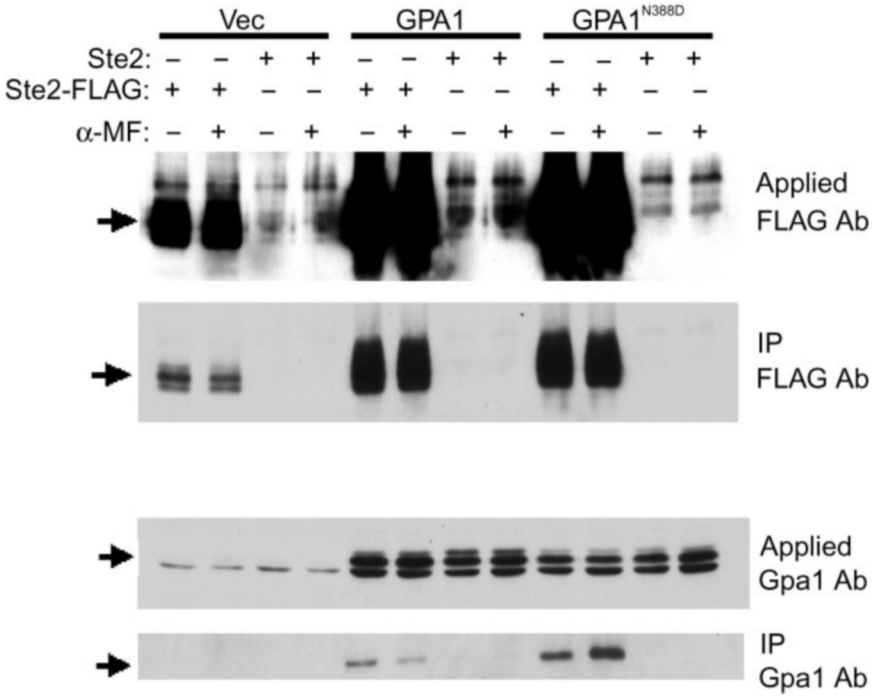

FIG. 11. **Pheromone-dependent complex formation of Gpa1^{N388D} and receptor.** To determine whether Gpa1^{N388D} forms a stable complex with the receptor, diploid cells (strain YPH501) were transformed with plasmids containing either tagged or untagged *STE2*, *STE4/18* (Gβγ), and either wild-type or mutant *GPA1* and then treated with 2.5 μM α-factor (*α-MF*), as indicated. Detergent-solubilized cell lysates were immunoprecipitated with anti-FLAG antibodies. Samples of total cell lysates (*Applied*) and purified proteins (*IP*) were resolved by 7.5% SDS-PAGE and subjected to immunoblotting with antibodies that detect Ste2 (*FLAG Ab*) and Gpa1. Data shown are representative of three independent experiments. *Arrows* indicate the proteins specifically detected by the indicated antibodies.

of receptor coupling to G proteins. We have shown that the GαND mutant binds and hydrolyzes GTP, binds Gβγ in a guanine nucleotide-dependent manner, and binds receptor in an agonist-dependent manner. We have also demonstrated that the mutant is poorly expressed and rapidly degraded but expression is elevated by prolonged treatment with agonist. We conclude that GαND binds stably to the activated form of the receptor and thereby prevents activation of endogenous wild-type G protein.

Dominant-negative mutants have long been used to study a variety of signaling proteins, most notably monomeric G proteins such as Ras (49). At least three dominant-negative Ras mutants have been identified (50–52). Extensive biochemical analysis of the most widely used mutant, RasN17 (Ser-17 → Asn mutation), revealed that it competes with normal Ras for binding to guanine nucleotide exchange factors. More specifically, the mutant assumes an unactivable "dead-end" complex with the exchange factor, thereby preventing it from binding to the endogenous wild-type protein. This mechanism of action is analogous to the one proposed here, in which Gpa1^{N388D} is thought to act by competing with normal Gpa1 for binding to the receptor, thereby preventing activation of the pathway.

Although less widely used, dominant-negative mutations of heterotrimeric G proteins have also been described. The earliest report was from Osawa and Johnson (15), who showed that Gα$_s^{G226T}$ could partially inhibit β-adrenergic receptor-promoted stimulation of cAMP synthesis. Simon and co-workers (16, 53) described two other dominant-negative mutants, Gα$_o^{S47C}$ and Gα$_i^{S48C}$, and showed that these mutants lack GTP binding activity but retain Gβγ binding function. Another dominant-negative Gα$_s$ mutant was constructed using multiple substitutions (17) including A366S, which decreases affinity for GDP and causes the protein to spend more time in the empty state (54), as well as G226A and E268A, two substitutions that impair binding to GTP and the conformational changes required for dissociation of Gβγ (55, 56). More recently, Berlot and co-workers (57, 58) have described a dominant-negative Gα$_s$ mutant that combines G226A and A366S with multiple

substitutions in the $\alpha 3$ $\beta 5$ loop region that increase receptor affinity, decrease receptor-mediated activation, and impair activation of adenylyl cyclase. Expression of this mutant at close to wild-type levels blocked signaling from the luteinizing hormone receptor to $G\alpha_s$ by up to 97% (21).

Perhaps the best characterized dominant-negative Gα mutants are variants of $G\alpha_o$, $G\alpha_{11}$, and $G\alpha_{16}$ that were engineered to bind xanthine nucleotides instead of guanine nucleotides. Xanthine monophosphate is an intermediate in the biosynthesis of guanosine monophosphate. However, the cellular abundance of xanthine diphosphate and xanthine triphosphate is negligible, so the xanthine nucleotide-binding G proteins remain in the empty (nucleotide-free) state. Because it is the nucleotide-free form of Gα that has highest affinity for agonist-bound receptors, stable association with the mutant G protein makes the receptor unavailable to activate the endogenous wild-type G protein (18–20).

$Gpa1^{N388D}$ was originally reported to have no activity based on its inability to rescue a *gpa1*Δ mutant (41) but was later shown to promote recovery from pheromone-induced growth arrest (30). Thus, $Gpa1^{N388D}$ was long known to have properties of a dominant-negative mutant, but not recognized as such in part because of the earlier conclusion that $Gpa1^{N388D}$ is incapable of binding to G$\beta\gamma$. The evidence for lack of G$\beta\gamma$ binding, albeit negative, is as follows: (i) $Gpa1^{N388D}$ failed to prevent constitutive signaling in a cell lacking the *GPA1* gene (30, 41); (ii) $Gpa1^{N388D}$ displayed no binding to Ste4 in the two-hybrid assay (30); and (iii) $Gpa1^{N388D}$ did not (in purified recombinant form) bind to immobilized G$\beta\gamma$ (28). In contrast, we have shown that G$\beta\gamma$ associates in a guanine-nucleotide-dependent manner with both $G\alpha_{i2}{}^{N270D}$ and $Gpa1^{N388D}$. We believe that earlier failures to detect binding stem from the poor stability and poor expression of the mutant protein.

The mechanism of $Gpa1^{N388D}$ also was obscured by the long held supposition (which was never demonstrated) that $Gpa1^{N388D}$ lacks GTPase activity. Based on this assumption, Stone and co-workers (28–30) proposed that $Gpa1^{N388D}$ is an "activating" mutation that promotes recovery from growth arrest via a desensitization effector. However, their model did not account for the requirement for pheromone, which simultaneously requires binding of the receptor to all three subunits of the G protein. Their model is also inconsistent with our observation that $Gpa1^{Q323L}$ (demonstrated previously to bind but not hydrolyze GTP) has a phenotype opposite to that exhibited by $Gpa1^{N388D}$ (10, 13). This prompted us to revisit the question and test directly whether GTPase activity is affected. We found that the protein is highly unstable *in vitro* but is nevertheless able to bind and hydrolyze GTP. Based on our data, we propose that $Gpa1^{N388D}$ is rapidly degraded but forms a more stable complex with agonist-bound receptor and G$\beta\gamma$. This complex cannot be activated and therefore precludes further signaling via the endogenous Gα or G$\beta\gamma$. It is worth emphasizing that, whereas the behavior of Gα dominant-negative mutants has often been ascribed to stable association with the receptor, this is the first time such an association has been documented.

Asn-388 lies within the conserved "Asn-Lys-*X*-Asp" sequence that links the $\beta 5$ strand and the $\alpha 4$ helix of all G protein α subunits (59). All three conserved residues are present in Ras and bacterial elongation factor Tu as well as in heterotrimeric G proteins. In the available crystal structures Asn and Asp form hydrogen bonds with the guanine ring of the nucleotide, whereas the Lys methylene group provides a hydrophobic surface that lies over the purine ring (59). Mutational substitution of the Asp is sufficient to switch the nucleotide binding specificity from guanine to xanthosine in Gα (as discussed above) as well as in Ras (60) and elongation factor Tu (61). Thus one possibility is that substitution of the Asn relaxes nucleotide binding specificity or affinity sufficiently to diminish activation of the G protein by receptors. However, until a crystal structure of a receptor and G protein heterotrimer becomes available, the structural basis for the dominant-negative behavior will remain obscure.

Finally, distinct G proteins trigger a wide variety of signal responses. In some instances, a single G protein activates multiple effectors. Conversely, multiple G proteins activate a common effector. By disrupting the activity of cognate activators or effectors, dominant-negative G proteins could be used to identify upstream and downstream signaling components, regardless of how signaling specificity is achieved. If specificity depends on intrinsic differences in receptor or effector binding, the dominant-negative mutant should exhibit a similar binding specificity and therefore as a consequence selectively block that interaction. If a G protein has broad specificity for receptors but is restricted in its activity through subcellular compartmentalization, the dominant-negative version of that protein should have a similar subcellular distribution and would therefore leave other signaling pathways unaffected.

G protein-coupled receptors clearly play an important role in cellular physiology. Our ability to understand (and modulate) receptor signaling has been hampered by a lack of pathway-specific inhibitors, particularly inhibitors that act downstream of receptors. In this regard, dominant-negative G proteins hold tremendous promise for understanding how signaling networks are organized. Given that Asn-388 in Gpa1 is faithfully conserved in all Gα proteins, comparable inhibitory mutants should have a similar function in other G protein subtypes.

Acknowledgments—We are grateful to Paul Flanary, Ming Guo, Duane Jenness, David Stone, and Malcolm Whiteway for providing valuable reagents.

REFERENCES

1. Lefkowitz, R. J. (1998) *J. Biol. Chem.* **273,** 18677–18680
2. Gether, U., and Kobilka, B. K. (1998) *J. Biol. Chem.* **273,** 17979–17982
3. Ji, T. H., Grossmann, M., and Ji, I. (1998) *J. Biol. Chem.* **273,** 17299–17302
4. Hollinger, S., and Hepler, J. R. (2002) *Pharmacol. Rev.* **54,** 527–559
5. Robishaw, J. D., and Berlot, C. H. (2004) *Curr. Opin. Cell Biol.* **16,** 206–209
6. Ribeiro-Neto, F. A., Mattera, R., Hildebrandt, J. D., Codina, J., Field, J. B., Birnbaumer, L., and Sekura, R. D. (1985) *Methods Enzymol.* **109,** 566–572
7. Landis, C. A., Masters, S. B., Spada, A., Pace, A. M., Bourne, H. R., and Vallar, L. (1989) *Nature* **340,** 692–696
8. Masters, S. B., Miller, R. T., Chi, M. H., Chang, F. H., Beiderman, B., Lopez, N. G., and Bourne, H. R. (1989) *J. Biol. Chem.* **264,** 15467–15474
9. Graziano, M. P., and Gilman, A. G. (1989) *J. Biol. Chem.* **264,** 15475–15482
10. Apanovitch, D. M., Iiri, T., Karasawa, T., Bourne, H. R., and Dohlman, H. G. (1998) *J. Biol. Chem.* **273,** 28597–28602
11. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) *Science* **265,** 1405–1412
12. Kleuss, C., Raw, A. S., Lee, E., Sprang, S. R., and Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9828–9831
13. Guo, M., Aston, C., Burchett, S. A., Dyke, C., Fields, S., Rajarao, S. J., Uetz, P., Wang, Y., Young, K., and Dohlman, H. G. (2003) *Mol. Cell* **12,** 517–524
14. Herskowitz, I. (1987) *Nature* **329,** 219–222
15. Osawa, S., and Johnson, G. L. (1991) *J. Biol. Chem.* **266,** 4673–4676
16. Slepak, V. Z., Katz, A., and Simon, M. I. (1995) *J. Biol. Chem.* **270,** 4037–4041
17. Iiri, T., Bell, S. M., Baranski, T. J., Fujita, T., and Bourne, H. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 499–504
18. Yu, B., Slepak, V. Z., and Simon, M. I. (1997) *J. Biol. Chem.* **272,** 18015–18019
19. Yu, B., and Simon, M. I. (1998) *J. Biol. Chem.* **273,** 30183–30188
20. Yu, B., Gu, L., and Simon, M. I. (2000) *J. Biol. Chem.* **275,** 71–76
21. Berlot, C. H. (2002) *J. Biol. Chem.* **277,** 21080–21085
22. Dohlman, H. G. (2002) *Annu. Rev. Physiol.* **64,** 129–152
23. Xue, Y., Batlle, M., and Hirsch, J. P. (1998) *EMBO J.* **17,** 1996–2007
24. Yun, C. W., Tamaki, H., Nakayama, R., Yamamoto, K., and Kumagai, H. (1997) *Biochem. Biophys. Res. Commun.* **240,** 287–292
25. Batlle, M., Lu, A., Green, D. A., Xue, Y., and Hirsch, J. P. (2003) *J. Cell Sci.* **116,** 701–710
26. Harashima, T., and Heitman, J. (2002) *Mol. Cell* **10,** 163–173
27. Apanovitch, D. M., Slep, K. C., Sigler, P. B., and Dohlman, H. G. (1998) *Biochemistry* **37,** 4815–4822
28. Cismowski, M. J., Metodiev, M., Draper, E., and Stone, D. E. (2001) *Biochem. Biophys. Res. Commun.* **284,** 247–254
29. Metodiev, M., Matheos, D., Rose, M. D., and Stone, D. E. (2002) *Science* **296,** 1483–1486
30. Stratton, H. F., Zhou, J., Reed, S. I., and Stone, D. E. (1996) *Mol. Cell. Biol.* **16,** 6325–6337

31. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1987) *Current Protocols in Molecular Biology*, Wiley-Interscience, New York
32. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
33. Dohlman, H. G., Apaniesk, D., Chen, Y., Song, J., and Nusskern, D. (1995) *Mol. Cell. Biol.* **15,** 3635–3643
34. Song, J., Hirschman, J., Gunn, K., and Dohlman, H. G. (1996) *J. Biol. Chem.* **271,** 20273–20283
35. Giniger, E., and Ptashne, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 382–386
36. Kozasa, T., and Gilman, A. G. (1995) *J. Biol. Chem.* **270,** 1734–1741
37. Parker, E. M., Kameyama, K., Higashijima, T., and Ross, E. M. (1991) *J. Biol. Chem.* **266,** 519–527
38. Whiteway, M., Hougan, L., and Thomas, D. Y. (1990) *Mol. Cell. Biol.* **10,** 217–222
39. Hoffman, G., Garrison, T. R., and Dohlman, H. G. (2002) *Methods Enzymol.* **344,** 617–631
40. Dohlman, H. G., Goldsmith, P., Spiegel, A. M., and Thorner, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9688–9692
41. Kurjan, J., Hirsch, J. P., and Dietzel, C. (1991) *Genes Dev.* **5,** 475–483
42. Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J. H., Gorwa, M. F., Colavizza, D., and Thevelein, J. M. (1998) *EMBO J.* **17,** 3326–3341
43. Dietzel, C., and Kurjan, J. (1987) *Cell* **50,** 1001–1010
44. Miyajima, I., Nakafuku, M., Nakayama, N., Brenner, C., Miyajima, A., Kaibuchi, K., Arai, K., Kaziro, Y., and Matsumoto, K. (1987) *Cell* **50,** 1011–1019
45. Zhou, J., Arora, M., and Stone, D. E. (1999) *Cell Biochem. Biophys.* **30,** 193–212
46. Chang, F., and Herskowitz, I. (1990) *Cell* **63,** 999–1011
47. Schauber, C., Chen, L., Tongaonkar, P., Vega, I., and Madura, K. (1998) *Genes Cells* **3,** 307–319
48. Neer, E. J. (1995) *Cell* **80,** 249–257
49. Feig, L. A. (1999) *Nat. Cell Biol.* **1,** E25–E27
50. Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., Temeles, G. L., Wolanski, B. S., Socher, S. H., and Scolnick, E. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 952–956
51. Feig, L. A., and Cooper, G. M. (1988) *Mol. Cell. Biol.* **8,** 3235–3243
52. Powers, S., O'Neill, K., and Wigler, M. (1989) *Mol. Cell. Biol.* **9,** 390–395
53. Slepak, V. Z., Quick, M. W., Aragay, A. M., Davidson, N., Lester, H. A., and Simon, M. I. (1993) *J. Biol. Chem.* **268,** 21889–21894
54. Iiri, T., Herzmark, P., Nakamoto, J. M., van Dop, C., and Bourne, H. R. (1994) *Nature* **371,** 164–168
55. Miller, R. T., Masters, S. B., Sullivan, K. A., Beiderman, B., and Bourne, H. R. (1988) *Nature* **334,** 712–715
56. Lee, E., Taussig, R., and Gilman, A. G. (1992) *J. Biol. Chem.* **267,** 1212–1218
57. Berlot, C. H., and Bourne, H. R. (1992) *Cell* **68,** 911–922
58. Grishina, G., and Berlot, C. H. (2000) *Mol. Pharmacol.* **57,** 1081–1092
59. Sprang, S. R. (1997) *Annu. Rev. Biochem.* **66,** 639–678
60. Zhong, J. M., Chen-Hwang, M. C., and Hwang, Y. W. (1995) *J. Biol. Chem.* **270,** 10002–10007
61. Hwang, Y. W., and Miller, D. L. (1987) *J. Biol. Chem.* **262,** 13081–13085