Second Site Suppressor Mutations of a GTPase-deficient G-Protein α-Subunit

SELECTIVE INHIBITION OF Gβγ-MEDIATED SIGNALING*

(Received for publication, June 30, 1998)

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G proteins transmit signals from cell surface receptors to intracellular effectors. The intensity of the signal is governed by the rates of GTP binding (leading to subunit dissociation) and hydrolysis. Mutants that cannot hydrolyze GTP (e.g. GαQ227L, GαR209S) are constitutively active and can lead to cell transformation and cancer. Here we have used a genetic screen to identify intragenic suppressors of a GTPase-deficient form of the Ga in yeast, Gpa1Q323L. Sequencing revealed two-site mutations in three conserved residues, K34E, R327S, and L353Δ (codon deletion). Each mutation alone results in a complete loss of the βγ-mediated mating response in yeast, indicating a dominant-negative mode of inhibition. Likewise, the corresponding mutations in a mammalian Gaα (K46E, R209S, L235Δ) lead to inhibition of Gβγ-mediated mitogen-activated protein (MAP) kinase phosphorylation in cultured cells. The most potent of these βγ inhibitors (R209S) has no effect on Gaα-mediated regulation of adenyl cyclase. Despite its impaired ability to release βγ, purified recombinant Gpa1Q32278 is fully competent to bind and hydrolyze GTP. These mutants will be useful for uncoupling Gβγ- and Ga-mediated signaling events in whole cells and animals. In addition, they serve as a model for drugs that could directly inhibit G protein activity and cell transformation.

The actions of many hormones and neurotransmitters are mediated through a cell surface receptor, heterotrimeric “G protein,” and an intracellular effector that propagates the signal. Upon agonist binding to the receptor, Ga undergoes guanine nucleotide exchange and a conformational change leading to dissociation from βγ. The subunits remain in the active state until GTP is hydrolyzed, at which time they reassociate and signaling stops.

Not surprisingly, disturbances in the cycle of G protein activation and inactivation can lead to disease. The severe and often fatal diarrhea associated with cholera is caused by the pathogenic exotoxin of Vibrio cholerae (cholera toxin) which promotes ADP-ribosylation of Gα in the gut epithelium. Similarly, whooping cough is caused by a toxin from Bordetella pertussis and ADP-ribosylation of Gα (1). A number of germ line and somatic cell defects in Ga proteins have also been described. These include both activating and inactivating mutations, which can sometimes lead to cell transformation and cancer (2–4). An early example of an activated G protein allele was described by Landis, et al. (5), who showed that certain types of human pituitary tumors are associated with GTPase-deficient mutants of Gα. Another inherited disorder, known as pseudohypoparathyroidism type IA, is associated with loss-of-function mutations in Gα (R231H, A366S). Interestingly, GαR366S is unstable and inactive at 37 °C but is stable and constitutively active at the slightly lower temperature of the male testis, resulting in a paradoxical combination of pseudohypoparathyroidism and testotoxicosis in affected individuals (6).

G proteins can transform cells in at least two ways. First, mutations in Gα can lead to direct activation of certain effector enzymes, which promote cell proliferation. However, this mechanism appears to be operative in only a minority of cases (3). Alternatively, activating mutations in Ga can lead to constitutive dissociation from Gβγ. The ability of βγ subunits to promote cell proliferation occurs through activation of small GTP-binding proteins such as Rac and Ras, which in turn lead to the activation of Jun and mitogen-activated protein (MAP)1 kinases, respectively (3).

A strikingly similar signaling cascade has been identified through genetic analysis of the mating response pathway in yeast Saccharomyces cerevisiae. Haploid yeast cells secrete small peptide pheromones that bind to G protein coupled receptors. The G protein α subunit (GPA1 gene product, Gpa1) does not directly activate any known effector; rather it is βγ (Ste4/Stei18) that activates a signaling cascade that includes a MAP kinase homologue, Fus3. This in turn triggers a coordinated series of events required for mating, including cell fusion, new gene transcription, and cell cycle arrest in G1 (7).

A principal goal of pharmacology is to identify drugs that bypass or otherwise compensate for the molecular defects that lead to disease. One emerging strategy is to use genetics to identify protein binding partners of a dysfunctional gene product because any interacting proteins represent alternative

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* This work was supported by National Institutes of Health Grants GM 55316 (to H. G. D.), GM27800, and CA54427 (to H. R. B.) and by American Cyanamid Company (to H. G. D.). 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.

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1 The abbreviations used are: MAP, mitogen-activated protein; HA, hemagglutinin; IgG, chonic gonadotropin; Quin, quinpirole; PTX, pertussis toxin; Carb, carbacol; CAMP, cyclic adenosine monophosphate; MAPK, mitogen-activated protein kinase; IP, inositol phosphate; CHO, Chinese hamster ovary.
Gpa1 and mammalian Gα mutants were constructed by oligonucleotide-directed mutagenesis (Altered Sites, Promega) and expressed in plasmids pRS316 and pG1501 (Gpa1). Construction of Gpa1Q323L was described previously (19). Other mutant oligonucleotide sequences are as follows: GαR205S, 5'-GGC CCC CAG GAT GAA GAC TCT SAC GAT GCT ATT TCA TGT GAG GAC GGA GAA AGT ACT CTC CCC TGA TTC TCC AGC ACC; GαR231S, 5'-CTT GCT CTC AGA TCT CTG ACC ACC; G i2 R207S, 5'-GCA G GG C AA A GCA AGG TCT CAG; G i2 R231S, 5'-GCA G GG C AA A GCA AGG TCT CAG.

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MATERIALS AND METHODS

Strains, Plasmids, Mutagenesis—Established methods were used for the growth of bacteria Escherichia coli and the manipulation of plasmids (10). All molecular biology reagents were purchased from New England Biolabs and used according to manufacturer instructions. Yeast expression plasmids were pRS316, pRS316-GPA1 (11), pG1501, and pG1501-GPA1 (12). The transcription reporter plasmid was pET15b (Novagen) (16). The mammalian Gα expression plasmid was pCDNA1amp (containing EE-epitope tagged versions of Gα, Gβ, and Gγ) (17, 18). Other mammalian receptor expression vectors are described in the references provided below.
Inositol Phosphate Accumulation—Inositol phosphate (IP) accumulation in intact cells was assayed as described (26, 27). Briefly, 24 h after transfection, cells were replated in 24-well plates at $1.5 \times 10^5$ cells/well and labeled with myo-$[^3H]$inositol ($6 \mu$Ci/ml, Amersham Pharmacia Biotech) for 24 h. After washing with a medium containing 5 mM LiCl for 10 min, cells were incubated with the appropriate agonist in the presence of 5 mM LiCl for 45 min. IP and total inositol fractions were resolved on a Dowex AG 1-X8 formate column (Bio-Rad), and IP accumulation was estimated by determining the ratio of IP radioactivity to the sum of radioactivity of IP and total inositol.

Measurement of p44 HA-MAPK Activity—HA-MAPK activity was assayed as described (26, 28) with modifications. Cells were transfected in 6-well plates at $7 \times 10^5$ cells/well, placed in serum-free medium after 28 h, and assayed after an additional 20 h. After pretreatment with PTX (where indicated, 200 ng/ml for 4 h), cells were stimulated with the appropriate agonist for 8 min. HA-MAPK was immunoprecipitated from cell lysates (300 μl, representing $4 \times 10^6$ cells) with 2 μg of 12CA5 antibody and 35 μl of protein A-agarose (50% slurry). After washing once with lysis buffer and once with kinase buffer, the agarose beads were incubated at 22 °C for 20 min in 50 μl of kinase buffer (28) containing 250 μg/ml myelin basic protein and 50 μM [γ-$^32$P]ATP (2 μCi/tube, 700 cpm/pmol, NEN Life Science Products). The reaction was stopped with 5 μl of 88% formic acid, and radioactivity incorporated into myelin basic protein was determined by filtration on Whatman P81 membranes (28).

RESULTS

Screening for Intragenic Suppressors of gpa1 Q323L—Our objective here was to identify dominant-negative-type inhibitors of βγ-mediated signaling. Our approach was to screen for intragenic suppressors of a GTPase-deficient allele, gpa1 Q323L.

To this end, the gpa1 Q323L mutant was expressed using the galactose-inducible GAL1/10 promoter (plasmid pG1501) in cells lacking GPA1 (gpa1 Δ, strain YGS5). The gpa1 Δ mutation ordinarily leads to constitutive signaling and cell division arrest; however, YGS5 is viable at 34 °C because of a temperature-sensitive mutation that blocks the signal downstream of βγ (ste11ts). Cells were initially maintained in galactose medium at 34 °C and then plated and shifted to 24 °C. Rare colonies that grew under these restrictive conditions were picked, patched, and tested for galactose-dependent growth (Fig. 1, top). Identical results were obtained for the Q323L + K54E and Q323L + L553Δ mutants (data not shown).
mutants to inhibit receptor-dependent signaling in a GPA1 mutant to bind and data not shown). All three Gi2 mutations were prepared and expressed using a low copy plasmid. In this case, all three single-site mutants led to a complete inhibition of the pheromone response (determined by the halo assay, Fig. 2A). The double mutants reduced basal MAP kinase activity by 20%, as compared with control (empty vector) transfected COS-7 cells. Upon stimulation with a D2 agonist quinpirole, phosphorylation was attenuated 20–40% and quinpirole-stimulated activity by 50–60%. Similar mutations and transfected these together with the D2-dopamine receptor, hCG-receptor, M2-muscarinic receptor, and either Gα12R207S (α12RS), Gα12R231S (α12RS), or Gα12L353A (α12LA), as described for Fig. 3B. Quinpirole (Quin) inhibition of hCG-stimulated adenyl cyclase activity (panel A), hCG-stimulated adenyl cyclase activity (panel B), or carbachol-stimulated inositol phosphate (IP) production (panel C) was measured, as detailed under “Materials and Methods.”

Gα1 Mutants Inhibit the Mating Response Pathway in Yeast—Complementation of gpa1Δ reflects an ability of each mutant to bind βγ in vivo. We then tested the ability of the mutants to inhibit receptor-dependent signaling in a GPA1+ strain. In this case, all three single-site mutants led to a complete inhibition of pheromone response (determined by the growth inhibition “halo assay,” Fig. 2A). The double mutants can also inhibit pheromone signaling (and will even complement gpa1Δ) but only when overexpressed (data not shown). In comparison, a 2-fold overexpression of wild-type GPA1 led to a more modest inhibition of the pheromone response, and the double-site mutants were without effect (Fig. 2A). The mutant and wild-type forms of Gpa1 were expressed at equal levels, as determined by immunoblotting (data not shown). Similar results were obtained using a pheromone-induced transcription reporter assay (FUS1 promoter, lacZ reporter; Fig. 2B).

Inhibition of Gβγ-mediated MAP Kinase Activity in COS-7 Cells—The results presented in Figs. 1 and 2 reveal that all three single-site mutants will complement a gpa1Δ mutant and can inhibit signaling even in the presence of wild-type Gpa1, consistent with a dominant-negative mode of action. We then examined if the corresponding mutations in a mammalian Gα subunit (α) are known inhibitors of Gβγ signaling. Pertussis toxin (PTX) is a known inhibitor of Gα.

As shown in Fig. 3, all three Gα12 mutants reduced basal MAP kinase activity by 20%, as compared with control (empty vector) transfected COS-7 cells. Upon stimulation with a D2 agonist quinpirole, phosphorylation was attenuated 20–40% by the mutants, with R207S being the most potent inhibitor. In comparison, two known inhibitors of βγ binding—the βγ-binding domain of β-adrenergic receptor kinase (βARKΔ) and the transducin α subunit (α1)—inhibited basal phosphorylation by ~30% and quinpirole-stimulated activity by 50–60%. Similar
results were obtained using transfected CHO cells (Fig. 3B). Wild-type G\(_{\text{i2}}\)a does not inhibit bg signaling (data not shown), presumably because the wild-type protein responds normally to activation by the receptor.

Having determined that G\(_{\text{i2}}\)a\(_{\text{R209S}}\) potently inhibits bg-mediated signaling, we also examined if the mutant has any effect on Ga-mediated signaling events. CHO cells were transfected with G\(_{\text{i2}}\)a\(_{\text{R209S}}\), the D2 dopamine receptor, and the luteinizing hormone/hCG receptor. Whereas, G\(_{\text{i2}}\)a\(_{\text{R209S}}\) inhibited agonist-dependent MAP kinase activity by 40% (Fig. 3B), the same mutant had no effect on hCG-mediated stimulation (via G\(_{\text{s}}\)) or quinpirole-mediated inhibition (via Gi) of adenylyl cyclase (Fig. 4A). In comparison, signaling via Gi was potently and selectively inhibited by PTX treatment.

The results presented in Figs. 3 and 4A reveal that G\(_{\text{i2}}\)a\(_{\text{R209S}}\) can inhibit signaling through bg but not through Ga. We also examined whether the corresponding mutations in G\(_{\text{s}}\)a and G\(_{\text{q}}\)a would behave similarly, at least with respect to Ga-mediated signaling events. CHO cells were transfected with G\(_{\text{s}}\)a\(_{\text{R231S}}\), the D2 dopamine receptor, and the luteinizing hormone/hCG receptor. Whereas, G\(_{\text{s}}\)a\(_{\text{R231S}}\) inhibited agonist-dependent MAP kinase activity by 40% (Fig. 3B), the same mutant had no effect on hCG-stimulated (Gs-mediated) production of cAMP (Fig. 4B); and G\(_{\text{q}}\)a\(_{\text{R207S}}\) had no effect on carbachol-stimulated (Gq-mediated) inositol phosphate production (Fig. 4C). Thus the Arg-to-Ser mutation does not interfere with coupling between receptors and the endogenous (wild-type) Ga, or between Ga and its downstream effectors, in all three cases tested.

**Biochemical Properties of the Gpa1\(_{\text{R327S}}\) Mutant**—The Arg-to-Ser mutation leads to a dramatic, dominant-negative-type inhibition of G\(_{\beta}\gamma\) activity, at least in the two cases where this could be measured (Gpa1\(_{\text{R327S}}\), G\(_{\alpha}\)a\(_{\text{R209S}}\)). There are at least two ways that such a mutant could block G\(_{\beta}\gamma\) signaling. First, it could alter the conformation or subunit binding affinity of Ga for G\(_{\beta}\gamma\). Second, it could simply prevent binding to GTP. To rule out this more trivial explanation, two types of experiments were performed using purified recombinant Gpa1\(_{\text{R327S}}\). First, we measured the rate of pseudo-irreversible binding of \[^{[35]S}\]GTP, which is limited by the rate of GDP dissociation (16, 29). As shown in Fig. 5A, Gpa1\(_{\text{R327S}}\) is able to exchange GDP for GTP, as a second measure of GTP binding, we determined the steady state rate of GTP hydrolysis by Gpa1, which also reflects the rate of guanine nucleotide exchange.
term goal is to express these mutants in animals, to determine how inhibition of Gβγ signaling affects their growth and behavior in vivo. These experiments will help to distinguish Gα versus Gβγ-mediated signaling processes in a variety of systems, from yeast to humans.

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