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A $G_{s\alpha}$ mutant designed to inhibit receptor signaling through G_s

(dominant negative/adenylyl cyclase/trimeric G proteins)

TAROH IIRI*†, SEAN M. BELL*, THOMAS J. BARANSKI*, TOSHIRO FUJITA†, AND HENRY R. BOURNE*‡

*Departments of Cellular and Molecular Pharmacology and Medicine and Cardiovascular Research Institute, University of California, San Francisco, CA 94143; and †Fourth Department of Internal Medicine, University of Tokyo School of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan

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ABSTRACT **Hormonal signals activate trimeric G proteins by substituting GTP for GDP bound to the G protein α subunit (Gα), thereby generating two potential signaling molecules, Gα–GTP and free G$\beta\gamma$. The usefulness of dominant negative mutations for investigating Ras and other monomeric G proteins inspired us to create a functionally analogous dominant negative Gα mutation. Here we describe a mutant α subunit designed to inhibit receptor-mediated hormonal activation of G_s, the stimulatory regulator of adenylyl cyclase. To construct this mutant, we introduced into the α subunit (α_s) of G_s three separate mutations chosen because they impair α_s function in complementary ways: the $A^{366}S$ mutant reduces affinity of α_s for binding GDP, whereas the $G^{226}A$ and $E^{268}A$ mutations impair the protein's ability to bind GTP and to assume an active conformation. The triple mutant robustly inhibits (by up to 80%) G_s-dependent hormonal stimulation of adenylyl cyclase in cultured cells. Inhibition is selective in that it does not affect cellular responses to expression of a constitutively active α_s mutant (α_s–$R^{201}C$) or to agonists for receptors that activate G_q or G_i. This α_s triple mutant and cognate Gα mutants should provide specific tools for dissection of G protein-mediated signals in cultured cells and transgenic animals.**

Heterotrimeric G proteins are GTP-dependent molecular switches that relay signals from receptors for sensory stimuli, hormones, and neurotransmitters to effector enzymes and ion channels (1–3). Receptors activate trimeric G proteins by promoting exchange of GTP for GDP bound to the G protein's α subunit (Gα) (4–7), generating two potential signaling molecules, α–GTP and free $\beta\gamma$. Distinct families of G protein trimers, each distinguished by structures of their Gα subunits, trigger different sets of signaling pathways. It can be difficult, nonetheless, to identify which of the many different trimeric G protein families expressed in an individual cell or tissue transmits a specific signal. For this purpose it would be useful to create dominant negative Gα mutants analogous to the dominant negative mutants of monomeric GTPases (8, 9), kinases (10), and other proteins, all of which have proved extremely useful in dissecting other signaling pathways.

Such dominant negative proteins often interrupt signal transmission by stoichiometrically sequestering a second protein in the same signaling pathway. Thus, dominant negative monomeric GTPases sequester guanine nucleotide exchange factors, the proteins that catalyze replacement of GDP by GTP in the nucleotide-binding pocket of the GTPase. By analogy, a dominant negative Gα mutant would specifically sequester its guanine nucleotide exchange factor, the hormone receptor. One such Gα mutant has been reported, but its dominant negative activity was quite weak (11). In that case, a $G^{225}T$ mutation in the α subunit of G_s (the stimulatory regulator of adenylyl cyclase) produced a protein whose expression induced a rather modest decrease in the cAMP accumulation stimulated by an agonist for a G_s-coupled receptor; in addition, overexpression of this mutant caused constitutive stimulation of cAMP synthesis (11). Some loss-of-function mutations in α_i also appear to exert dominant negative effects on signaling, but these effects are probably mediated by sequestering G$\beta\gamma$ subunits rather than activated receptors (see *Discussion*).

Why have attempts to create receptor-sequestering Gα mutants proved unsuccessful? To prevent receptor activation of the endogenous Gα, the dominant negative mutant Gα must bind to and sequester not only G$\beta\gamma$ but also the activated receptor, interrupting the GTPase cycle by stabilizing or "freezing" an unproductive receptor–Gα–$\beta\gamma$ complex. For the complex to be sufficiently stable, the guanine nucleotide-binding pocket of the mutant Gα should be empty (4, 12); this is difficult to accomplish because "empty" Gα (the α_e state) is extremely unstable (13–15) and because GDP, like GTP, can induce dissociation of the receptor–Gα–$\beta\gamma$ complex (12). In addition, an overexpressed Gα mutant that sequesters the receptor weakly can actually promote constitutive stimulation of the effector, even if it sequesters G$\beta\gamma$ reasonably well, because a stoichiometric excess of free mutant Gα retains the ability to bind and to be activated by GTP (16, 17).

To bypass these difficulties, we sought to create a Gα mutant with impaired affinities for both GDP and GTP, hoping that the mutant's α_e state would be stabilized in a complex that sequesters G$\beta\gamma$ and activated receptors, thereby inhibiting signal transmission by wild-type Gα. We created an α_s triple mutant in which the $A^{366}S$ mutation reduces affinity for binding GDP (13) and promotes formation of the α_e state (13, 18), whereas the $G^{226}A$ and $E^{268}A$ mutations probably impair receptor-dependent binding of GTP- and GTP-induced conformational change. The triple mutant selectively inhibits hormonal signals that act by activating G_s.

MATERIALS AND METHODS

Cell Culture and Transfection. COS-7 cells maintained in DME-H21 medium containing 10% fetal calf serum were transiently transfected by the DEAE-adenovirus method (17, 19) with pcDNAI containing DNA encoding either wild-type or mutant α_s tagged with a hemagglutin (HA) epitope and the indicated receptors, except that the luteinizing hormone receptor (LH-R) was in the PSG5 vector. Membranes of COS-7 cells were prepared after nitrogen cavitation as described (17, 20).

cAMP Assay. cAMP accumulation in intact cells was assayed as described (13, 21). Briefly, 24 hr after transfection, cells

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Abbreviations: MAPK, mitogen-activated protein kinase; HA, hemagglutinin; Gα, G protein α subunit; LH-R, luteinizing hormone receptor; IP, inositol phosphate; IBMX, 3-isobutyl-1-methylxanthine-human; hCG, human chorionic gonadotropin.

‡To whom reprint requests should be addressed at: S-1212, Box 0450, University of California Medical Center, San Francisco, CA 94143. e-mail: h_bourne@quickmail.ucsf.edu.

were replated in 24-well plates at 1.5×10^5 cells per well and labeled with [^{3}H]adenine (4 μCi/ml, Amersham; 1 Ci = 37 GBq) for an additional 24 hr. Cells were stimulated with the indicated agonist in the presence of 3-isobutyl-1-methylxanthine (IBMX) for 25 min. cAMP and ATP fractions were resolved, and cAMP accumulation was estimated by determining the ratio of cAMP radioactivity to the sum of radioactivity of cAMP and ATP.

Inositol Phosphate Accumulation. Accumulation of inositol phosphates (IPs) in intact cells was assayed as described (22, 23). Briefly, 24 hr after transfection, cells were replated in 24-well plates at 1.5×10^5 cells per well and labeled with myo-[^{3}H]inositol (6 μCi/ml, Amersham) for 24 hr. After washing with a medium containing 5 mM LiCl for 10 min, cells were incubated with 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–Mitogen-Activated Protein Kinase (MAPK) Activity. HA–MAPK activity was assayed as described (23, 24) with modifications. Cells were transfected in 6-well plates at 7×10^5 cells per well, placed in serum-free medium after 28 hr, and assayed after an additional 20 hr. Cells were stimulated with appropriate agonist for 8 min. HA–MAPK was immunoprecipitated from cell lysate (300 μl, representing the extract from 4×10^5 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 (24) containing 250 μg/ml myelin basic protein and 50 μM [γ-^{32}P]ATP [2 μCi/tube, 700 cpm/pmol, Dupont/NEN]. The reaction was stopped with 5 μl of 88% formic acid. Radioactivity incorporated into myelin basic protein was separated by using Whatman P81 filters (24).

Immunoblots. Membrane fractions were solubilized with 1% (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) CHAPS (20) on ice for 60 min with mixing every 10 min. After centrifugation for 30 min at 100,000 $\times$ *g*, supernatant fractions were subjected to SDS/PAGE and visualized by using Western blot analysis with 12CA5 anti-HA antibody (25).

RESULTS

We sought to design a mutant α_s that strongly inhibits receptor-dependent activation of G_s but does not exhibit constitutive activity on its own. To assess effects of candidate mutants, we transiently cotransfected COS-7 cells with cDNAs encoding the mutant α_s tagged with an internal hemagglutin epitope (25) and LH-R, which can mediate activation of G_s but is not found in COS-7 cells. We then measured cellular cAMP accumulation in the presence or absence of the LH-R ligand, human chorionic gonadotropin (hCG). We asked whether candidate mutants inhibit hCG-stimulated cAMP accumulation and whether they constitutively stimulate cAMP accumulation.

This test system revealed rather weak dominant negative effects of several loss-of-function α_s mutants that are defective in GTP binding or GTP-induced activation (Fig. 1). As previously reported (11), expression of α_s-G^{225}T increases basal cAMP accumulation in the absence of receptor agonist and inhibits hormone-dependent cAMP accumulation quite weakly (by 30%–35%; Fig. 1*A*). A second loss-of-function mutant, α_s-G^{226}A (26), causes similarly weak inhibition of cAMP accumulation in the presence of hCG, and less (albeit still reproducible) stimulation of basal cAMP accumulation in the absence of hCG (Fig. 1*A*). A third loss-of-function mutant, α_s-R^{231}H, is thought to lack a conserved arginine–glutamate salt bridge that stabilizes the GTP-bound activated conformation of wild-type Gα subunits (17); this mutant exhibits no dominant negative activity (Fig. 1*B*). Mutation of the glutamate partner in the salt bridge with R^{231} (27) produces another mutant α_s (E^{268}A) with weak dominant negative activity (Fig. 1*B*). A candidate α_s containing two mutations, G^{226}A and E^{268}A, shows no greater dominant negative activity, although its constitutive stimulation of cAMP accumulation may be somewhat decreased (Fig. 1*A*).

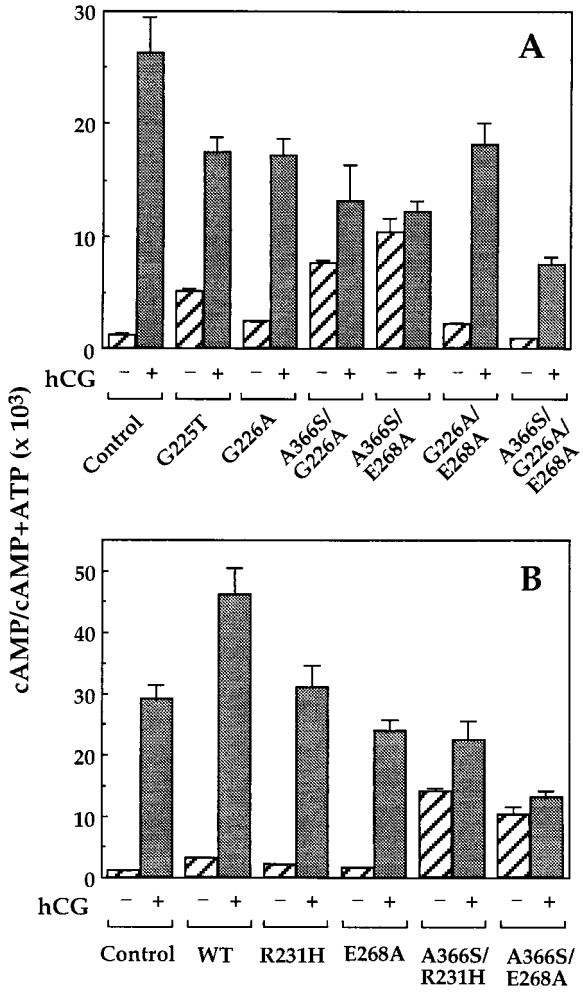

FIG. 1. Inhibition of receptor-stimulated cAMP accumulation by α_s mutants. COS-7 cells transfected with control plasmid pcDNAI or plasmids encoding wild-type or mutant HA-α_s (1.2 μg per 1.6×10^6 cells per 60-mm dish) and the LH-R in PSG5 (0.2 μg per 1.6×10^6 cells per 60-mm dish), were incubated at 37°C for 25 min with 100 μM IBMX and 75 ng/ml hCG (shaded bars) or no drug (hatched bars), and cAMP accumulation was measured. *A* and *B* represent results of two separate experiments, and all values represent means ± SD of triplicate determinations. Each set of results is representative of at least two additional experiments.

To increase the tendency of mutant α_s to assume the empty α_e state, we added to three of these loss-of-function mutations a different mutation, A^{366}S, which accelerates release of GDP and, by itself, constitutively activates adenylyl cyclase (data not shown; ref. 13). The A^{366}S mutation slightly augments dominant negative activities of mutants containing the G^{226}A (Fig. 1*A*), the R^{231}H, or the E^{268}A mutation (Fig. 1*B*), but at the same time increases basal constitutive activity of all three double mutants.

In contrast, a mutant carrying three mutations (A^{366}S, G^{226}A, and E^{268}A) substantially reduces hCG-stimulated cAMP accumulation (by 65–70%; Fig. 1*A*). Moreover, the α_s-A^{366}S/G^{226}A/E^{268}A mutant shows no tendency to increase basal cAMP accumulation in the absence of hormonal stimulation (Figs. 1*A* and 2*A*). The α_s triple mutant also reduces

cAMP accumulation stimulated by an agonist acting on the endogenous β_2-adrenoreceptor (Fig. 2*A*); thus, its dominant negative effect is exerted against multiple G_s-coupled receptors. Dominant negative activity of the triple mutant against the β_2-adrenoreceptor appears a bit weaker (50–60% inhibition) than the triple mutant against the LH-R (compare Figs. 2*A* and 1*A*); this may reflect the fact that the endogenous β_2-adrenoreceptor activates adenylyl cyclase in the entire COS-7 cell population, whereas the mutant α_s is expressed in a subpopulation of cells (17, 19); in contrast, the mutant α_s appears to exert a stronger negative effect on stimulation via the LH-R, because both the mutant α_s and the LH-R are expressed in the same subpopulation of cells.

We expected that the α_s triple mutant, like α_s-$A^{366}S$ (13), would be thermolabile. Indeed, the triple mutant does act more efficiently as a dominant negative in cells incubated at 33°C than in cells incubated at 37°C: inhibition was 65–70% at 37°C and ≈80% at 33°C (Fig. 3 *A* and *B*; compare cAMP accumulation in cells transfected with 0.2 μg LH-R). Immunoblots showed that expression of recombinant wild-type α_s is greater than that of the triple mutant at either temperature, although the mutant is better expressed at 33°C than at 37°C (Fig. 3*C*).

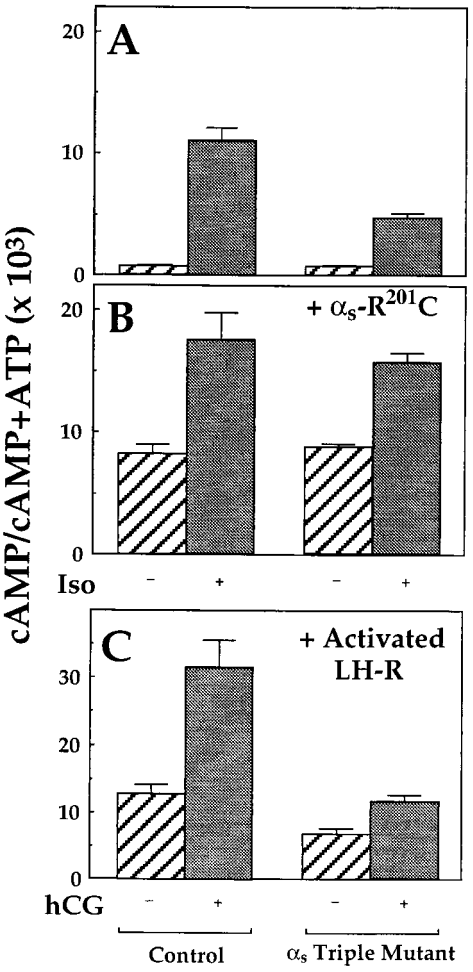

FIG. 2. α_s-$A^{366}S/G^{226}A/E^{268}A$ does not inhibit cAMP accumulation stimulated by constitutively active α_s but does inhibit cAMP accumulation stimulated by constitutively active LH-R. (*A* and *B*) COS-7 cells cotransfected with control plasmid pcDNAI or with plasmids encoding HA–α_s-$A^{366}S/G^{226}A/E^{268}A$ (1.2 μg per 1.6×10^6 cells per 60-mm dish) and a second plasmid without (*A*) or with (*B*) a constitutively active α_s mutant, HA–α_s-$R^{201}C$ (0.2 μg per 1.6×10^6 cells per 60-mm dish) were incubated at 37°C for 25 min with 100 μM IBMX and 1 μM isoproterenol (shaded bars) or no drug (hatched bars), and cAMP accumulation was measured. (*C*) COS-7 cells transfected with control plasmid pcDNAI or with plasmids encoding HA–α_s-$A^{366}S/G^{226}A/E^{268}A$ (1.2 μg per 1.6×10^6 cells per 60-mm dish) without or with a plasmid encoding a constitutively active LH-R (LH-R-$D^{578}G$, 0.2 μg per 1.6×10^6 cells per 60-mm dish) were incubated at 37°C for 25 min with 100 μM IBMX and 75 ng/ml hCG (shaded bars) or no drug (hatched bars), and cAMP accumulation was measured. Values represent means ± SD of triplicate determinations. Each set of results is representative of at least two additional experiments.

If the triple mutant acts by sequestering activated receptors, then its dominant negative effect will be counteracted either by increasing expression of the relevant receptor or by increasing fractional occupancy of the receptor by agonist. We tested these predictions in COS-7 cells transfected with different concentrations of DNA encoding the LH-R and α_s-$A^{366}S/G^{226}A/E^{268}A$; the results confirm the predictions (Fig. 3). At both 33°C and 37°C (Fig. 3 *A* and *B*), the dominant negative effect of the α_s triple mutant on hCG-stimulated cAMP accumulation is substantially greater in cells transfected with low concentrations of LH-R DNA (75–80% inhibition, even at 37°C, with 0.04 μg of LH-R DNA); conversely, at all LH-R DNA concentrations, increasing the concentration of α_s-$A^{366}S/G^{226}A/E^{268}A$ DNA augments inhibition of hCG-stimulated cAMP accumulation. Similarly, the α_s triple mutant exerts a more powerful dominant negative effect in cells treated with low concentrations of hCG, which occupy a smaller fraction of receptors (Fig. 3*D*): the mutant inhibits cAMP accumulation by ≈90% at 5 ng/ml hCG, but only by ≈60% at 500 ng/ml hCG.

If α_s-$A^{366}S/G^{226}A/E^{268}A$ acts by sequestering activated hormone receptors, it should not inhibit cAMP accumulation stimulated by a constitutively active α_s mutant, α_s-$R^{201}C$ (28). This proved to be the case (compare *A* and *B* in Fig. 2), indicating that the inhibition of cAMP accumulation does not result from sequestration of the constitutively α_s or from inhibition of adenylyl cyclase; instead, the triple mutant probably targets the receptor rather than the G protein's ability to stimulate the effector. Accordingly, the triple mutant does inhibit cAMP accumulation stimulated by a constitutively active LH-R, carrying the $D^{578}G$ mutation (ref. 29; Fig. 2*C*).

Finally, α_s-$A^{366}S/G^{226}A/E^{268}A$ does not block signals transmitted by either G_q or G_i, as assessed, respectively, by measurements of phosphoinosotide accumulation (stimulated by the M1 muscarinic acetylcholine receptor; Fig. 4*A*) or activation of MAPK (stimulated by the D2 dopamine receptor; Fig. 4*B*). Thus the triple mutant exerts its dominant negative effect specifically on signals transmitted by receptors coupled to G_s.

DISCUSSION

We have designed a dominant negative α_s mutant that selectively inhibits receptor-mediated stimulation of adenylyl cyclase. This triple mutant, α_s-$A^{366}S/G^{226}A/E^{268}A$, selectively inhibits receptor-stimulated cAMP accumulation by up to ≈80% but does not block signaling by a constitutively active α_s mutant or by agonists that act through G_q or G_i. In addition to its selectivity for G_s-mediated functions, this triple mutant shows no constitutive activity in the absence of hormonal stimulation. As in the case of dominant negative Ras mutants that sequester guanine nucleotide exchange proteins (8, 9), the selective negative effect of the triple mutant suggests that it works by sequestering receptors—the catalysts of guanine nucleotide exchange on trimeric G proteins. This mode of action is feasible because most cells contain many more copies of a trimeric G protein than of the receptors that stimulate it; the ratio of G protein to receptor may be as high as 10:1 (30).

In contrast, dominant negative effects of other mutant Gα proteins probably depend on sequestration of Gβγ rather than of receptors; most of these other mutants block actions of hormones coupled to G_i, which transmits many signals via βγ rather than via the α_i subunit. The wild-type α subunit of

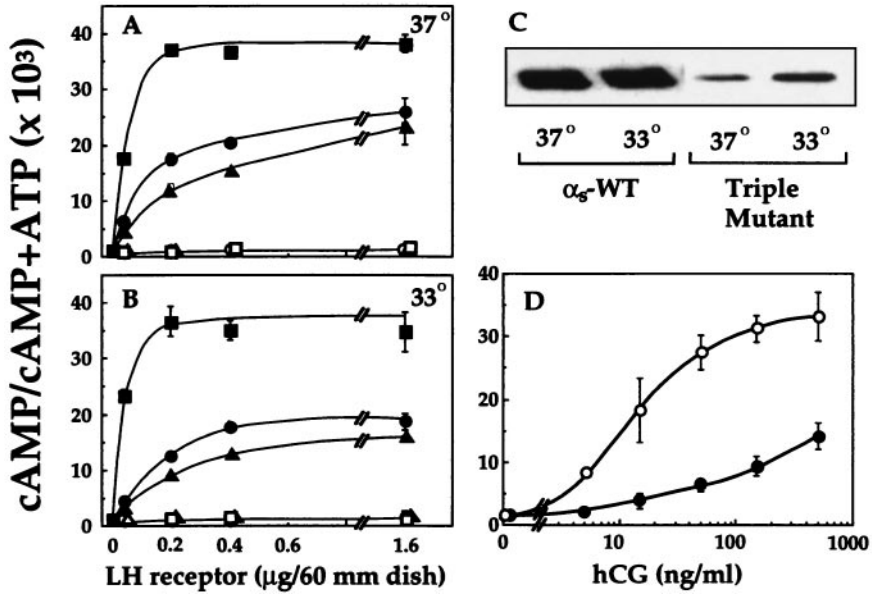

FIG. 3. Inhibition by α_s-$A^{366}S/G^{226}A/E^{268}A$ depends on concentrations of transfected α_s and LH-R DNA as well as concentration of agonist ligand. (*A* and *B*) COS-7 cells were transfected with control plasmids (open symbols) or a plasmid containing LH-R DNA (filled symbols; 0.04–0.16 μg per 1.6×10^6 cells per 60-mm dish, as indicated) and cotransfected with a second control plasmid (squares) or a plasmid encoding the α_s triple mutant (0.6 μg (circles) or 2.4 μg (triangles) per 1.6×10^6 cells per 60-mm dish). After labeling with [^{3}H]adenine for 24 hr either at 37°C (*A*) or at 33°C (*B*), cells were further incubated either at 37°C or at 33°C for 25 min with 100 μM IBMX and 75 ng/ml hCG (filled symbols) or no ligand (open symbols), and cAMP accumulation was measured. (*C*) Membranes (0.25 mg/ml) of COS-7 cells transfected with plasmids encoding either HA–α_s-wild type or HA–α_s-triple mutant (2.4 μg per 1.6×10^6 cells per 60-mm dish) and incubated either at 37°C or at 33°C (as indicated) for the ensuing 24 hr were solubilized with 1% CHAPS on ice for 60 min. Extracts (each representing 12.5 μg of membrane protein) were subjected to SDS/PAGE and immunoblotted with 12CA5 antibody (see *Materials and Methods*). (*D*) Cells were transfected with plasmids encoding the LH-R (0.2 μg per 1.6×10^6 cells per 60-mm dish) with (filled symbols) or without (open symbols) the α_s triple mutant (1.6 μg per 1.6×10^6 cells per 60-mm dish). After labeling with [^{3}H]adenine for 24 hr at 33°C, cells were further incubated at 33°C for 25 min with 100 μM IBMX and the indicated concentration of hCG, and accumulation of cAMP was measured. Values represent means ± SD of triplicate determinations. Each set of results is representative of at least two additional experiments.

retinal transducin, α_t, serves as a prototype dominant negative of this type: this protein binds $\beta\gamma$ but cannot be activated by many G_i-coupled receptors. Consequently, overexpression of α_t in cultured cells inhibits G_i- (and $\beta\gamma$-) mediated hormonal stimulation of adenylyl cyclase, type II (31) and of the MAPK pathway (24, 32, 33). Strikingly, however, α_t does not prevent a G_i-dependent effect that is mediated by the α subunit of G_i—i.e., inhibition of adenylyl cyclase (33). Similarly, dominant negative effects have been reported for two loss-of-function $G\alpha_{i2}$ mutants, cognate either to the $G^{226}A$ (34) or to the $G^{225}T$ (35) mutation of α_s. Both mutant proteins should bind $\beta\gamma$ normally but be less susceptible to GTP-induced conformational change than the corresponding wild-type α_i; accordingly, it is likely that sequestration of $\beta\gamma$ accounts for their ability to inhibit proliferation of fibroblasts (34) or activation of phospholipase A2 (35).

Although the $S^{17}N$ Ras mutant (8) acts by sequestering Ras-specific guanine nucleotide exchange proteins, the cognate Gα mutants probably exert dominant negative effects by sequestering $\beta\gamma$. An α_{i2} mutant with such a cognate mutation inhibited $\beta\gamma$-dependent activation of phospholipase β2 (36). A second cognate mutant, α_o-$S^{47}C$, identified by screening random α_o mutants for inability to bind a GTP analog, blocked opening of Cl^- channels in *Xenopus* oocytes stimulated by thyrotropin releasing hormone (37); this effect may be mediated by $\beta\gamma$, rather than by α_o–GTP. In contrast, the cognate mutations in αq and α_{16} showed no dominant negative activity against hormones that activate phospholipase β via receptors coupled to G proteins in the G_q family (36); the cognate α_s mutant, $S^{54}N$ (38), similarly fails to inhibit effects of hormones that activate adenylyl cyclase via receptors coupled to G_s (M. Faure and H.R.B., unpublished data). In each of the three latter cases, the GTP-bound Gα—α_q, α_{16}, or α_s—stimulates its effector directly. Thus, Gα mutations cognate to the S17N Ras mutation exert dominant negative effects when sequestration of $\beta\gamma$ can prevent activation of an effector but do not block effects mediated by Gα–GTP.

Abundant evidence indicates that hormone receptors activate G protein trimers containing α and $\beta\gamma$ subunits but cannot activate Gα subunits alone (39, 40). Thus, sequestration of $\beta\gamma$ by a loss-of-function Gα mutant might be expected to interrupt hormonal signals mediated by any G protein. Why, then, do loss-of-function Gα mutants block $\beta\gamma$-mediated signals but fail to inhibit hormonal signals mediated by α–GTP? We suspect that the difference reflects different roles of $\beta\gamma$. In the first case, $\beta\gamma$ mediates the hormonal signal stoichiometrically by binding to and activating an effector; sequestration of $\beta\gamma$ would be expected to inhibit such a signal in proportion to its ability to reduce the concentration of free $\beta\gamma$. In the case of signals mediated by α–GTP, however, the role of $\beta\gamma$ is catalytic rather than stoichiometric, and signal transmission probably requires substantially lower concentrations of free $\beta\gamma$; if so, free $\beta\gamma$ would have to disppear almost completely to interrupt the hormonal signal. Thus, interruption of the hormonal signal mediated by Gα–GTP requires a mutant Gα that sequesters activated receptors, probably in complexes that also contain $\beta\gamma$.

Several dominant negative Gα mutations identified in a genetic screen of *Saccharomyces cerevisiae* (41) probably act by sequestering $\beta\gamma$, the G protein subunit that mediates pheromone signals in this yeast species (42). Two of these yeast mutations affect codons for either the glutamate or the arginine (equivalent to E^{268} and R^{231} in α_s) that participate in a salt bridge necessary for receptor-catalyzed GTP binding (17); neither cognate mutation in α_s exerts a substantial dominant negative effect on G_s signaling, and the $R^{231}H$ mutant actually causes modest constitutive activation (Fig. 1). Similarly, α_q mutations cognate to these yeast mutations lack

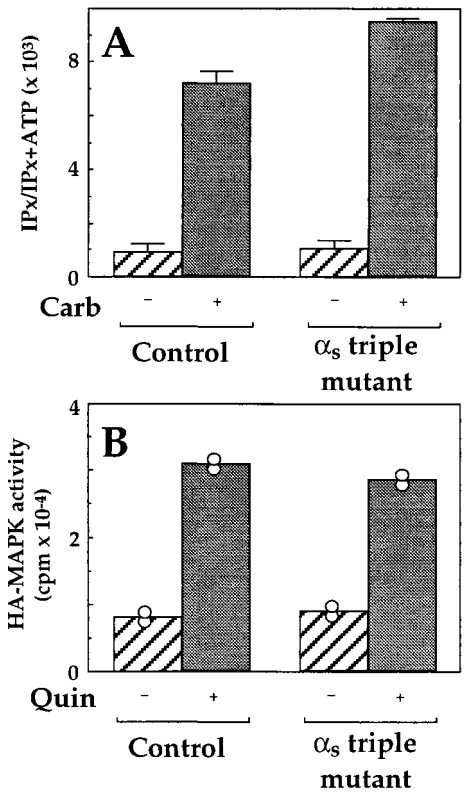

FIG. 4. α_s-$A^{366}S/G^{226}A/E^{268}A$ does not inhibit agonist ligands that act by activating G_q or G_i. (*A*) COS-7 cells were transfected with control plasmid pcDNAI or plasmids encoding HA–α_s-$A^{366}S/G^{226}A/E^{268}A$ (1.2 μg per 1.6×10^6 cells per 60-mm dish) and the M1 muscarinic receptor (0.2 μg per 1.6×10^6 cells per 60 mm dish) and labeled with myo-[^{3}H]inositol. Cells were incubated for 45 min with 5 mM LiCl and 200 μM carbachol (shaded bars) or no drug (hatched bars) and, phosphatidylinositol accumulation was measured. Values represent means ± SD of triplicate determinations. (*B*) COS-7 cells were transfected with vector plasmid pcDNAI or plasmids encoding wild-type HA–α_s-$A^{366}S/G^{226}A/E^{268}A$ (triple mutant, 1.2 μg per 1.6×10^6 cells per 60-mm dish) and the D2 dopamine receptor (0.2 μg per 1.6×10^6 cells per 60-mm dish) plus HA–MAPK (0.4 μg per 1.6×10^6 cells per 60-mm dish). Cells were incubated for 8 min with 10 μM quinpirole (shaded bars) or no drug (hatched bars), and HA-MAPK activity was measured; the small circles indicate the values of duplicate determinations. Each set of results is representative of at least two additional experiments.

dominant negative activity against hormones that act via G_q (T.I., unpublished data).

Thus, to block hormonal stimulation of adenylyl cyclase, mediated by α_s–GTP, it was necessary to create a mutant protein that sequesters activated receptors rather than just $\beta\gamma$. The α_s triple mutant appears to do just this, probably because the combination of mutations stabilizes the $\alpha_e\beta\gamma$ state of the trimer, which forms a tight complex with receptors (6). The mutations stabilize the α_e state by different mechanisms: the A366S mutation impairs binding of GDP (13)—perhaps by mimicking the α_e state—as suggested by the crystal structure of a cognate α_{i1} mutant (18); conversely, the $E^{268}A$ and $G^{226}A$ mutations impair GTP binding and GTP-induced conformational change (16, 17).

Because each of the three amino acids mutated in the triple mutant is highly conserved, we predict that mutations cognate to those of α_s-$A^{366}S/G^{226}A/E^{268}A$ will confer dominant negative activity on other Gα subunits. Such mutants—lacking constitutive activity in the absence of hormonal stimulation and specific for receptors that act via a particular subset of G proteins—may selectively block hormonal signals mediated by the appropriate G protein family, G_i, G_q, G_t, or $G_{12/13}$. In cultured cells and transgenic mice, the mutants could serve as useful probes for determining whether a particular G protein mediates a regulatory signal even when the relevant receptor or agonist is not known.

Susceptibility to thermal denaturation conferred by the $A^{366}S$ mutation (13) represents a potential limitation of the α_s triple mutant; a more thermostable protein, expressed in higher abundance, would probably sequester receptors more effectively. The thermolability may be unavoidable because the $A^{366}S$ mutation reduces the protein's affinity for binding GDP and the empty α_e state is presumably necessary for sequestering activated receptors. Indeed, we suspect that the triple mutant acts as strongly as it does because activated receptors stabilize the $\alpha_e\beta\gamma$ complex (6). Even though we do not know why the α_e state unfolds, it may be useful to devise genetic screens for a mutation that stabilizes it; such a fourth mutation could transform the α_s triple mutant into a more stable protein with stronger dominant negative activity.

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