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

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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. Gpa1G252D cannot hydrolyze GTP and permanently activates the pheromone response pathway. Gpa1N29BD was also proposed to lack GTPase activity, yet it has an inhibitory effect on pheromone responsiveness. We have characterized this inhibitory mutant (designated GαND) 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 GαND. We conclude that agonist binding to the receptor promotes the formation of a nondissociable complex with Gα2ND, 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) 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 the 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 Go-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 Goα, resulting in inactivation of its GTPase activity, thus maintaining Goα in the active GTP-bound state. Pertussis toxin catalyzes ADP-ribosylation of Goi, and this modification blocks G protein coupling to GPCRs (6). However, pertussis toxin does not modify all members of the Goi subfamily, and no toxins have been identified that modify members of the Goa and Golar 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 Gαο. Another GTPase-deficient mutant replaces Gln-204 in Gαo (Gln-277 in Gαl and Gln-323 in Gpa1) (8–10). A crystal structure of GαoG204S and additional biochemical analysis suggest that the catalytic Gln acts by stabilizing the tringonal-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 Go subunits, and we recently used this approach to show that the yeast Go 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 Go 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 Goα (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

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mediates the cellular response to glucose and environmental stressors such as high osmolarity and heat shock. Components of this pathway include the Ga protein Gpa2 working in conjunction with the putative glucose receptor Gpr1 (23, 24). Recent studies of Gpr1 signaling have identified two candidate Ω subunits, Gpb1 and Gpb2, and a candidate Gτ, Ggp1 (25, 26). The Gpb12 proteins lack the seven WD40 repeats found in classical Ω 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 Ga subunit have been described. Gpa1Q232L binds but does not hydrolyze GTP (13, 27). Gpa1N388D 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 Gpa1Q232L (13, 27), and here we characterize the inhibitory Gpa1N388D mutant. We report that Gpa1N388D binds and hydrolizes 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 DH5α was used for plasmid maintenance and amplification. The strains of yeast Saccharomyces cerevisiae used in this study and their origins are as follows: BY4741 (MATa his3Δ1 leu2Δ0 ura3Δ0) (32), YGS5 (YHP499 MATa ura3Δ1) (32), YGS5 (YHP499 gpa1Δ:his5 ste11Δ1) (33), BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; from Research Genetics, Huntsville, AL), and a BY4741-derived gpa1Δ ste11Δ1 mutant strain (MATa ste11Δ7:KanMX gpa1Δ:his5, provided by Paul Planany, University of North Carolina). Yeast cells were grown in synthetic medium supplemented with adenine, amino acids, 2% glucose (SC), or 2% galactose plus 0.2% sucrose (SCG) to express GAL1-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 Gpa1Q232L signaling and growth arrest; however, these cells can be maintained at 30 °C by inactivation of the temperature-sensitive ste11Δ1 mutant.

Several expression plasmids used in this study have been described previously: pRS15 (CEN LEU2), pRS423 (2 μM H3S), pRS424 (2 μM, TRP1) (32), pADAM (2 μM, LEU2, ADH1 promoter and terminator), and pADAM-GST and pADAM-GPA1-GST (34). pGAL1 (2 μM, LEU2) and pGAL1 (2 μM, LEU2) (35) (provided by Ming Guo, Yale University). pRS315-GPA1 contains TTTTATCCAGAAAGAGTACGACAGA-3′ which introduced an in-frame hexahistadine tag at the N terminus of this construct.

Expression of GαND in Insect Cells—A baculovirus encoding human GαND with an N-terminal hexahistidine tag was prepared and amplified using plasmid his6-GαND-pFastBacHta according to the manufacturer’s instructions (Invitrogen). Four liters of Sf9 cells at a density of 1.5 × 10⁸ 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 × 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 MgCl2, 9.8 mM β-mercaptoethanol, 0.01 mM GDP, 500 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 10 μM t-1-tosylamido-2-phenylethyl chloromethyl ketone) and lysed by passage through a pressurized Emulsifier (Avestin, Ottawa, Canada). The lysate was centrifuged at 500 × g for 15 min to remove intact cells and nuclei. The cleared lysate was further centrifuged at 150,000 × 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 imidazole in cell lysis buffer. His-GαND was eluted with 180 mL of 50 mM imidazole in cell lysis buffer, diluted 1:4 in Buffer A (20 mM HEPES, pH 8, 2 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EDTA, 0.01 mM GDP, 500 mM LiCl, 0.01 μM aprotinin, 0.01 μM leupeptin, 0.01 μM pepstatin, 0.01 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 10 μM 1-1-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 [35S]GTPγS binding and GTPase activity.

Expression of GαND—Expression of GαND, or GαND::KanMX, in pYES2.1/V5-His-TOPO was digested with HindIII and subject to centrifugation at 3,000 × g for 1 h. The remaining plasmid was subjected to centrifugation at 15,000 × g for 1 h.

Three different constitutive mutants in the yeast Ga subunit have been described. Gpa1Q232L binds but does not hydrolyze GTP (13, 27). Gpa1N388D 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 Gpa1Q232L (13, 27), and here we characterize the inhibitory Gpa1N388D mutant. We report that Gpa1N388D binds and hydrolizes GTP but is unable to dissociate effectively from receptors and therefore acts as a potent dominant-negative inhibitor of receptor signaling.

Expression of GαND, or GαND::KanMX, in pYES2.1/V5-His-TOPO was digested with HindIII and subject to centrifugation at 15,000 × g for 1 h. The remaining plasmid was subjected to centrifugation at 15,000 × g for 1 h.
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 A600nm ~ 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 1:50000 dilution of K-Nap (K-Nap is a substrate for β-galactosidase) to each well and incubating it for 60 min at 30°C. The bound proteins were eluted by heating at 100°C for 10 min. The resultant lysates were harvested by centrifugation at 1,000g for 10 min and the supernatant was subjected to centrifugation and activity in the supernatant was quantitated by scintillation counting to determine free phosphate.

Fraction numbers are indicated above each lane, and hatched bars, mean ± S.E. Note that a wider range of fractions are included in B and C than in A.

**Gpa1 and Gpa1**<sup>N388D</sup> **Expression Assay—**To compare expression of Gpa1 versus Gpa1<sup>N388D</sup>, protein concentration was examined in the gpa1Δ 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 A600nm ~ 0.6 and incubation continued for an additional 2 h. Cells were grown to mid-log phase in SCG (A600nm ~ 1.0), and cell growth was stopped by addition of 10 mM (final concentration) NaN3. Cells were harvested by centrifugation at 1,000 × g for 10 min. Cells were washed once with 10 mM NaN3 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<sup>N388D</sup>, 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 Ga (Gpa1) or Ga<sup>N388D</sup> (Gpa1<sup>N388D</sup>) 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 A600nm units of cells were resuspended in purification lysis buffer (40 mM triethanolamine, pH 7.2, 2 mM EDTA, 150 mM NaCl, 2 mM diethiothreitol, 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 μg/ml alarcin 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 MgCl2 and 10 μM GDP (condition 1, "-AlF<sub>4</sub>"), 3 mM MgCl2, 10 μM GDP, 30 μM AlCl<sub>3</sub>, and 10 mM NaF (condition 2, "-AlF<sub>4</sub>"), 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<sup>N388D</sup> and receptor (Ste2) tagged or nontagged with the FLAG epitope were grown in selective SCG medium to A600nm ~ 1.0. All the following procedures were conducted at 4°C. Twenty five A600nm units of cells were harvested 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 diethiothreitol, 1 × of protease inhibitor mixture (catalog number 1873588; 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
resulting zone of growth inhibition was documented after 2–3 days.

1 h of rocking to liberate membrane-bound proteins. The samples were then centrifuged at 10,000 × g for 10 min, and the resulting supernatant was incubated with 40 μl of a 50% slurry of EZViewTM Red anti-FLAG M2 affinity gel (Sigma). After 2 h of gentle agitation, the gel was centrifuged at 10,000 × 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 3X 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 × 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 Go12 (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

GoND Can Bind and Hydrolyze GTP—Gpa1N388D 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 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 Gpa1N388D from bacteria yielded a product unable to hydrolyze GTP, but which was also unable to bind to GTP or Gβγ (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 Go12 expressed in insect cells. Go 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 Go12N270D fused at the N terminus to a hexahistidine affinity tag (his6-Go12N270D). 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 Go12 (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 his6-Go12N270D 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 his6-Go12N270D (data not shown).

To further purify his6-Go12N270D, 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. 1A, his6-Go12N270D represented greater than 50% of the total protein in fractions with significant [35S]GTPγS binding activity (see below). his6-
Fig. 4. Gpa2<sup>N270D</sup> 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<sup>N386D</sup>, or GPA2<sup>N270D</sup> under the control of the GAL<sup>+</sup> 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.

Gα<sub>o</sub><sup>N270D</sup> immunoreactivity did not elute as a discrete species but rather in two broad peaks over a wide range of NaCl concentrations (Fig. 1B). These results suggest that the protein was not monodisperse, consistent with our observations that protein solubility and GTPase activities were not monodisperse, consistent with our observations that protein solubility and GTPase activities were not monodisperse, consistent with our observations.

The Q-Sepharose eluate was assayed for <sup>[35]S</sup>GTPγS binding and GTPase activities. As shown in Fig. 1C, substantially more <sup>[35]S</sup>GTPγS binding and GTPase activity was observed in the first peak (fractions 32–36) than in the second peak, suggesting that the his<sub>6</sub>-G<sub>α</sub><sub>o</sub><sup>N270D</sup> 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 G<sup>N270D</sup> (determined by protein staining).

Wild-type his-G<sub>α</sub><sub>o</sub> purified under the same conditions eluted as a single discrete peak at ~200 mM NaCl, and these fractions exhibited nearly stoichiometric <sup>[35]S</sup>GTPγS binding (data not shown).

Significant loss of <sup>[35]S</sup>GTPγS binding activity of G<sub>α</sub><sub>o</sub><sup>N270D</sup> occurred as a function of time and temperature. For this reason all purification steps were carried out at 4 °C, and all assays of <sup>[35]S</sup>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<sub>1</sub> and Gγ<sub>2</sub> increased the proportion of G<sub>α</sub><sub>o</sub><sup>N270D</sup> associated with the membrane fraction, suggesting that the mutant protein interacts with Gβγ dimers and is recruited to the membrane by this interaction (data not shown). We have also co-expressed untagged G<sub>α</sub><sub>o</sub><sup>N270D</sup> and pentahistidine-tagged G<sub>γ</sub><sub>2</sub> with Gβ<sub>1</sub> and purified the heterotrimeric complex by Ni-NTA and ion exchange chromatography. We found that G<sub>α</sub><sub>o</sub><sup>N270D</sup> co-eluted with his<sub>6</sub>-Gβγ in this procedure, further suggesting that the mutant associates with Gβγ (data not shown).

**Gpa1<sup>N388D</sup> Is a Receptor- and Gα Subtype-selective Dominant-negative Mutant**—The data presented in Fig. 1 indicate that Gα<sup>NP</sup> binds and hydrolyzes GTP normally. This makes it unlikely that Gpa1<sup>N388D</sup> functions by activating any effector protein, because effectors recognize only the GTP-bound form of Ga. 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<sup>N388D</sup> 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<sup>N388D</sup> specifically inhibits the signaling activity of the pheromone...
We first compared pheromone signaling in wild-type cells to that in the dominant-negative strain. A, gpa1Δ ste7Δ mutant strain was transformed with plasmids containing STE7 under the control of the GAL1/10 promoter and either wild-type GPA1 or GPA1N388D under the control of the GALH 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 GPA1N388D (GALH 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.

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 Gpa2N365D would likewise enhance heat shock sensitivity. As shown in Fig. 4, Gpa2N365D significantly reduced the survival rate of cells grown at 50 °C for 45 min (Fig. 4A). In contrast, wild-type Gpa2, Gpa1, or Gpa1N388D exhibited no effect on heat shock sensitivity. Likewise, no difference was observed among any of the tested strains maintained at 30 °C (Fig. 4B). Taken together, these data indicate that Gpa2N365D (but not GPA1N388D) acts in the Gpr1-Gpa2 pathway, whereas Gpa1N388D (but not Gpa2N365D) diminishes Ste2-Gpa1-mediated pheromone signaling. Stated differently, GαND functions as a receptor-selective dominant-negative mutant.

Receptor Coupling Is Required for Gpa1N388D Dominant-negative Activity—The data above indicate that Gpa1N388D 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 Gpa1N388D 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. As shown in Fig. 5, the Gpa1N388D-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. 5A). Equal expression of each protein was confirmed by immunoblotting (Fig. 5B). These data indicate that the dominant-negative activity of Gpa1N388D requires direct coupling to its receptor.

Dominant-negative Activity of Gpa1N388D Depends on Receptor Activation—Gpa1N388D evidently couples to its receptor (Ste2), yet appears unable to undergo receptor activation. If Gpa1N388D and the receptor indeed form an unproductive complex, it remains unclear why any response to pheromone occurs at least initially. One possibility is that Gpa1N388D 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 Gpa1N388D to bind and thereby prevent further activation of wild-type Gpa1.

To test this aspect of the model, Gpa1N388D was expressed in the absence of wild-type Gpa1 (i.e. a gpa1Δ 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 Gpa1N388D. A, to measure Gpa1N388D expression, a gpa1 mutant (strain YG55) was transformed with a plasmid containing either no insert (Vec), wild-type GPA1, or GPA1N388D under the control of the GAL1 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 (α/MF). Another addition of α-factor was made 2 h prior to collecting the cells, as indicated (α/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 Gβγ-dependent expression of Gpa1N388D. To determine the relative contribution of receptor and Gβγ to stable Gpa1N388D expression, wild-type diploid cells (strain YPH501) were transformed with plasmids containing no insert (Vec), wild-type GPA1, or GPA1N388D, alone or in combination with plasmids that express receptor (STE2), Gβγ (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, Gpa1N388D blocked cell division arrest in pheromone-treated cells (those nearest the source of pheromone) (Fig. 6A) (45). Cells grew poorly at the perimeter of the halo, where pheromone concentrations are reduced (Fig. 6A). 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 Gpa1N388D 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 Gpa1N388D can form a stable complex with pheromone-occupied receptor and Gβγ. Alternatively, Gpa1N388D could subserve a role in cell division cycling independent of its ability to bind Gβγ. 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), Gpa1N388D (in the absence of wild-type protein) produced a high basal transcription activity and a dose-dependent inhibition of the response (Fig. 6B). These data mirror that seen in the growth arrest assay, and further suggest that Gpa1N388D binds to pheromone-activated receptor and Gβγ. However, rather than leading to activation, the mutant appears to remain stably bound to receptor and Gβγ.

Pheromone Stimulation Increases Gpa1N388D Stability and Expression—The above results suggest that pheromone triggers the association of Gpa1N388D with Gβγ and receptor. Thus, we investigated the mechanism by which pheromone treatment can unmask the apparent ability of Gpa1N388D to sequester Gβγ. One possibility is that pheromone is required for stable expression of Gpa1N388D (47). Indeed, purification of GαS-N270D or Gpa1N388D yielded protein that was unstable in vitro (28). Thus we considered whether Gpa1N388D is also unstable in vivo.

To determine whether Gpa1N388D is unstable, we monitored its rate of loss in cells following treatment with the protein synthesis inhibitor cycloheximide. As shown in Fig. 7A, overexpressed wild-type Gpa1 protein was quite stable, with almost no change after a 90-min treatment with cycloheximide. In contrast, Gpa1N388D was expressed at much lower levels, and expression was undetectable after only 30 min of translation inhibition (Fig. 7A). 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 Gpa1N388D expression was again measured by immunoblotting. As shown in Fig. 7B, addition of pheromone resulted in elevated expression of Gpa1N388D but had no effect on the already high levels of wild-type Gpa1. These data suggest that pheromone promotes more stable expression of Gpa1N388D. The elevated expression might allow the mutant protein to associate with Gβγ 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, Sat2, 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 Go 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^Q323LN388D 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 AlF₄⁻ 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.

Discussion

We have characterized a novel mutant (designated Gα₁^ND) and found that it acts as a potent dominant-negative inhibitor...
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 Gα1N388D 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 Gα2G226T could partially inhibit β-adrenergic receptor-stimulated cAMP synthesis. Simon and co-workers (16, 53) described two other dominant-negative mutants, GαoS47C and GαiS48C, and showed that these mutants lack GTP binding activity but retain Gβγ binding function. Another dominant-negative Gαq 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 Gαi mutant that combines G226A and A366S with multiple

Fig. 10. Gβγ binds preferentially to Gα1N388D in the GDP-bound conformation. To determine whether Gα1N388D 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, Gα1N388D-GST, or GST alone. The cells were disrupted in the presence of GDP (−) or GDP and AlF4 (−), 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 Gpa1N388D and receptor. To determine whether Gpa1N388D 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.
substitutions in the α3 β5 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 Gaα by up to 97% (21).

Perhaps the best characterized dominant-negative Ga mutants are variants of Gaα3, Gaα11, and Gaα16 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 Gaα 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).

Gpa1N388D was originally reported to have no activity based on its inability to rescue a gpa1Δ mutant (41) but was later shown to promote recovery from pheromone-induced growth arrest (30). Thus, Gpa1N388D was known to have properties of a dominant-negative, but not recognized as such in part because of the earlier conclusion that Gpa1N388D is incapable of binding to Gβγ. The evidence for lack of Gβγ binding, albeit negative, is as follows: (i) Gpa1N388D failed to prevent constitutive signaling in a cell lacking the GPA1 gene (30, 41); (ii) Gpa1N388D displayed no binding to Ste4 in the two-hybrid assay (30); and (iii) Gpa1N388D did not (in purified recombinant form) bind to immobilized Gβγ (28). In contrast, we have shown that Gβγ associates in a guanine-nucleotide-dependent manner with both Gaα3N270D and Gpa1N388D. We believe that earlier failures to detect binding stem from the poor stability and poor expression of the mutant protein.

The mechanism of Gpa1N388D also was obscured by the long held supposition (which was never demonstrated) that Gpa1N388D lacks GTPase activity. Based on this assumption, Stone and co-workers (28–30) proposed that Gpa1N388D 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 Gpa1N388D (demonstrated previously to bind but not hydrolyze GTP) has a phenotype opposite to that exhibited by Gpa1N388D (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 Gpa1N388D is rapidly degraded but forms a more stable complex with agonist-bound receptor and Gβγ. This complex cannot be activated and therefore precludes further signaling via the endogenous Gaα or Gβγ. It is worth emphasizing that, whereas the behavior of Gaα 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 β5 strand and the α4 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 Gaα (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 Gaα proteins, comparable inhibitory mutants should have a similar function in other G protein subtypes.

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