Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 5656–5661, May 1997
Cell Biology

Conditional activation defect of a human $G_{s\alpha}$ mutant

(trimeric G proteins/G_s/pseudohypoparathyroidism/guanosine triphosphate/conformational change)

TAROH IIRI*, ZVI FARFEL†, AND HENRY R. BOURNE*‡

*Departments of Cellular and Molecular Pharmacology and Medicine and the Cardiovascular Research Institute, S-1212, Box 0450, University of California, San Francisco, CA 94143; and †Laboratory of Biochemical Pharmacology and Department of Medicine E, Sheba Medical Center, Tel Aviv University, Tel Hashomer 52621, Israel

Contributed by Henry R. Bourne, March 20, 1997

ABSTRACT **Hormonal signals activate trimeric G proteins by promoting exchange of GTP for GDP bound to the G protein's α subunit (Gα). Here we describe a point mutation that impairs this activation mechanism in the α subunit of G_s, producing an inherited disorder of hormone responsiveness. Biochemical analysis reveals an activation defect that is paradoxically intensified by hormonal and other stimuli. By substituting histidine for a conserved arginine residue, the mutation removes an internal salt bridge (to a conserved glutamate) that normally acts as an intramolecular hasp to maintain tight binding of the γ-phosphate of GTP. In its basal, unperturbed state, the mutant α_s binds guanosine 5′-[γ-thio]triphosphate (GTP[γS]), a GTP analog, slightly less tightly than does normal α_s, but (in the GTP[γS]-bound form) can stimulate adenylyl cyclase. The activation defect becomes prominent only under conditions that destabilize binding of guanine nucleotide (receptor stimulation) or impair the ability of α_s to bind the γ-phosphate of GTP (cholera toxin, AlF_4^- ion). Although GDP release is usually the rate-limiting step in nucleotide exchange, the biochemical phenotype of this mutant α_s indicates that efficient G protein activation by receptors and other stimuli depends on the ability of Gα to clasp tightly the GTP molecule that enters the binding site.**

The α subunits of heterotrimeric G proteins (Gα) are GTP-dependent molecular switches that relay signals from cell surface receptors to effector enzymes and ion channels (1, 2). Hormone-activated receptors turn α_s on by catalyzing release of otherwise tightly bound GDP, which is rapidly replaced by GTP. The switch reverts to the "off" position when its intrinsic GTPase activity converts bound GTP to GDP. In this cycle the activity of Gα is directly controlled by the presence or absence of the γ-phosphate of bound guanine nucleotide. Although crystal structures of α-5′-[γ-thio]triphosphate (GTP[γS]) and α-GDP are similar (3–5), limited regions of Gα (switches 1, 2, and 3) undergo a GTP-dependent conformational change that is induced by interactions between Gα residues and the γ-phosphate of GTP.

This conformational change controls interactions of Gα with receptors, $\beta\gamma$, effectors, and regulator of G protein signaling proteins. The γ-phosphate also serves as a driving force to ensure unidirectionality of the Gα cycle. Gα binds GTP much more tightly than GDP because its interactions with the γ-phosphate provide additional binding energy. Consequently, GTP does not dissociate from Gα before it can be hydrolyzed to GDP.

In turning a G protein on, the hormone–receptor complex not only accelerates dissociation of GDP but also apparently accelerates GTP binding (2, 6), increasing the preference of Gα for binding GTP, relative to GDP. GDP competes against GTP more effectively in the G protein's resting state than when GDP–GTP exchange is accelerated by receptor stimulation (7). Indeed, the concentration of GTP[γS] required for association with unstimulated pure Gα (8, 9) is much greater than that required for association with the receptor-activated G protein trimer (10).

We recently reported (11) that a new α_s mutation, found in patients with pseudohypoparathyroidism, type I (PHP-I), impairs the ability of the mutant α_s to mediate hormonal stimulation of cAMP accumulation in transiently transfected cells. Here we report a detailed biochemical analysis of the mutant α_s phenotype, which shows that both GDP–GTP exchange and GTP-induced conformational change depend on the ability of Gα to clasp GTP tightly in the guanine nucleotide binding pocket. The mutation, which replaces Arg-231 with histidine, causes an activation defect that is paradoxically intensified by hormonal and other stimuli. Thus the mutant α_s sabotages its own activation by making the activation process work against itself.

MATERIALS AND METHODS

Cell Culture and Transfection. S49 *cyc*$^-$ cells, maintained in DMEM containing 10% horse serum, were transfected with the retroviral vector pMV7 containing DNA encoding hemagglutinin (HA)-tagged wild-type (wt) (12) or mutant α_s (α_s-R231H), and stable clones were selected as described (12, 13). The *cyc*$^-$ clones studied in these experiments expressed nearly identical amounts of normal or mutant α_s. HEK293 cells were maintained in DMEM containing 10% fetal calf serum. Stable clones expressing the β_2-adrenergic receptor (AR) (14) were cotransfected by calcium phosphate precipitation with pSVneo and the pcDNAI vector containing either HA-tagged wt or mutant α_s, and stable clones were selected (15). COS-7 cells, maintained in DME-H21 medium containing 10% calf serum, were transiently transfected by the DEAE/adenovirus method (16) with pcDNAI vector containing either HA-tagged wt or mutant α_s. Membranes of S49 *cyc*$^-$ cells or COS-7 cells were prepared after nitrogen cavitation as described (17).

Immunofluorescence. Localization of HA-tagged α_s and β_2-AR was assessed in HEK293 cells as described (14, 15).

Purification of α_s. α_s was purified from cytosol of Sf9 cells infected with baculovirus encoding α_s (18). Sf9 cells (1.5×10^6 cells per ml), maintained in Sf-900II medium at 28°C, were infected with baculoviruses encoding α_s (three plaque-forming units per cell). After cell lysis using nitrogen cavitation, the supernatant fraction was sequentially chromatographed on

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/945656-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

Abbreviations: Gα, α subunits of heterotrimeric G proteins; PHP-1, pseudohypoparathyroidism, type 1; AR, adrenergic receptor; wt, wild type; HA, hemagglutinin; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

‡To whom reprint requests should be addressed. e-mail: h_bourne@quickmail.ucsf.edu.

columns of HiTrap Q (Pharmacia; 5 ml bed volume × 2) (13), Econo-Pac HTP (Bio-Rad; 5 ml bed volume) with a potassium phosphate gradient (19), and Mono Q (1 ml bed volume) (20) in the absence of detergents.

GTP[γS] Binding and GTPase Assays. GTP[γS] binding and GTP hydrolysis were quantitated as described (13, 20). Apparent on rates (k_{app}) of GTP[γS] binding were determined as described (19).

cAMP Assay. cAMP accumulation in intact S49 *cyc*$^-$ cells (21) and cAMP synthesis by recombinant α_s in membranes of S49 *cyc*$^-$ cells (13) were assayed as described. Before the latter assay, the α_s proteins were incubated with 100 μM GTP[γS] for 60 min.

Receptor Binding Assay. Competition between isoproterenol and [^{125}I]pindolol for binding to β-adrenergic receptors was determined in membranes of S49 *cyc*$^-$ cells expressing HA-tagged wt or mutant α_s as described (22, 23).

Trypsin Protection Assay. Hormonal activation of α_s in COS-7 cell membranes was assessed as described for rhodopsin-dependent activation of α_t (16), with modifications. Membranes (0.2 mg/ml) of COS-7 cells expressing HA-α_s or HA-α_s-R231H with the β_2-AR and G protein β_2 and γ_2 subunits were incubated at 22°C for 3 min in buffer containing 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol, in the presence or in the absence of 10 μM isoproterenol before the reaction was initiated by adding 10 μM GTP[γS]. At the times indicated, the samples were mixed with 0.6 mg/ml trypsin, 1% Lubrol-PX (Sigma), and 0.5 mM GDP and further incubated on ice for 60 min. Trypsin-resistant fragments of α_s were visualized and quantitated by SDS/PAGE and Western blot analysis using 12CA5 antibody (12).

To assess protection of recombinant protein from trypsin, 0.7 μM α_s or α_s-R231H were incubated at 22°C for 60 min in buffer containing 20 mM Na-Hepes, 2 mM $MgSO_4$, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 0.025% Lubrol, and the indicated guanine nucleotides. Samples were then incubated on ice for 60 min with 0.1 mg/ml of trypsin and trypsin-resistant fragments of α_s were visualized by SDS/PAGE followed by Coomassie blue staining.

RESULTS

R231H Mutation Impairs Activation. Activation of α_s-R231H is impaired in intact cells, as assessed after stable expression in S49 *cyc*$^-$ cells, which genetically lack endogenous α_s. Stimulation of cAMP accumulation by isoproterenol and cholera toxin was reduced by 80% relative to cAMP accumulation in cells expressing similar amounts of α_s-wt (Fig. 1*A*). The mutant protein's abundance (not shown) and plasma membrane localization (see below) were similar to α_s-wt, however.

Immunofluorescence showed that the R231H mutation blocks a second effect of α_s activation, agonist-induced translocation from plasma membrane to cytosol (Fig. 2). Stimulation of the β_2-AR in HEK293 cells stably expressing the receptor and HA-tagged α_s caused α_s-wt (15), but not the mutant protein, to appear in cytosol; the agonist caused the β_2AR to translocate normally (14, 15) in cells expressed either wt or mutant α_s. Cholera toxin also failed to cause the cytosolic translocation of α_s-R231H that was observed with α_s-wt (15) (not shown).

Despite its loss of function, α_s-R231H interacts normally with receptors, as assessed by its ability to enhance the affinity of the β_2-AR for binding the β-adrenergic agonist, isoproterenol. In membranes of cells expressing α_s-wt, the receptor shows high affinity for binding the agonist, and GTP[γS], a hydrolysis-resistant analog of GTP, abolishes high affinity binding (Fig. 1*B*), as described (22, 23). The high affinity binding of agonist is thought to reflect interaction of the

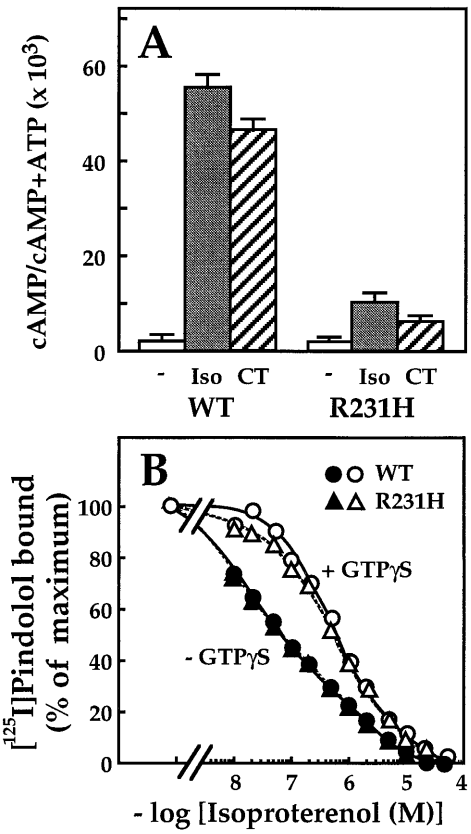

FIG. 1. cAMP accumulation and β-adrenergic receptor binding in S49 *cyc*$^-$ cells transfected with wild type or mutant α_s. (*A*) cAMP accumulation in intact cells. S49 *cyc*$^-$ cells stably transfected with HA-α_s or HA-α_s-R231H were incubated at 37°C for 30 min with 100 nM 3-isobutyl-1-methylxanthine and 10 μM isoproterenol (■) or no drug (□ and ▨) and cAMP accumulation was measured (21). Cholera toxin (1 μg/ml; ▨) was added to the culture medium 3 h before adding IBMX. Values represent mean ± SD of triplicate determinations. (*B*) Competition between isoproterenol and [^{125}I]pindolol for binding the β-adrenergic receptor. Membranes (0.15 mg/ml) of S49 *cyc*$^-$ cells stably transfected with HA-α_s or HA-α_s-R231H were incubated with [^{125}I]pindolol (72 pM) and the indicated concentrations of isoproterenol in the presence (open symbols) or absence (filled symbols) of 30 μM GTP[γS]; incubations and binding assays were performed as described (22).

receptor with G_s in the empty state, and is prevented by either binding of guanine nucleotide or absence of α_s (e.g., in *cyc*$^-$ membranes). The ability of α_s-R231H to increase the affinity of agonist binding was identical to that of α_s-wt (Fig. 1*B*), indicating that the receptor-G_s interaction is largely intact. Because receptor binding is measured at equilibrium, however, this result does not necessarily reflect possible changes in agonist-induced rates of GDP dissociation or GTP[γS] binding. In addition, this assay is not a valid measure of conformational change in Gα, because both GDP and GTP[γS] abolish high affinity binding (24).

Behavior of Pure α_s-R231H Under Basal Conditions. Despite its functional defect in cells, pure recombinant α_s-R231H can bind GTP and undergo GTP-induced conformational change. GTP[γS] associated with α_s-R231H almost as rapidly as with α_s-wt (k_{app} = 0.13 min^{-1} vs. 0.28 min^{-1}; Fig. 3*A*). Because the rate of association of GTP[γS] is limited by dissociation of GDP, this result suggests that the R231H mutation does not accelerate dissociation of GDP from α_s.

Although the mutation apparently does not destabilize binding of GDP, it does impair the stability of GTP[γS] binding. Although GTP[γS] did not measurably dissociate from α_s-wt (19), GTP[γS] dissociated from α_s-R231H at a low

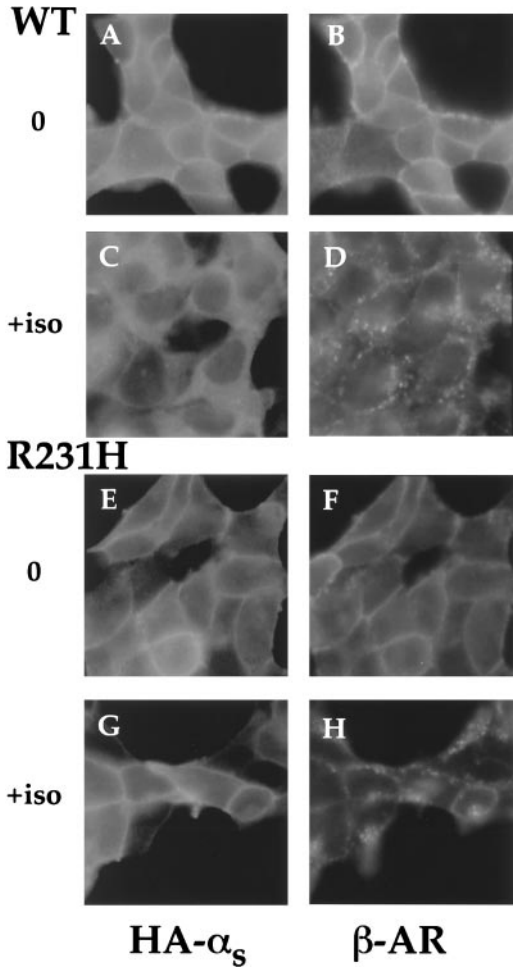

FIG. 2. Immunofluorescence of wt or mutant α_s. HEK293 cells stably expressing the β_2-AR and HA-α_s or HA-α_s-R231H were incubated with (*C, D, G*, and *H*) or without (*A, B, E,* and *F*) 10 μM isoproterenol for 20 min and then fixed with 4% formaldehyde. α_s was detected by incubation with mAb 12CA5, followed by fluorescein isothiocyanate-conjugated anti-mouse antibody (15). The β_2-AR was detected by incubation with a polyclonal rabbit antiserum against its C terminus (14), followed by Texas red-conjugated anti-rabbit antibody (15). Images were visualized as described (14, 15).

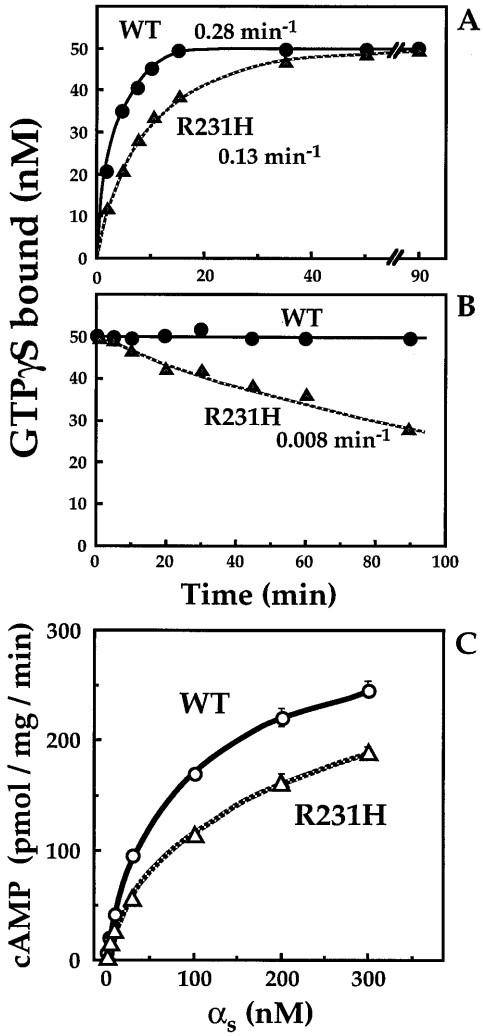

FIG. 3. Biochemical properties of recombinant wt and mutant α_s. (*A*) Rates of GTP[γS] binding. α_s or α_s-R231H (● and ▲, respectively; 50 nM each) were incubated at 22°C with 1 μM [^{35}S]GTP[γS] (3×10^5 cpm/pmol). At the times indicated, the reaction was terminated and GTP[γS] binding was quantitated by filtration on nitrocellulose filters (13, 20) and apparent on-rates of GTP[γS] binding (k_{app}) were calculated (19). (*B*) Rates of dissociation of GTP[γS]. α_s or α_s-R231H (● and ▲, respectively; 50 nM each) were incubated at 22°C with 1 μM [^{35}S]GTP[γS] as described in *A* for 45 min. Dissociation of bound [^{35}S]GTP[γS] was assessed by adding 200 μM unlabeled GTP[γS] (at time zero in *B*). At the times indicated, the reaction was terminated and GTP[γS] binding was quantitated as described in *A*. (*C*) cAMP synthesis stimulated by different concentrations of α_s or α_s-R231H in the presence of GTP[γS]. Reactions were conducted at 22°C for 15 min in 50 μl volumes containing 15 μg *cyc*$^-$ membranes, as described (13). Before the assay, the α_s proteins were incubated with 100 μM GTP[γS] for 60 min.

but easily measurable rate (0.008 min^{-1}; Fig. 3*B*), as assessed by measuring the rate at which nonradioactive GTP[γS] (200 μM) replaced [^{35}S]GTP[γS] bound to recombinant protein.

α_s-R231H can nonetheless assume an active conformation, as indicated by resistance to proteolysis and ability to activate effector. When activated by GTP[γS], Gα proteins are cleaved by trypsin near their N termini but the proteolytic products are resistant to further proteolysis. GTP[γS] protected α_s-R231H and α_s-wt from trypsin, while GDP did not (Fig. 4*C*). We also tested activation of adenylyl cyclase by adding α_s to *cyc*$^-$ membranes. In the presence of GTP[γS], α_s-R231H activated adenylyl cyclase almost as effectively as α_s-wt, over α_s concentrations from 0–300 nM (Fig. 3*C*).

The R231H mutation does not impair the interaction of α_s with $\beta\gamma$, as assessed by measuring the ability of $\beta\gamma$ to reduce the rate of association of GTP[γS] with purified α_s. $\beta\gamma$ inhibited the apparent rates of association of GTP[γS] with α_s-wt and α_s-R231H at similar concentrations and to a similar extent (data not shown).

Activation Defect of α_s-R231H. Despite normal activation by GTP[γS] under basal conditions *in vitro*, α_s-R231H showed defective activation by receptors, cholera toxin, and AlF_4^-.

Because isoproterenol failed to stimulate cAMP accumulation or translocation to the cytosol in intact cells expressing α_s-R231H, we asked more directly whether the mutation impairs receptor-mediated activation of α_s. As assessed by a trypsin protection assay (16) in membranes from COS-7 cells cotransfected with the β_2-AR and G_s subunits, isoproterenol promoted binding of GTP[γS] to α_s-R231H, but at a rate 30-fold less than α_s-wt (Fig. 4*A*). In contrast, GTP[γS] protected mutant and wt α_s at similar (slower) rates in the basal state, in the absence of isoproterenol.

Because cholera toxin failed to stimulate cAMP accumulation in intact cells expressing α_s-R231H, we used the trypsin protection assay to test whether R231H mutation impairs activation by cholera toxin. HEK293 cells stably expressing α_s-wt or α_s-R231H were cultured in the presence or absence of cholera toxin. Treatment with cholera toxin prevented

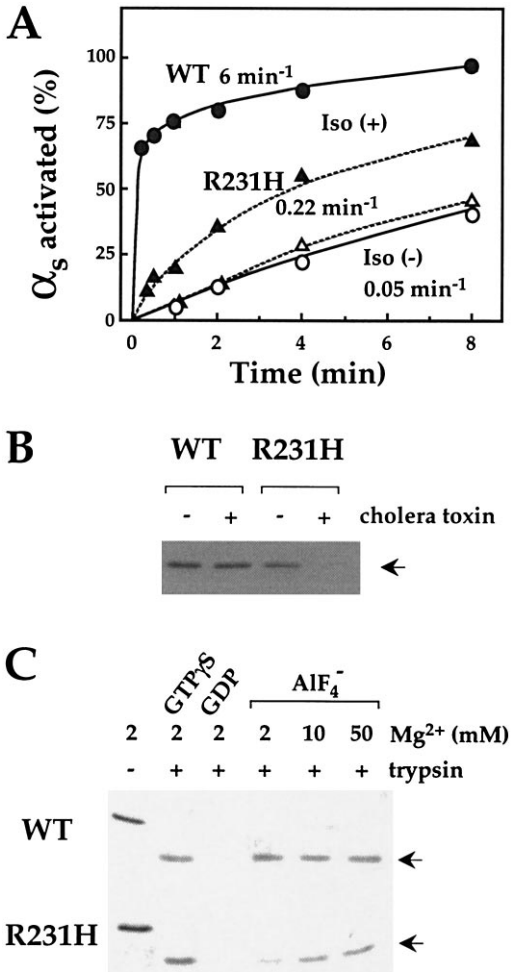

FIG. 4. Effect of activation on tryptic cleavage of wt and mutant α_s. (*A*) Receptor dependent activation of wt and mutant α_s. Membranes (0.2 mg/ml) of COS-7 cells expressing recombinant HA-α_s (●) or HA-α_s-R231H (▲) plus the β_2-AR and G protein β_2 and γ_2 subunits were incubated at 22°C with (filled symbols) or without (open symbols) 10 μM isoproterenol plus 10 μM GTP[γS]. At the times indicated, the reaction was terminated and samples were treated with trypsin (0.6 mg/ml) as described in *Materials and Methods*. Trypsin-resistant fragments of α_s were visualized and quantitated by Western blot analysis using 12CA5 antibody (12). (*B*) Effect of modification by cholera toxin on protection by GTP[γS] against cleavage by trypsin. HEK293 cells stably transfected with HA-α_s or HA-α_s-R231H were cultured in the absence or in the presence of 1 μg/ml of cholera toxin for 3 h. Membranes were incubated with 10 μM GTP[γS] at 22°C for 10 min. Samples were incubated with trypsin (10 μg/ml) and trypsin-resistant fragments of α_s (indicated by arrow) were visualized by Western blot analysis as described in *A*. (*C*) Effect of GTP[γS] and GDP/AlF$_4^-$ on tryptic cleavage. α_s or α_s-R231H (0.7 μM each) were incubated with 10 μM GTP[γS], 10 μM GDP, or 10 μM GDP plus 20 μM $AlCl_3$ and 10 mM NaF at 22°C for 60 min. Samples were further incubated in the absence or presence of trypsin (0.1 mg/ml) on ice for 60 min and trypsin-resistant fragments of α_s (arrows) were visualized by SDS/PAGE followed by Coomassie blue staining.

GTP[γS] from protecting α_s-R231H but not α_s-wt from trypsin (Fig. 4*B*). The toxin catalyzed ADP ribosylation of α_s-R231H to the same extent as α_s-wt, however, in membranes exposed to activated toxin and radioactive NAD^+ (result not shown).

AlF_4^-, which activates Gα by mimicking the γ-phosphate of GTP, protected pure recombinant α_s-R231H only weakly from tryptic proteolysis (Fig. 4*C*), in contrast to α_s-wt. Because Gα crystal structures show that Mg^{2+} links AlF_4^- to Gα residues in the nucleotide binding pocket (25, 26), we tested the effect of increasing the Mg^{2+} concentration. High concentrations of Mg^{2+} restored protection (Fig. 4*C*). This result was in accord with measurements of adenylyl cyclase activity stimulated by α_s-R231H in *cyc*$^-$ membranes; although the mutant protein stimulated cAMP synthesis weakly in the presence of AlF_4^-, increasing the Mg^{2+} concentration restored activation to a level comparable to that seen with AlF_4^--stimulated α_s-wt (not shown).

DISCUSSION

In this report we show that Gα activation requires a tight grasp of the protein on bound GTP. The R231H mutation causes a conditional defect that is intensified by activating stimuli. In its basal state, activation of α_s-R231H is almost normal, because the mutant protein can assume an active conformation, even though it binds GTP[γS] less rapidly and less tightly than does α_s-wt. Upon stimulation by receptor, cholera toxin, or AlF_4^-, however, the activation defect becomes much more prominent. Here we discuss how the R231H mutation impairs tight binding of GTP, how this causes a conditional activation defect, and implications of these findings for PHP-I.

An Intramolecular Hasp. R231 in α_s is cognate to R204 of α_t, which is located in the switch 2 region (the α2 helix) of Gα. Upon binding of GTP, switch 2 undergoes a conformational change, forming a coordinated complex with residues in the α3 helix and the switch 3 region, as previously noted (3, 4). R204 in α_t, and by inference R231 in α_s, is one of the residues that interacts with α3, forming a salt bridge with a conserved glutamate (E241 in α_t, E268 in α_s) in the α3 helix. The biochemical phenotype of α_s-R231H suggests that this salt bridge serves as an intramolecular hasp to fasten switch 2 to the α3 helix; this hasp helps Gα to hold GTP more tightly and to maintain an active conformation (Fig. 5).

In keeping with the postulated essential function of the intramolecular hasp in G protein signaling, this arginine-

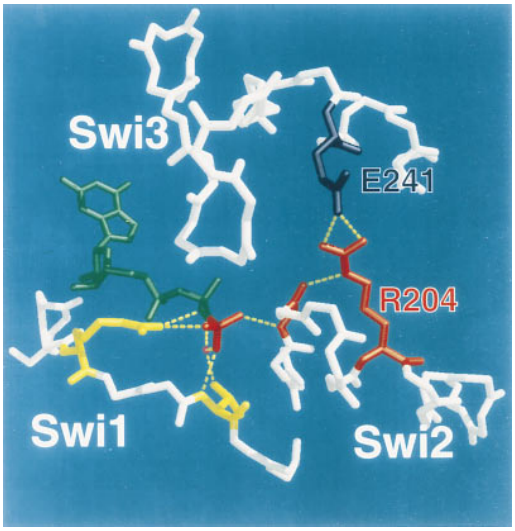

FIG. 5. The arginine-glutamate hasp of Gα. Switch (Swi) regions 1–3 in the crystal structure of α_t·GTP[γS] (3) are shown in relation to bound GTP (green; γ-phosphate colored red) and Mg^{2+} ion (magenta). Except for side chains of key amino acids, only main chains are depicted. The γ-phosphate of GTP (red) is linked to two amino acids (yellow, not labeled) in switch 1; these include R174 (on the left side of the picture) and T177. R174 corresponds to R201 of α_s, the target of ADP ribosylation by cholera toxin. T177 (T 204 in α_s) is linked to the γ-phosphate via bound Mg^{2+} (magenta). The γ-phosphate is linked to switch 2 by the main chain amide of G199 in α_t (red, not labeled; corresponding to G226 in α_s). The intramolecular hasp is the salt bridge between R204 (red; cognate to R231 in α_s) in switch 2 (the α2 helix) and E241 (blue; E268 of α_s) in α3. The hasp helps to maintain both the tight binding of GTP and the GTP-induced active conformation by fastening switch 2 and α3, thus reinforcing linkage of the glycine residue in switch 2 to the γ-phosphate of GTP.

glutamate pair is completely conserved among Gα proteins. In addition, mutational replacement of the glutamate member of this pair impairs G protein signaling in *Saccharomyces cerevisiae*. The E364K mutation in the yeast Gα subunit (E364 is cognate to E241 in α_t) makes yeast cells resistant to otherwise lethal doses of pheromones that act on G protein-coupled receptors (27). Biochemical analysis (David Stone, personal communication) suggests that the putative hasp in the yeast Gα stabilizes binding of GTP and the GTP-induced active conformation: The yeast mutant binds GTP[γS] less tightly at low concentrations of Mg^{2+} than does the wt protein.

The position of R204 in the crystal structure of α_t also explains why mutation of the cognate arginine in α_s does not prevent the mutant protein from interacting normally with βγ, the β_2-AR, or the effector. The side chain of this arginine does not interact with βγ in crystals of $\alpha_t\beta\gamma$-GDP, despite its location in the region that provides most of the Gα surface for interacting with βγ (28). In the same crystals (28), the location of this arginine relative to the presumed plane of the plasma membrane (28–30) makes an interaction with the receptor very unlikely. Finally, participation of the arginine in a salt bridge with its paired glutamate (3, 4) should prevent it from forming part of an effector-interacting surface.

The Activation Defect. Absence of the intramolecular hasp loosens the grip of α_s-R231H on GTP, slowing the rate at which the mutant protein binds and is activated by GTP[γS] (Fig. 3*A*). In response to stimulation by cholera toxin, AlF_4^-, or hormone receptor, the activation defect becomes much more severe (Figs. 1*A*, 2, and 4). This is because each of these stimuli activates α_s by a mechanism (different for each stimulus; see below) that also destabilizes the binding of guanine nucleotide by normal or mutant α_s. Synergy between this stimulus-induced instability and the intrinsic instability of GTP binding in α_s-R231H causes conditional exacerbation of the activation defect because the intramolecular hasp is not available to reinforce binding of the γ-phosphate.

From crystal structures we infer that both cholera toxin and AlF_4^- would destabilize binding of GTP, by interfering with the ability of Gα to bind the γ-phosphate of GTP (Fig. 5). In the crystal structure of αt (3), the arginine that is modified by cholera toxin directly interacts with the γ-phosphate of GTP. Although AlF_4^- mimics the γ-phosphate of GTP, the GDP·AlF_4^--bound form of Gα is inevitably less stable than the GTP[γS]-bound form, simply because AlF_4^- is not covalently attached to GDP. Thus the combination of the R231H mutation with either of these stimuli would severely destabilize the interaction of α_s with GTP, even though neither the mutation nor the stimuli would do so by themselves.

Conversely, a higher concentration of Mg^{2+} (another link between the γ-phosphate and Gα; Fig. 5) is expected to counteract the destabilizing effect of the R231H mutation on activation by AlF_4^-. This proved to be the case (Fig. 4*C*). The idea that two destabilizing influences may potentiate one another explains the earlier observation (31) that AlF_4^- activates adenylyl cyclase weakly when α_s has been modified by cholera toxin. In this case toxin-induced modification of a residue that interacts directly with the γ-phosphate presumably reduces stability of the α_s·GDP·AlF_4^- complex, thereby inhibiting its ability to activate the effector.

To promote release of bound GDP, receptors must destabilize the guanine nucleotide binding site also. We have postulated (13, 30) that receptors do so by altering interactions between Gα and the guanine ring of GDP. The receptor interaction should similarly destabilize binding of GTP; indeed, receptors can increase rates of dissociation of GTP analogs from G proteins (10, 32). GTP can replace GDP in the binding site, however, because GTP rescues normal Gα from receptor-induced instability, in two ways: First, the instability is offset by the additional binding energy furnished by the γ-phosphate, allowing Gα to bind GTP in preference to GDP. Second, GTP (but not GDP) induces a conformational change in Gα that causes it to dissociate from βγ and from the receptor; separation from the receptor definitively removes its destabilizing effect. Efficiency of both mechanisms in α_s-R231H will be reduced, because the disrupted arginine-glutamate hasp weakens the protein's ability to clasp GTP and to stabilize the conformation that disengages it from βγ and receptor.

An alternative possibility is that the R231H mutation inhibits receptor-induced release of GDP rather than binding of GTP. Because it fails to explain the defective responses to cholera toxin and AlF_4^-, we infer that this explanation is not correct.

Implications for PHP-I. Finally, the biochemical phenotype of the α_s-R231H mutant suggests a possible modification in the criteria we and others have used as a basis for inferring the locus of genetic mutations in families of PHP-I patients.

Classical PHP-I is a syndrome characterized by multiple abnormalities, including Albright hereditary osteodystrophy and resistance to several hormones that act by stimulating G_s (33–35). Erythrocyte membranes of most PHP-I patients contain only 50% of the normal complement of G_s activity (36, 37). The G_s-deficient phenotype, classified as PHP-Ia (36), indicates that the affected patients carry inactivating mutations in an α_s gene (the residual activity represents the intact α_s allele); DNA sequencing has revealed α_s mutations in many PHP-Ia patients (11, 35). In contrast, the PHP-Ib phenotype is assigned to a smaller number of PHP-I patients whose erythrocytes contain normal (or nearly normal) G_s activity (36). Most PHP-Ib patients, but not all (33), lack several of the clinical features of classical PHP-I, including Albright hereditary osteodystrophy; PHP-Ib patients have been thought to carry mutations in genes other than α_s.

The three affected patients in the family carrying the α_s-R231H mutation showed classical clinical features of the disease, including Albright hereditary osteodystrophy, but G_s activities in their erythrocytes were nearly normal (ranging between 60–90% of normal; results not shown). The G_s assay (36, 38) measures the ability of erythrocyte extracts to stimulate the adenylyl cyclase of *cyc*$^-$ membranes in the presence of isoproterenol and GTP[γS]. Because G_s activity in this crude assay reflects stimulation by GTP[γS] as much as that induced by the hormonal agonist (38), α_s-R231H activity was scored as nearly normal, despite the dramatic hormone-response defect produced by the mutant protein in cultured cells and in affected patients.

Thus the α_s-R231H patients show that results of the erythrocyte G_s assay can lead to an incorrect inference with respect to the genetic basis of the disease. Instead, PHP-I patients with apparently normal or nearly normal erythrocyte G_s activities merit careful investigation, especially when they display the classical clinical phenotype, including Albright hereditary osteodystrophy. Although such patients may inherit mutations in genes distinct from α_s, their α_s genes may encode mutant proteins with instructive qualitative defects, including impairment of conformational change, subcellular localization, or interaction with other proteins, including receptors, βγ, effectors, and regulator of G protein signaling proteins.

We thank Mark von Zastrow for anti β-AR antibody, Philip B. Wedegaertner and members of the Bourne laboratory for useful advice, and Helen Czerwonka for secretarial support. This work was supported by an National Institutes of Health (National Institute of General Medical Sciences) Grant GM27800 (to H.R.B.). T.I. was supported by a Julius H. Comroe Jr. Award by the Cardiovascular Research Institute. Z.F. was supported by a grant from the Israeli Ministry of Health.

1. Conklin, B. R. & Bourne, H. R. (1993) *Cell* **73,** 631–641.

2. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649.
3. Noel, J. P., Hamm, H. E. & Sigler, P. B. (1993) *Nature (London)* **366,** 654–663.
4. Lambright, D. G., Noel, J. P., Hamm, H. E. & Sigler, P. B. (1994) *Nature (London)* **369,** 621–628.
5. Mixon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G. & Sprang, S. R. (1995) *Science* **270,** 954–960.
6. Brandt, D. R. & Ross, E. M. (1986) *J. Biol. Chem.* **261,** 1656–1664.
7. Florio, V. A. & Sternweis, P. C. (1989) *J. Biol. Chem.* **264,** 3909–3915.
8. Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C. & Gilman, A. G. (1993) *J. Biol. Chem.* **268,** 14367–14375.
9. Singer, W. D., Miller, R. T. & Sternweis, P. C. (1994) *J. Biol. Chem.* **269,** 19796–19802.
10. Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H. & Ross, E. M. (1992) *J. Biol. Chem.* **267,** 8081–8088.
11. Farfel, Z., Iiri, T., Shapira, H., Roitman, Z., Mouallem, M. & Bourne, H. R. (1996) *J. Biol. Chem.* **271,** 19653–19655.
12. Levis, M. J. & Bourne, H. R. (1992) *J. Cell Biol.* **119,** 1297–1307.
13. Iiri, T., Herzmark, P., Nakamoto, J. M., Van Dop, C. & Bourne, H. R. (1994) *Nature (London)* **371,** 164–168.
14. von Zastrow, M. & Kobilka, B. K. (1992) *J. Biol. Chem.* **267,** 3530–3538.
15. Wedegaertner, P. B., Bourne, H. R. & von Zastrow, M. (1996) *Mol. Biol. Cell* **7,** 1225–1233.
16. Garcia, P. D., Onrust, R., Bell, S. M., Sakmar, T. P. & Bourne, H. R. (1995) *EMBO J.* **14,** 4460–4469.
17. Iiri, T., Homma, Y., Ohoka, Y., Robishaw, J. D., Katada, T. & Bourne, H. R. (1995) *J. Biol. Chem.* **270,** 5901–5908.
18. Iiri, T., Backlund, P. B., Jones, T. L. Z., Wedegaertner, P. B. & Bourne, H. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14592–14597.
19. Graziano, M. P., Freissmuth, M. & Gilman, A. G. (1989) *J. Biol. Chem.* **264,** 409–418.
20. Iiri, T., Ohoka, Y., Ui, M. & Katada, T. (1992) *J. Biol. Chem.* **267,** 1020–1026.
21. Wong, Y. H., Federman, A., Pace, A. M., Zachary, I., Evans, T., Pouysségur, J. & Bourne, H. R. (1991) *Nature (London)* **351,** 63–65.
22. Bourne, H. R., Kaslow, D., Kaslow, H. R., Salomon, M. R. & Licko, V. (1981) *Mol. Pharmacol.* **20,** 435–441.
23. Miller, R. T., Masters, S. B., Sullivan, K. A., Beiderman, B. & Bourne, H. R. (1988) *Nature (London)* **334,** 712–715.
24. Haga, K., Haga, T. & Ichiyama, A. (1986) *J. Biol. Chem.* **261,** 10133–10140.
25. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G. & Sprang, S. R. (1994) *Science* **265,** 1405–1412.
26. Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E. & Sigler, P. B. (1994) *Nature (London)* **372,** 276–279.
27. Stratton, H. F., Zhou, J., Reed, S. I. & Stone, D. E. (1996) *Mol. Cell. Biol.* **16,** 6325–6337.
28. Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E. & Sigler, P. B. (1996) *Nature (London)* **379,** 311–9.
29. Lichtarge, O., Bourne, H. R. & Cohen, F. E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7507–7511.
30. Onrust, R., Herzmark, P., Chi, P., Garcia, P. D., Lichtarge, O., Kingsley, C. & Bourne, H. R. (1997) *Science* **275,** 381–384.
31. Cassel, D. & Selinger, Z. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3307–3311.
32. Cassel, D. & Selinger, Z. (1977) *J. Cyclic Nucleotide Res.* **3,** 11–22.
33. Farfel, Z. & Bourne, H. R. (1982) *Miner. Electrolyte Metab.* **8,** 227–236.
34. Spiegel, A. M., Shenker, A. & Weinstein, L. S. (1992) *Endocr. Rev.* **13,** 536–565.
35. Ringel, M. D., Schwindinger, W. F. & Levine, M. A. (1996) *Medicine (Baltimore)* **75,** 171–184.
36. Farfel, Z., Brickman, A. S., Kaslow, H. R., Brothers, V. M. & Bourne, H. R. (1980) *N. Engl. J. Med.* **303,** 237–242.
37. Levine, M. A., Downs, R. W., Jr., Singer, M., Marx, S. J., Aurbach, G. D. & Spiegel, A. M. (1980) *Biochem. Biophys. Res. Commun.* **94,** 1319–1324.
38. Farfel, Z., Brothers, V. M., Brickman, A. S., Conte, F., Neer, R. & Bourne, H. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3098–3102.