Activation of the α Subunit of G_s in Intact Cells Alters Its Abundance, Rate of Degradation, and Membrane Avidity

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Abstract. Binding of GTP induces α subunits of heterotrimeric G proteins to take on an active conformation, capable of regulating effector molecules. We expressed epitope-tagged versions of the α subunit (α_s) of G_s in genetically α_s-deficient S49 *cyc*$^-$ cells. Addition of a hemagglutinin (HA) epitope did not alter the ability of wild type α_s to mediate hormonal stimulation of adenylyl cyclase or to attach to cell membranes. The HA epitope did, however, allow a mAb to immunoprecipitate the recombinant protein (HA-α_s) quantitatively from cell extracts. We activated the epitope-tagged α_s in intact cells by: (*a*) exposure of cells to cholera toxin, which activates α_s by covalent modification; (*b*) mutational replacement of arginine-201 in HA-α_s by a cysteine residue, to create HA-α_s-R201C; like the cholera toxin-catalyzed modification, this mutation activates α_s by slowing its intrinsic GTPase activity; and (*c*) treatment of cells with the β-adrenoceptor agonist, isoproterenol, which promotes binding of GTP to α_s, thereby activating adenylyl cyclase. Both cholera toxin and the R201C mutation accelerated the rate of degradation of α_s (0.03 h^{-1}) by three- to fourfold and induced a partial shift of the protein from a membrane bound to a soluble compartment. At steady state, 80% of HA-α_s- R201C was found in the soluble fraction, as compared to 10% of wild type HA-α_s. Isoproterenol rapidly (in <2 min) caused 20% of HA-α_s to shift from the membrane-bound to the soluble compartment. Cholera toxin induced a 3.5-fold increase in the rate of degradation of a second mutant, HA-α_s-G226A, but did not cause it to move into the soluble fraction; this observation shows that loss of membrane attachment is not responsible for the accelerated degradation of α_s in response to activation. Taken together, these findings show that activation of α_s induces a conformational change that loosens its attachment to membranes and increases its degradation rate.

HETEROTRIMERIC G proteins transduce signals from cell-surface receptors to membrane-bound effector molecules, including adenyly cyclase, phospholipase C, and ion channels (2, 8, 32). Agonist-bound receptors activate G proteins by promoting exchange of GTP for GDP bound to the α subunit of the heterotrimer. α-GTP rapidly dissociates from the $\beta\gamma$ complex and can interact with the effector until its intrinsic GTPase converts it to α-GDP, allowing re-association with $\beta\gamma$. Experiments in broken cells, confirmed and refined in studies using biochemically pure components, show that the nucleotide-bound state of the α subunit determines its interactions with other proteins.

How do changes in the active state of G protein α subunits affect their behavior in the more complex environment of an intact cell? Attempts to answer this question require experimental models that allow manipulation of the nucleotide-bound state of intracellular α subunits and assessments of their number, subcellular distribution, turnover, and associations with other proteins. Here we report an attempt to devise and exploit such a model.

The model focuses on the behavior of the α subunit (α_s)[1] of G_s, a well-characterized G protein that mediates hormonal stimulation of cAMP synthesis by membrane-bound adenyly cyclase. A key element of the model, the genetically α_s-deficient *cyc*$^-$ S49 mouse lymphoma cell (3, 13) provides a null background for analyzing the behavior of recombinant α_s, normal or mutant; the nucleotide-bound state of the recombinant α_s can be readily altered by β-adrenoceptor (β-AR) stimulation and by cholera toxin-catalyzed ADP-ribosylation. Two α_s mutations provide independent ways of manipulating the protein's activity: replacement by cysteine of the arginine residue at position 201 of α_s activates the protein by reducing its GTPase activity, as shown by studies of the oncogenic R201C mutation in human pituitary adenomas (17); R201 is also the residue covalently modified

1. *Abbreviations used in this paper*: α_s, α subunit of stimulatory G protein; βAR, β adrenergic receptor; CTX, cholera toxin; GTPγS, guanosine 5'-(γ-thio)-triphosphate; HA, hemagglutinin; RIPA, radioimmunoprecipitation assay.

by cholera toxin (17, 34). Another mutation, which replaces glycine-226 with alanine, prevents G_s from mediating stimulation of cAMP synthesis by cholera toxin and β-AR agonists (4, 22, 30), apparently because α_s-G226A cannot readily dissociate from $\beta\gamma$ (18).

A lack of suitable antibodies has slowed experimental studies of α subunit proteins in intact cells. An ideal antibody for such a purpose would specifically and quantitatively immunoprecipitate the native, undenatured α subunit. Because none of the polyclonal anti-α_s antisera available to us met this stringent criterion, we adopted an alternative approach, which should prove applicable to any G protein α subunit. We tagged recombinant α_s with a peptide epitope, derived from the hemagglutinin (HA) protein of influenza virus (11), which is the target of a high-affinity mAb.

The resulting experimental model, using epitope-tagged α_s expressed in the S49 *cyc*$^-$ cell, reveals that activating mutations, like cholera toxin-catalyzed ADP-ribosylation, produce two changes in the intracellular behavior of α_s: Much of the protein redistributes from a membrane bound into a soluble compartment, and its rate of degradation increases. The first of these changes does not cause the second.

Materials and Methods

Materials

Cholera toxin was obtained from List Biologicals. [^{35}S]methionine/cysteine (Tran^{35}Slabel) was obtained from ICN Radiochemicals (Irvine, CA). Radioactive ATP and cAMP were from DuPont NEN. ^{125}I-labeled protein A was from Amersham Corp. (Arlington Heights, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of Expression Vectors

The normal rat α_s cDNA was available in the pMV7 vector as described (33). Mutagenesis was carried out using the Bio-Rad Mutagene kit according to the manufacturer's instructions (Bio-Rad Laboratories, Cambridge, MA). Briefly, the α_s cDNA was ligated into the pTZ vector for production of phagemid. Single-stranded (coding) DNA was annealed to a 41-base oligomer containing the information (noncoding strand, 5′ CCATCGCTGTTGGACGCGTAATCCGGCACGTCCTCTTCGCC 3′) for the hemagglutinin epitope (see Fig. 1) flanked on both sides by 12 bases of wild type α_s sequence. An identifying MluI restriction site was also included. The reaction product was used to transform *E. coli* MV1190, and the mutagenized cDNA (HA-α_s) derived from an ampicillin-resistant colony was re-ligated back into pMV7 for transfection into S49 cells. To create HA-α_s-R201C, a 5′ SalI-EcoRI restriction fragment from HA-α_s was ligated to an EcoRI-AflII fragment from a rat α_s cDNA encoding the R201C substitution (provided by C. Landis; see reference 17). The resulting SalI-AflII fragment was re-ligated into pMV7. HA-α_s-G226A was generated by ligating the 5′ HindIII-EcoRI fragment from the HA-α_s cDNA to the 3′ EcoRI-HindIII fragment from an α_s cDNA encoding the G226A substitution (22). cDNA constructs were expressed in S49 *cyc*$^-$ cells via packaging cell lines, as described (33).

Antibodies

The mAb 12CA5 (generously provided by Dr. Ian Wilson) was raised against a 13 amino acid peptide sequence derived from the HA protein of influenza virus (11). The antibody was purified from ascites using a BioRad Affi-gel protein A column. The column eluate was dialyzed against several changes of coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3), then coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's directions. Normal mouse ascites (Sigma Chemical Co.) was used as a source of control antibody. For immunoblotting, affinity-purified rabbit polyclonal antisera raised against residues 27–41 of the wild type α_s sequence was used at a concentration of 10 μg/ml, as described (5).

Quantitation Standard

A recombinant fragment of HA-α_s was used as an immunoblot standard in quantitation studies. For its preparation, the cDNA encoding residues 77–213 of HA-α_s was expressed in *E. coli* and purified to homogeneity using conventional chromatography. The polypeptide was the generous gift of Dr. David Markby (manuscript in preparation).

Cell Culture and Metabolic Labeling

The wild type and *cyc*$^-$ S49 cell lines used in this paper were originally designated 24.3.2 and 94.15.1, respectively (3). An additional cell line, 3E, was generated (33) by transfecting 94.15.1 cells with the normal rat α_s cDNA by the method described above. Cells were maintained under 5% CO_2 in DME supplemented with 10% heat-inactivated horse serum. For metabolic labeling, cells (at 10^7/ml) were starved for 3 h in methionine- and cysteine-free DME with 10% dialyzed horse serum. [^{35}S]methionine/cysteine (Tran^{35}Slabel, 1,200 Ci/mmol; ICN Radiochemicals) was then added, at 250 μCi/ml. Cells were pulse-labeled for 20 min and then pelleted at 200 *g* and resuspended in chase medium (DME/horse serum supplemented with 1 mM each cold methionine and cysteine). The pulse-labeled cells were divided into equal aliquots and incubated (3×10^5 cells/ml) in chase medium in T75 culture flasks at 37°C, 5% CO_2. After pulse labeling, the cells continued to divide normally. For each time point of a turnover analysis, all cells from a given flask (in 50 ml medium) were harvested.

Turnover Studies

Whole-cell detergent extracts of metabolically labeled cells were prepared by pelleting cells (typically 20×10^6 per sample) at 200 *g*, washing once with ice-cold PBS, and then lysing in 0.5 ml RIPA buffer (1% NP-40, 0.5% Na deoxycholate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 8 μg/ml PMSF, 2 μg/ml each leupeptin and aprotinin) on ice for 1 h. The mixture was then centrifuged at 14,000 rpm in an Eppendorf centrifuge (Brinkman Instruments Inc., Westbury, NY) for 5 min. The clarified supernatant was used as the whole-cell lysate, while the pellet (mostly nuclei, and containing no immunodetectable α_s) was discarded. Metabolically labeled extracts were first pre-cleared by tumbling end-over-end at 4° C for 2 h with 20-μl vol sepharose beads coupled to normal mouse ascites. Pre-clearing beads were pelleted from the extract in a microfuge, then 20-μl vol beads coupled to antibody 12CA5 were added to each sample. After each sample was tumbled end-over-end overnight at 4° C, the beads were pelleted, washed twice with RIPA adjusted to 0.3% SDS, and once with 0.1% NP-40, 10 mM Tris-HCl, pH 7.4. The beads were then suspended in 80 μl gel sample buffer (125 mM Tris-HCl, pH 6.8, 15% sucrose, 2% SDS, 50 mM DTT, 0.01% Bromphenol blue), boiled 5 min, and subjected to SDS-PAGE. This was performed as described by Laemmli (16), using 10% acrylamide gels. Following electrophoresis, gels were fixed and stained in 50% methanol, 10% acetic acid, 0.1% Coomassie blue, soaked in Amplify (Amersham Corp.) for 20 min, dried, and subjected to autoradiography at −70° C. The band corresponding to HA-α_s was excised and radioactivity was quantitated by liquid scintillation spectroscopy.

Cell Fractionation

For analysis of subcellular distribution of α_s, cells were allowed to swell in hypotonic dounce buffer (20 mM Hepes, pH 7.4, 20 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, plus protease inhibitors) for 10 min on ice. Samples were then dounce-homogenized using a Wheaton 1-ml tissue grinder (>90% cell disruption by light microscopy), and centrifuged for 30 min at 150,000 *g* in a TL-100 tabletop ultracentrifuge (Beckman Instruments). The supernatant (S150) fraction was adjusted to 1% NP-40, 0.5% Na deoxycholate, 150 mM NaCl, while the pellet (P150) was first resuspended in dounce buffer and then likewise adjusted with detergent and NaCl and incubated on ice for 45 min. Detergent-insoluble material from the P150 extract was removed by centrifugation in a microfuge (Beckman Instruments) at 14,000 rpm for 5 min. Immunoprecipitation was carried out as described above.

Preparation of Membranes and Adenylyl Cyclase Assay

For adenylyl cyclase assays, membranes were prepared by nitrogen cavitation of S49 cells, as described (28). In cell fractionation studies, cells were lysed by douncing in the hypotonic buffer described above, then centrifuged

for five minutes at 1000 *g* to remove nuclei. The post-nuclear supernatant was then centrifuged for 30 min at 150,000 *g* to pellet the membranes. Adenylyl cyclase activity was measured by the method of Salomon et al. (29), with small modifications (5).

Western Blotting

Immunoblot analysis was carried out as described by Chang and Bourne (5), except for quantitation studies, in which proteins were electroblotted at low voltage (25 V) overnight to 0.2 μm pore-sized PVDF membrane (Bio-Rad Laboratories). Using these conditions, we obtained a uniform transfer of proteins ranging in size from 14 to 46 kD, as assessed by quantitating transfer and retention of ^{14}C-labeled molecular weight standards (Amersham Corp.). Densitometry was performed using an Ultroscan XL (LKB Instruments Inc., Bromma, Sweden) on films that had been "preflashed" before exposure to the probed membrane.

Results

Construction and Expression of Epitope-tagged α_s

Alternative splicing of α_s transcripts produces α_s proteins that migrate at rates corresponding to 45- and 52-kD; compared to the 45-kD form, the 52-kD form contains a 14-amino acid insert (27). No substantial difference in function of the two forms has been detected (10). We modified the 52-kD form at a site (residues 77–81) within the insert to confer upon it recognition by a mAb, 12CA5, which is directed against a well-defined (14) peptide epitope of the influenza HA (Fig. 1). Alteration of five residues of wild type α_s sequence generates a unique epitope "tag" in the α subunit (Fig. 1).

If HA-tagged α_s is to serve as a valid stand-in for untagged α_s, the two types of protein should be expressed in similar amounts and distributed similarly in subcellular fractions; they should also function similarly as regulators of adenylyl cyclase and should be similarly affected by mutations.

Tagged and untagged α_s proteins were expressed in similar amounts in S49 cells (Fig. 2). A Western blot of membrane proteins (Fig. 2 *A*), probed with an affinity-purified anti-α_s antiserum, shows that epitope-tagged α_s (lane *3*) is expressed in *cyc*$^-$ cells at a level comparable to that of the 52-kD form of α_s in wild type S49 cells (lane *1*). The tagged α subunit migrates slower than its untagged counterpart, probably owing to the addition of a negative charge. Two mutant forms of α_s, containing the R201C of G226A mutations, were also epitope-tagged and expressed in *cyc*$^-$ cells (Fig. 2 *A*, lanes *4* and *5*, respectively). The low expression of the α_s-R201C protein in membranes has been observed previously, both in transfected *cyc*$^-$ cells and in pituitary adenomas (L. Vallar, personal communication). Epitope-tagged α_s is hereafter referred to as HA-α_s. *Cyc*$^-$ cells expressing the different tagged α_s constructs are designated as HA cells (wild type α_s), HARC cells (R201C), or HAGA (G226A) cells.

αs	D	P	Q	A	A	R	S
		|	|	|	|	|	
HA-αs	D	V	P	D	Y	A	S
	76	77	78	79	80	81	82

Figure 1. α_s vs. HA-α_s. Amino acid residues 76-82 in HA-α_s comprise the hemagglutinin epitope for monoclonal antibody 12CA5. Vertical lines identify residues that differ between normal α_s (52-kDα form) and HA-α_s.

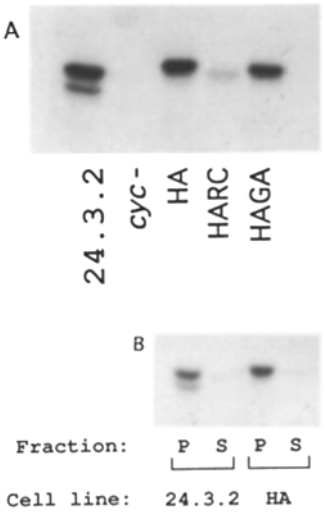

Figure 2. Western blot analysis of S49 clones. (*A*) Wild type S49 (*24.3.2*) and *cyc*$^-$ (94.15.1) cells are compared to clones generated by transfecting *cyc*$^-$ cells with cDNAs encoding HA-α_s, HA-α_s-R201C, and HA-α_s-G226A (*HA*, *HARC*, and *HAGA* cells, respectively). Membranes were harvested from S49 cells by nitrogen cavitation. 50 μg of membrane protein from each cell line was electrophoresed, transferred to nitrocellulose, and probed with a rabbit anti-peptide antibody specific for α_s. The antibody probe was visualized with ^{125}I-labeled protein A and autoradiography (24-h exposure). (*B*) Membrane-bound vs. soluble α_s. Cells were harvested by centrifugation at 200 *g*, resuspended in ice-cold hypotonic buffer, and lysed by dounce homogenization (see Materials and Methods). The post-nuclear supernatant was centrifuged at 15,000 *g*. 50-μg protein from the pellet (*P*) and supernatant (*S*) fractions of wild type (24.3.2) and HA cells were analyzed by SDS-PAGE and Western blotting, exactly as in *A*.

Fig. 2 *B* compares membrane-bound (P150) vs. soluble (S150) α_s, with and without the epitope tag, as detected in immunoblots probed with an anti-α_s antiserum. The distribution of HA-α_s between P150 and S150 fractions is identical to that of untagged α_s, suggesting that the epitope does not disturb targeting of the protein to the plasma membrane. This experiment does not accurately reflect the true ratio of membrane bound to soluble α_s, however; this is because the immunoblots compare equal quantities of membrane and soluble protein, despite the fact that the cells are quite unlikely to contain equal amounts of membrane and soluble protein. Quantitative immunoprecipitation provides a better way of assessing distribution of α_s between particulate and soluble fractions (see below).

The epitope tag did not alter regulation of adenylyl cyclase (Fig. 3). Isoproterenol, GTPγS, forskolin, and cholera toxin increased adenylyl cyclase in membranes from HA cells (Fig. 3 *B*) in a fashion indistinguishable from that seen in membranes of 3E cells (Fig. 3 *A*), which express recombinant wild type α_s lacking the epitope tag. Adenylyl cyclase in membranes from HAGA or HARC cells was unresponsive to all stimuli or constitutively active, respectively (Fig. 3, *C* and *D*); this is exactly the behavior expected of α_s with the G226A (22) and R201C (17) mutations, suggesting that the epitope does not substantially alter function of either mutant.

Thus, assays of subcellular distribution and signaling function show that the epitope tag does not substantially affect normal or mutant α_s.

Immunoprecipitation and Quantitation of Tagged α_s

We used immunoprecipitates from metabolically labeled cells to compare turnover of epitope-tagged normal and mu-

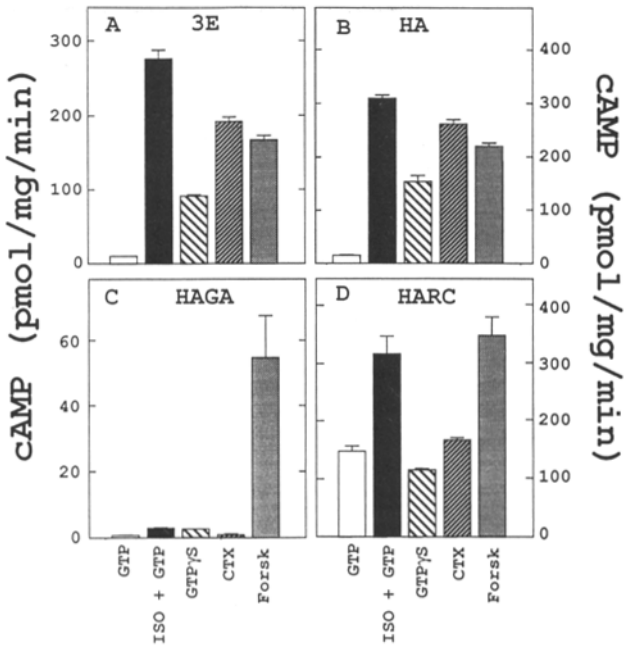

Figure 3. Adenylyl cyclase stimulated by normal and epitope-tagged α_s. Adenylyl cyclase activities were measured in membranes prepared from 3E cells, generated (33) by transfecting *cyc*$^-$ cells with wild-type, non-epitope-tagged α_s (*A*) and HA, HAGA, and HARC cells (*B–D*, respectively). Conditions were as follows: 10 μM GTP; 10 μM GTP + 10 μM isoproterenol (*ISO*); 100 μM GTPγS; 10 μg/ml cholera toxin + 100 μM NAD$^+$ (30 min treatment); 10 μM forskolin. Values represent the means ± SD of triplicate determinations.

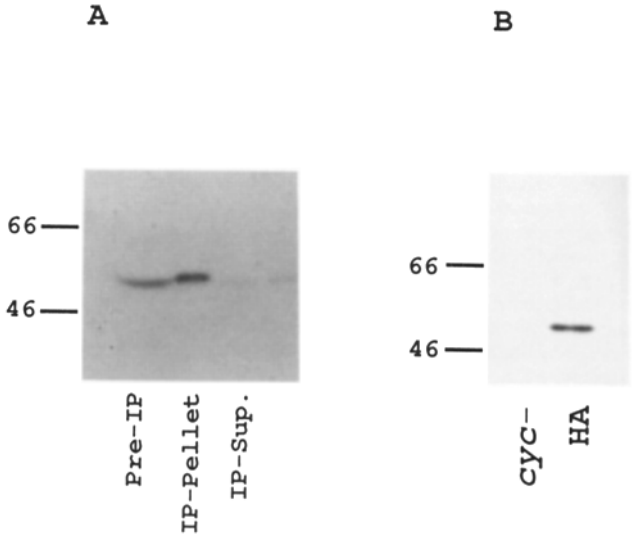

Figure 4. Quantitative immunoprecipitation and metabolic labeling of HA-α_s. (*A*) 10^7 HA cells were extracted in RIPA buffer. 10% of this extract was retained as "pre-IP" (lane *1*). The remainder was subjected to immunoprecipitation with 12CA5-coupled sepharose beads. 10% of both the immunoprecipitation pellet (lane *2*) and supernatant (lane *3*) fractions along with the "pre-IP" sample were subjected to SDS-PAGE, electroblotted to nitrocellulose and probed with an α_s-specific anti-peptide polyclonal antibody, followed by ^{125}I-labeled protein A. (*B*) Metabolic labeling. 2×10^7 HA cells (lane *2*) or *cyc*$^-$ cells (lane *1*) were starved for 3 h in methionine cysteine-free DME + 10% dialyzed horse serum, then incubated for 20 min in the same medium containing 250 μCi per ml ^{35}S-labeled methionine/cysteine. The cells were then pelleted, washed once in ice-cold PBS, and lysed in 0.5 ml RIPA buffer. The clarified, pre-cleared extracts were then subjected to immunoprecipitation with 12CA5-coupled sepharose beads and SDS-PAGE. The gel was soaked for 20 min in Amplify and analyzed by autoradiography (12-h exposure).

tant α_s. Validity of such comparisons requires quantitative extraction and immunoprecipitation of the protein. Using the detergent buffer described in Materials and Methods, we were able to render soluble >95% of the HA-α_s in HA cells (determined by Western blot and densitometry, data not shown). Fig. 4 *A* shows immunoblots of HA-α_s in a whole-cell detergent extract of HA cells (lane *1*) and in pellet and supernatant fractions produced by immunoprecipitation (lanes *2* and *3*, respectively); immunoblots were probed with polyclonal anti-α_s (see Materials and Methods). Densitometry of these immunoblots showed that >95% of the HA-α_s present in the extract is immunoprecipitated by the 12CA5 mAb. Fig. 4 *B* demonstrates specificity of the immunoprecipitation from metabolically labeled cells; the 12CA5 antibody immunoprecipitates HA-α_s from HA cells (lane *2*), but not from *cyc*$^-$ cells (lane *1*).

As a standard for immunoblot determination of the molar amount of HA-α_s in HA cells, we used a recombinant fragment of HA-α_s that contained the epitope and was readily available in the laboratory (see Materials and Methods). Known quantities of the recombinant standard were electrophoresed in lanes adjacent to lanes containing HA-α_s that was immunoprecipitated with the 12CA5 antibody from a detergent extract of 10^7 HA cells. The proteins were electroblotted to PVDF membrane and probed with the 12CA5 antibody (Fig. 5). Densitometry of the autoradiograph in Fig. 5 indicated that 10^7 HA cells contain 3.97 ± 0.47 pmol HA-α_s. Accordingly, each cell should contain ~240,000 HA-α_s molecules. A similar assay showed that a membrane preparation from HA cells (prepared by nitrogen cavitation, as detailed in Materials and Methods) contained 16.6 ± 2.0 pmol HA-α_s per mg protein (n = 3).

The 12CA5 antibody did not co-immunoprecipitate G protein β subunit with HA-α_s, as assessed by probing immunoprecipitates with an anti-β antiserum; in addition, immunoprecipitates from metabolically labeled cells revealed no labeled band at the location expected for β polypeptides (results not shown). Although it is impossible to rule out the possibility that the detergents we used disrupted the $\alpha\beta\gamma$ complex before or during immunoprecipitation, varying the nature and concentration of ionic and non-ionic detergents failed to produce immunoprecipitates containing $\beta\gamma$. It is unlikely that introduction of the epitope sequence into recombinant α_s prevented its binding to $\beta\gamma$, on two grounds: (*a*) The epitope does not detectably alter α_s-mediated signal transduction or subcellular distribution of HA-α_s, as shown above; and (*b*) the functional defect of the G226A mutant, which is thought to result from its tight attachment to $\beta\gamma$ in membranes (4, 18), is perfectly preserved in HA-α_s-G226A (Fig. 3, and see below). An alternative possibility is that the 12CA5 antibody itself interferes sterically with binding of $\beta\gamma$ to HA-α_s; in an overnight incubation, 12CA5-

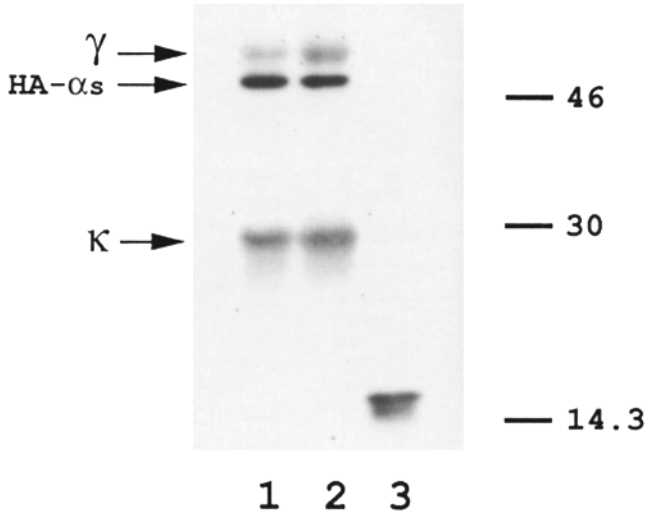

Figure 5. Western blot of HA-α_s and recombinant fragment. 10^7 HA cells (lane *1*) or 200 μg HA membranes (lane *2*) were extracted in RIPA buffer and immunoprecipitated with 12CA5-coupled sepharose beads (see Materials and Methods). Immunoprecipitates were subjected to SDS-PAGE, electroblotted to PVDF membranes, and probed with the 12CA5 ascites fluid diluted 1:50 in blotto (50 mM Tris, pH 7.4, 150 mM NaCl, 5% powdered nonfat milk, 0.05% Tween-20). The probe was visualized with ^{125}I-labeled goat anti-mouse antibody (New England Nuclear-DuPont, Boston, MA) and autoradiography. Five pmol of a recombinant fragment of HA-α_s was electrophoresed alongside (lane *3*), to serve as a densitometric standard for quantitation. Molecular weight standards are seen to the right. Before loading, the antigen-coupled 12CA5-Sepharose beads were boiled in reducing buffer, releasing both the bound antigen (*HA-α_s*) and a fraction of the γ and κ chains of the 12CA5 mAb. These chains were thus present on the PVDF membranes and reacted with the ^{125}I-labeled goat anti-mouse antibody (*arrows*).

coated beads could compete against $\beta\gamma$ for binding the α subunit. The competition is successful, as shown by the quantitative immunoprecipitation of HA-α_s from cell extracts (Fig. 4 *A*).

Turnover of Normal and Mutant α_s

For turnover studies, cells were labeled for 20 min by incubation with [^{35}S]methionine/cysteine (pulse) and then divided into fractions for immediate lysis (zero time) or continued culture in normal medium containing excess cold methionine and cysteine (chase). At appropriate times, cells were harvested and lysed, and the whole-cell detergent extracts were subjected to immunoprecipitation and SDS-PAGE (see Materials and Methods). Fig. 6 *A* shows representative autoradiographs for each cell line tested, and Figs. 6, *B* and *C* depict the time courses of α_s degradation, as quantitated by liquid scintillation spectroscopy of excised bands. All cell lines tested showed biphasic decay of α_s radioactivity, with a very rapid early phase and a slower, mono-exponential second phase. In the first phase, confined more or less to the first hour after pulse labeling, radioactive α_s decreased by 40–45%; the kinetics of this first phase were not further analyzed. Analysis of later time points revealed similar fractional rates of α_s degradation in HA in HAGA cells (0.03 h^{-1} for both). This rate corresponds to a half-life of 22 h and indicates that the G226A mutation in the α_s of HAGA cells does not accelerate or slow the turnover of α_s. In contrast, HA-α_s-R201C was degraded at a much faster rate (0.11 h^{-1}, half-life 6.2 h); the faster degradation may account, at least in part, for the lower amount of α_s detected in membrane preparations from HARC cells, as compared to α_s in membranes of HA and HAGA cells.

Fig. 6 *C* shows that cholera toxin treatment increased the rate of degradation of HA-α_s in HA cells. HA-α_s-G226A in HAGA cells showed a similar response to the toxin. This effect may be responsible for the marked reduction of immunodetectable α_s in membranes of cells exposed for several hours to cholera toxin, as reported by us (5) and others (21). Indeed, the fractional rate of degradation of HA-α_s in toxin-treated cells (0.14 h^{-1}) closely approximates that of HA-α_s-R201C (0.11 h^{-1}); the latter protein contains a cysteine at position 201 instead of the arginine that is ADP-ribosylated in HA-α_s. Both the covalently modified normal protein and the mutant protein hydrolyze GTP at reduced rates, relative to unmodified wild type α_s (7, 17). This suggests that the increased degradation rate induced by the toxin is not caused by ADP-ribosylation per se, but rather by an alteration in conformation or function that results from decreased GTPase activity. It is unlikely that the rapid degradation is caused by elevated intracellular cAMP, because we were unable to detect effects of stimulators of adenylyl cyclase (isoproterenol, forskolin) or an inhibitor of cAMP degradation (isobutylmethylxanthine) or turnover of α_s. These results are in keeping with the previously reported (5) failure of cAMP analogs or elevated cellular cAMP to lower α_s in membranes of treated cells.

α_s Content of Membrane vs. Soluble Fractions

Two observations led us to suspect that the R201C mutation may increase the relative amount of α_s in the soluble fraction of cell extracts. First, the strong signal seen with radiolabeled HA-α_s-R201C in pulse-chase studies, using whole cell extracts (Fig. 6 *A*), was not in keeping with the markedly reduced amount of HA-α_s-R201C in membranes prepared from HARC cells, as compared to the amount of HA-α_s in HA membranes (Fig. 2 *A*, lane *3* vs. lane *4*); the amount of α_s was 6.3-fold greater in HA than in HARC membranes, as determined by densitometry (Table I). Second, this marked decrease in membrane α_s was not fully accounted for by the 3.7-fold greater fractional degradation rate of HA-α_s-R201C compared to HA-α_s (0.11 vs. 0.03 h^{-1}, respectively).

Fig. 7 shows that the suspicion was correct: subcellular fractionation showed that in HARC cells most of the HA-α_s-R201C is in the soluble fraction. For each of the three cell lines (HA, HARC, and HAGA), 4×10^7 cells were divided into two equal aliquots. One was detergent lysed and immunoprecipitated with 12CA5 beads to provide a measure of all the α_s present in the cell. The other aliquot was suspended in hypotonic (non-detergent) buffer, dounce-homogenized, and centrifuged at 150,000 *g*. The pellet (P150) was extracted in detergent buffer, while the supernatant (S150) fraction was adjusted to buffer conditions identical to those in the P150 extract (see Materials and Methods). Both S150 and P150 were then subjected to immunoprecipitation with 12CA5 antibody, followed by SDS-PAGE, and immunoblot-

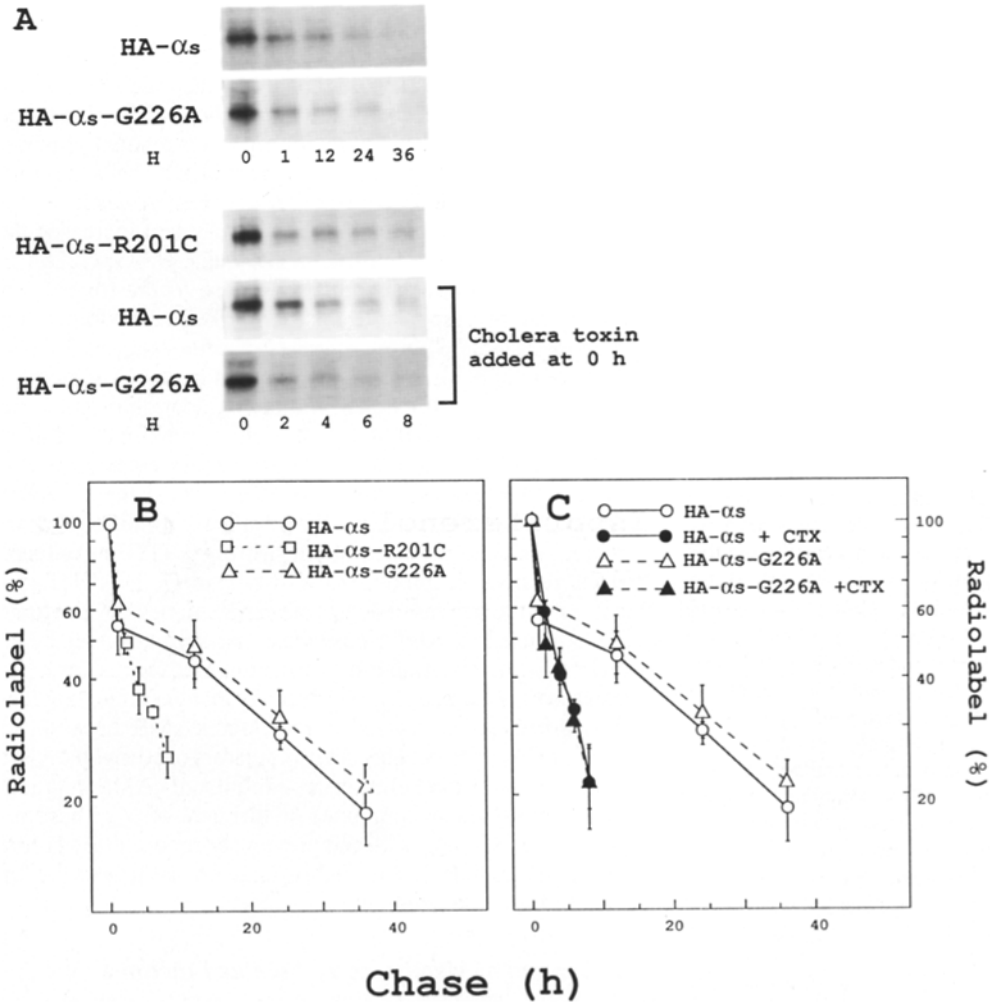

Figure 6. Degradation of normal and mutant HA-α_s. Representative autoradiographs are shown in *A*, degradation curves in *B* and *C*. Cells (2×10^8 per experiment) were starved in methionine/cysteine-free medium for 3 h, then pulse-labeled with 250 μCi/ml [^{35}S]methionine/cysteine for 20 min. The labeled cells were washed, divided into five equal aliquots, and incubated in chase medium (4×10^5 cells per ml). At the indicated times, the cells were lysed and subjected to immunoprecipitation, SDS-PAGE and autoradiography. (*A*) Autoradiographs of HA, HAGA, and HARC pulse-chase experiments are shown in rows *1*, *2*, and *3*, respectively. HA and HAGA cells were also chased in medium containing 100 ng/ml cholera toxin (rows *4* and *5*, respectively). Chase times were 0 to 36 h for HA and HAGA cells, and 0 to 8 h for HARC and cholera toxin-treated cells, as indicated. (*B*) Degradation curves for HA-α_s, HA-α_s-G226A, and HA-α_s-R201C. (*C*) Degradation curves for HA-α_s and HA-α_s-G226A, in the presence and absence of cholera toxin. Curves were constructed as follows: bands corresponding to normal or mutant HA-α_s were excised from gels and radioactivity was quantitated by liquid scintillation spectroscopy. Degradation was then plotted as percent radioactivity (compared to 100% at time 0) vs. time. Each time point represents the average (±SD) of values obtained from at least three independent pulse-chase experiments.

ting with a rabbit anti-α_s antiserum (Fig. 7). Both HA-α_s and HA-α_s-G226A were located predominantly in the particulate fraction, with a small signal in the S150 fraction. In contrast, most of the HA-α_s-R201C protein was found in the soluble fraction. Table I summarizes these results.

The presence of HA-α_s-R201C in the S150 fraction may reflect an increased relative amount of this protein in the

Table I. Cellular Content and Degradation Rates of Normal and Mutant Epitope-tagged α_s in Transfected Cell Lines

Cell line	α_s per cell*			Degradation rate (h^{-1})
	Whole cell	P150	S150	
HA	240,000	216,000(90)	24,000(10)	0.03
HARC	168,000	34,000(20)	134,000(80)	0.11
HAGA	144,000	108,000(75)	36,000(25)	0.03

* Numbers represent the number of α_s molecules in whole cells or fractions, as quantitated with the anti-epitope antibody, using a purified recombinant epitope-labeled fragment of α_s as a standard (see Materials and Methods). numbers in parentheses represent the percentage of total α_s in the soluble or pellet fractions.

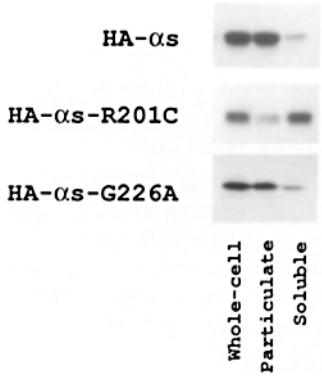

Figure 7. Distribution of normal and mutant HA-α_s between soluble and particulate fractions. For each experiment, 4×10^7 cells were divided into two equal aliquots. One aliquot was lysed in the detergent-based RIPA buffer ("whole-cell" extract) as described in Materials and Methods. The other aliquot was resuspended in hypotonic (non-detergent) buffer, dounce-homogenized, and centrifuged at 150,000 *g*. The pellet ("particulate") was resuspended in RIPA buffer for detergent extraction, while the supernatant ("soluble") fraction was adjusted with detergent and NaCl to concentrations equivalent to those in RIPA buffer. Thus, HA-α_s in the particulate and soluble fractions combined should equal that of the whole-cell extract. Samples were subjected to immunoprecipitation with 12CA5-coupled sepharose beads and SDS-PAGE, blotted and probed with anti-peptide antibody specific to α_s. Probes were visualized with ^{125}I-labeled protein A and autoradiography.

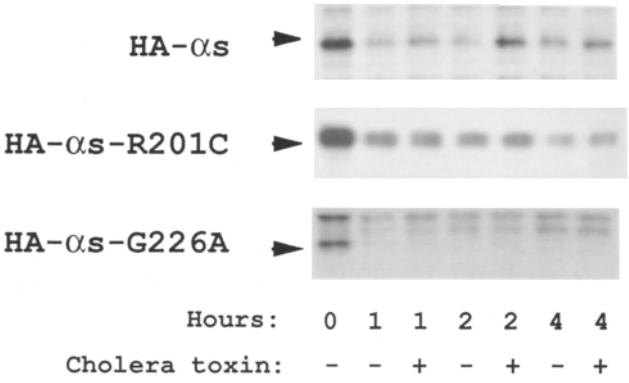

Figure 8. Effect of cholera toxin on soluble HA-α_s. Cells were pulse-labeled for 20 min with 250 μCi/ml ^{35}S-labeled methionine/cysteine, then chased in the presence (+) or absence (−) of 100 ng/ml cholera toxin. At the indicated times, cells were harvested, suspended in hypotonic buffer, lysed by dounce homogenization, and the soluble fraction was recovered by centrifuging the lysate for 30 min at 150,000 *g*. The soluble fractions from each time point were then subjected to immunoprecipitation with 12CA5-coupled sepharose beads and analyzed by SDS-PAGE and autoradiography. Arrows mark the position of the α subunit in each autoradiograph.

GTP-bound active form, caused by the GTPase-inhibiting mutation. Moreover, cholera toxin treatment in vitro is reported to induce dissociation of α_s from membranes (19, 24). Accordingly, we asked whether cholera toxin treatment of intact cells could elevate the amount of α_s in the S150 fraction (Fig. 8). Cells were pulse labeled with [^{35}S]methionine/cysteine and chased in the presence or absence of cholera toxin. At the times indicated, cells were lysed and the S150 fractions were subjected to immunoprecipitation with 12CA5 antibody. At 2 h, soluble HA-α_s was significantly increased in extracts of toxin-treated HA cells (Fig. 8, lanes *4* vs. *5* of row *1*). As expected, HA-α_s-R201C was not affected by the toxin, but was instead abundant in the S150 fraction of HARC cells throughout the chase (Fig. 8, row *2*). HA-α_s-G226A, however, was detected in the S150 fraction of HAGA cells only immediately following synthesis (Fig. 8, 0 h chase, lane *1* of row *3*). Although cholera toxin ADP-ribosylates α_s-G226A (4, 29) and accelerates its degradation (Fig. 6 *C*), the toxin does not induce re-entry of HA-α_s-G226A into the soluble pool.

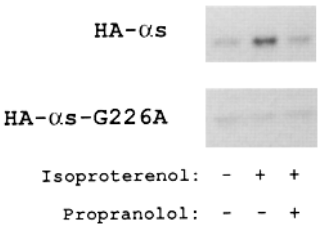

Figure 9. Effect of isoproterenol on soluble HA-α_s and HA-α_s-G226A. Cells (3×10^7 per sample) were suspended in phosphate buffered saline in the presence (lanes *2* and *3*) or absence (lane *1*) of 10 μM isoproterenol. The effect of antagonist was assessed by pre-incubating the cells for 120 s with 10 μM propranolol before adding isoproterenol (lane *3*). Cells were lysed within two min of exposure to isoproterenol by pelleting, resuspending in ice-cold hypotonic buffer, and dounce homogenization. The soluble fractions were recovered by centrifugation at 150,000 *g* for 30 min, then subjected to immunoprecipitation with 12CA5-coupled sepharose beads. The immunoprecipitates were analyzed by SDS-PAGE and Western blotting as described in Fig. 7.

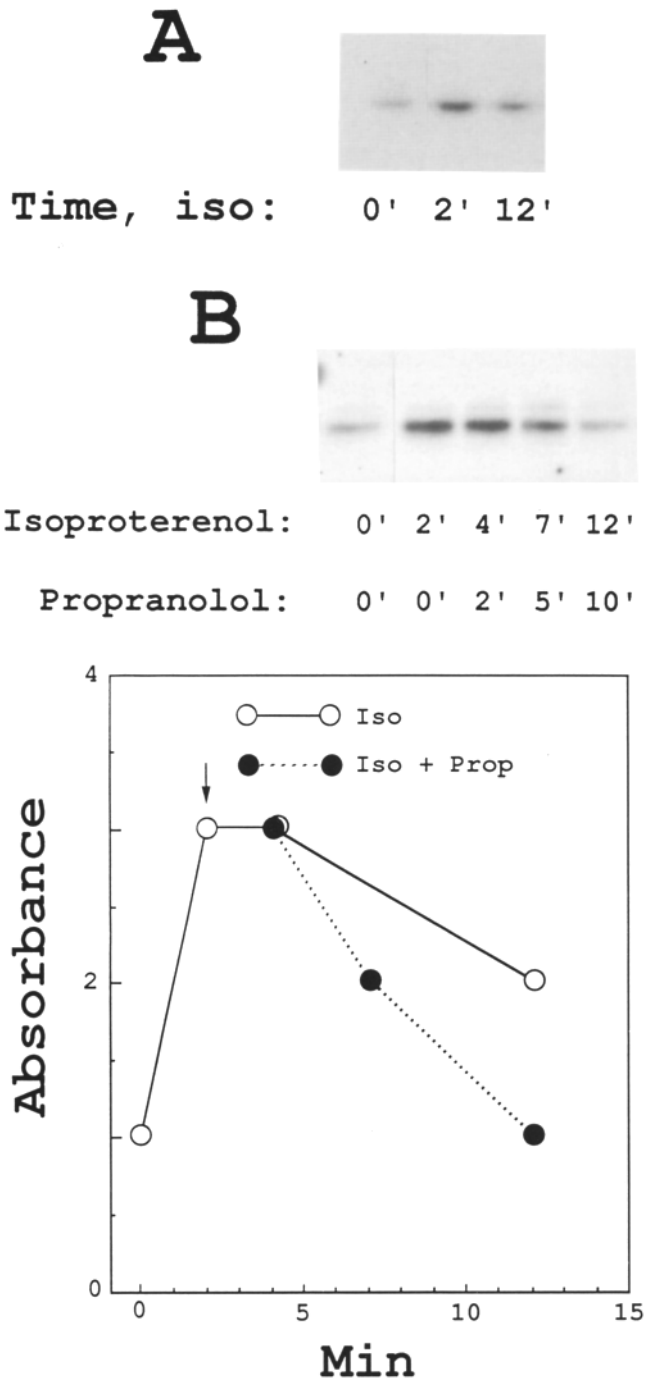

Figure 10. Time course of soluble HA-α_s after treatment with isoproterenol or isoproterenol followed by propranolol. (*A*) HA cells (3×10^7 per sample) were resuspended in 1 ml PBS with 10 μM isoproterenol and incubated at 37°C. At the indicated times, cells were rapidly pelleted, resuspended in ice-cold hypotonic buffer, and lysed by dounce homogenization. Soluble HA-α_s was isolated and analyzed as in Fig. 9. (*B*) HA cells were resuspended in PBS plus isoproterenol as in *A* but after two min, 10 μM propranolol was added to each sample. Cells were harvested at the indicated times and analyzed as in *A*). (*C*) Densitometry was performed on the blots shown in *A* and *B* and translated to a graph. The y-axis represents an arbitrary scale based on absorbance. The arrow marks the time point at which propranolol was added.

Isoproterenol Increases HA-α_s in the S150 Fraction

If an increase in the amount of GTP-bound α subunit is the mechanism by which cholera toxin induces HA-α_s to shift into the S150 fraction, then isoproterenol, a β-AR agonist, should induce a similar shift. Indeed, just such a shift has been reported in S49 (26) and mouse mastocytoma cells (24). In our hands, immunoblot analysis of S150 fractions, using a rabbit anti-α_s antibody, revealed in wild type S49 cells a reproducible increase in soluble α_s in response to isoproterenol; the effect was difficult to quantitate, however, because the maximal amount of S150 protein that can be subjected to SDS-PAGE contains little α_s protein. For this reason, we studied the phenomenon in HA cells, in which the pool of HA-α_s subunits in the S150 fraction is small but readily immunoprecipitated and quantitated (see Fig. 7, lane *3* of row *I*).

Exposure of HA cells to isoproterenol for two minutes substantially increased HA-α_s in the S150 fraction (Fig. 9); the isoproterenol-induced increase in soluble HA-α_s was blocked by prior addition of a β-AR antagonist, propranolol. Densitometry showed that the S150 fraction of unstimulated cells contained 10% of total cellular HA-α_s; immediately following stimulation with isoproterenol, 30% of HA-α_s was found in the S150 fraction—a threefold increase. In keeping with the functional defect of α_s-G226A (4, 18, 30), isoproterenol failed to induce movement of HA-α_s-G226A into the S150 fraction (Fig. 9).

Kinetic analysis showed that the isoproterenol-induced shift of HA-α_s into the S150 fraction was rapid (maximally detectable immediately after addition of the agonist); the amount of soluble HA-α_s declined slowly over the ensuing 10 min (Fig. 10 *A*). We deduce that the protein is not degraded, but rather is returned to the particulate fraction, because cells treated with isoproterenol did not display accelerated turnover of HA-α_s. Because isoproterenol treatment elevates soluble HA-α_s, it should reciprocally reduce HA-α_s in the particulate fraction. We could not reproducibly detect such a decrease (result not shown), probably because it is much more difficult to measure the ~20% decrease in the P150 fraction than the ~300% increase seen in the S150 fraction. In contrast to the rapid effect of isoproterenol, cholera toxin produced a maximal increase in soluble HA-α_s after a 2-h lag (Fig. 8); the lag is presumably required for the toxin to penetrate the cells and catalyze ADP-ribosylation of α_s.

To determine the rate of disappearance of HA-α_s from the soluble fraction after cessation of β-AR stimulation, HA cells were treated with isoproterenol (agonist) for 2 min and then "chased" with propranolol (Fig. 10 *B*). Although propranolol can be assumed to inhibit β-AR stimulation very rapidly (i.e., in seconds), soluble HA-α_s returned only partially toward baseline two min after addition of the antagonist; complete reversal of the isoproterenol effect required more than five min.

Fig. 10 *C* plots time vs. relative absorbance, obtained by densitometry of the α_s bands in Figs. 10, *A* and *B*. The temporal resolution of such an experiment is limited, owing to the unavoidable time delay required for "stopping" each incubation (at least 2 min for centrifugation of cells and preparation of a cell-free extract for immunoprecipitation; see Materials and Methods). Fig. 10 *C* shows the results of a single experiment. Additional experiments reproduced the observations that isoproterenol induces a relatively long-lived increase in soluble α_s, amounting to ~20% of total α_s, and that propranolol shifted α_s back to the membrane fraction.

Discussion

We have developed a useful model system for studying the behavior of α_s in an intact cell. Addition of an epitope did not detectably alter the protein's ability to mediate hormonal stimulation of adenylyl cyclase, but did enable us to immunoprecipitate it quantitatively from whole-cell detergent extracts and subcellular fractions; such quantitative immunoprecipitation was not feasible with polyclonal anti-α_s antibodies available to us. The epitope allowed us to examine the behavior of normal and mutant α_s in intact cells. Activation of α_s—by cholera toxin, mutation, or hormone—altered its rate of degradation, membrane avidity, and cellular content.

Activation Accelerates α_s Degradation

Pulse-chase studies revealed that degradation of HA-α_s is biphasic. Approximately 40% of the protein disappeared rapidly, within an hour or so; this newly synthesized HA-α_s is located exclusively in the soluble fraction of the cell. After transfer to the particulate fraction, the remaining HA-α_s decayed at a slow rate (0.03 h^{-1}, half-life 22 h). The biphasic degradation curve suggests that newly synthesized HA-α_s is unstable until it associates with the particulate fraction. Such behavior is not unusual for proteins targeted to the cytoplasmic face of membranes; glutamic acid decarboxylase (6) and α-spectrin (23) are two such examples. Protection of α_s from rapid degradation may result from association with another protein or proteins, such as the $\beta\gamma$ subunit or a chaperonin. If abundance of the putative protecting protein(s) is limiting, then the size of the rapidly degraded pool may be greater for HA-α_s than for endogenous α_s, because the promoter in the retroviral vector may induce relative overproduction of the recombinant α_s polypeptide.

Another group has reported (12) that endogenous α_s in S49 cells turns over with a half-life of 42 h, almost twice the half-life we found for the more stable (particulate) pool of HA-α_s. Does the substitution of five amino acids in HA-α_s cause the protein to turn over more rapidly than does its endogenous counterpart? We think it more likely that technical problems in the earlier experiments (12) account for the discrepancy. In those experiments the "pulse" of metabolic labeling required incubation for 24 h with radioactive amino acid, in contrast to the 20-min pulse used in our experiments. S49 cells tolerate 20-min exposure to Tran^{35}Slabel very well, as shown by their ability to proliferate at a normal rate upon transfer to normal medium; in our hands, however, prolonged exposure (that is, for several hours) to Tran^{35}Slabel causes significant cell death and a decrease in subsequent doubling rate. Indeed, in the previous study (12) incorporation of radiolabel into α_s was complete by 8 h of the 24-h pulse—a finding that is kinetically incompatible with a half-life of 42 h, but consistent with the possibility that by 8 h many of the radiolabeled cells had ceased to take up nutrients. Thus the 42-h half-life may have reflected behavior of cells that were moribund or dead. In addition, the same

study failed to show that the polyclonal antibody used could immunoprecipitate a significant fraction of cellular α_s. Incomplete immunoprecipitation may have accounted for the weak α_s signal in their immunoblots (autoradiographs in our experiments required 12-h exposure, vs. 1–4 wk in the other study).

Cholera toxin and the activating R201C mutation increased degradation of HA-α_s 3.5- to 5-fold and caused the α subunit to move from the particulate to the soluble fraction. These observations explain the greatly reduced amounts of α_s protein found in membranes of cells treated with cholera toxin (5, 21) and in particulate extracts from human pituitary tumors containing *gsp* mutations, such as R201C, in the α_s gene (L. Vallar, personal communication). It is possible that the acceleration of α_s degradation represents a physiologically relevant feedback response to activation of the G_s signalling pathway. In any case, activation of α_s, whether by mutation or by toxin-catalyzed ADP-ribosylation, promotes transfer of α_s into a soluble fraction and accelerates its degradation. It is appealing to infer that the soluble pool of α_s represents the route of accelerated degradation taken by the activated protein. The inference is wrong, however, as shown by the behavior of HA-α_s-G226A. The toxin accelerates turnover of HA-α_s-G226A, but does not push it into the soluble fraction (Fig. 8).

Activation Alters Membrane Avidity of α_s

It seems likely that a GTP-induced change in conformation of wild type HA-α_s is responsible for both the increase in turnover rate and the loosening of its attachment to membranes. If so, how can the toxin accelerate degradation of HA-α_s-G226A but fail to stimulate adenylyl cyclase in membranes of cells carrying the G226A mutation (Fig. 3; and see references 4 and 30)? Indeed, the S49 clone containing the α_s-G226a mutation was isolated by virtue of its inability to accumulate cAMP in response to cholera toxin (30). The apparent discrepancy can be accounted by recognizing that the G226A mutation does not absolutely block GTP-induced activation of α_s (18), but instead prevents GTP-induced dissociation of α_s-G226A from the $\beta\gamma$ subunit (18, 22). Thus, recombinant α_s-G226A, produced in *E. coli*, binds and hydrolyzes GTP, and mediates GTP-induced stimulation of adenylyl cyclase (18), while the same protein expressed in an S49 cell cannot stimulate cAMP synthesis, apparently because GTP-induced activation cannot pry it loose from binding to $\beta\gamma$. Accordingly, the evidence is consistent with the idea that a change in the conformation of α_s, induced by binding GTP and prolonged by the R201C mutation or by toxin-catalyzed ADP-ribosylation, suffices to accelerate degradation.

In keeping with the idea that the activated conformation reduces avidity with which α_s binds to the plasma membrane, isoproterenol, a β-AR agonist, rapidly (<2 min) induced a shift of approximately 20% of total HA-α_s from the particulate to the soluble fraction (Fig. 10). In a previous study (26), using different methods for cell disruption and quantitation, isoproterenol treatment caused as much as 50% of α_s in S49 cells to become soluble, with a time course that differed from what we observed. In our experiments, the isoproterenol-induced increase in soluble HA-α_s declined slowly (perhaps in association with β-AR desensitization); 10 min after addition of propranolol, a β-AR antagonist, soluble HA-α_s declined to baseline. Because isoproterenol did not accelerate HA-α_s degradation (result not shown), we assume that the soluble HA-α_s released from membrane attachment was not degraded during the course of treatment with isoproterenol, with or without propranolol. Thus we infer that the soluble HA-α_s seen after exposure to isoproterenol reflects continuing cycling of HA-α_s between the soluble pool, which it enters after activation of G_s by β-ARs, and the membrane, to which it returns via a pathway not yet identified. The return pathway may require deactivation of α_s (by GTP hydrolysis), although it occurs more slowly (over several minutes) than would be expected if the rate of return were solely limited by the α_s GTPase reaction, for which the reported k_{cat} is $\sim$4 min^{-1} (9, 10, 17).

Why should activation of α_s—whether induced by isoproterenol, ADP-ribosylation, or mutation—reduce its apparent affinity for membranes? The most obvious explanation is that the $\beta\gamma$ subunits of G proteins anchor inactive (GDP-bound) α_s subunits to the plasma membrane (31), and that GTP-induced activation simply reduces the affinity of α_s for $\beta\gamma$. In accord with this explanation, neither isoproterenol (Fig. 9) nor ADP-ribosylation (Fig. 8) released HA-α_s-G226A from the particulate fraction, probably because the G226A mutant is unable to dissociate from $\beta\gamma$ (4, 18). We have not yet tested this explanation, however, because the 12CA5 antibody does not co-immunoprecipitate $\beta\gamma$ with HA-α_s, as described in the Results section.

A second finding with HA-α_s-G226A, more difficult to explain, is that a larger fraction of HA-α_s-G226A, as compared to HA-α_s, is found in the soluble fraction of unstimulated cells (25% vs. 10%; Table I and Fig. 7). Perhaps, in addition to causing tight binding of $\beta\gamma$, the G226A mutation partially impairs some (as yet unidentified) process required for attaching α_s (or even $\alpha_s\beta\gamma$) to membranes.

Quantitation of α_s

To understand how G proteins amplify hormonal signals in intact cells, we need precise information regarding the subcellular location and numbers of receptor and effector molecules, as well as G protein subunits. Table I summarizes our quantification of HA-α_s, which refines and extends earlier studies in S49 cells (1, 25, 26). In HA-α_s-transfected S49 *cyc*$^-$ cells, we found 16.6 $\pm$ 2.0 pmol HA-α_s per mg membrane protein and 240,000 HA-α_s molecules per cell. These values are in excellent agreement with earlier estimates (25) for endogenous α_s in wild type S49 cells; this agreement reflects our choice to study an HA-α_s-transfected clone in which the complement of HA-α_s, detected with a polyclonal anti-α_s antiserum, was similar to that of wild type S49 cells (Fig. 2). Previous studies indicate that wild type S49 cells contain, relative to α_s, much smaller numbers of β-ARs (15) and adenylyl cyclase molecules (1)—1,200 and 3,000 per cell, respectively. If these estimates are correct, an S49 cell contains 200 α_s molecules for every β-AR and 80 α_s molecules for every adenylyl cyclase molecule.

Although others have reported agonist-induced release of α_s from attachment to membranes (24), the number of α_s molecules released from membranes of intact cells has not previously been precisely quantitated. Our quantitative estimate shows that hormone treatment activates very many

more α_s molecules than we might imagine are required for regulating adenylyl cyclase. Figs. 9 and 10 indicate that isoproterenol can induce transfer of ~48,000 HA-α_s molecules (that is, 20% of 240,000 per cell) from the particulate to the soluble fraction. Thus, each of the 1,200 β-ARs per cell altered the membrane attachment of 40 HA-α_s molecules. (This is probably an underestimation, because some of the HA-α_s molecules presumably recycled back to the membrane, as noted above.) The 48,000 HA-α_s molecules released by isoproterenol is 16-fold greater than the estimated number (1) of adenylyl cyclase molecules per cell.

Although we do not know what proportion of the newly soluble HA-α_s molecules are in the GTP-bound active state, it need not be very large. Indeed, if the released HA-α_s molecules were evenly dispersed throughout the cell (an unlikely proposition) they would reach a concentration of 80 nM in cell water; adjacent to the plasma membrane, the concentration of HA-α_s molecules is probably much higher. In an in vitro study with purified proteins (10), the concentration of active α_s required for half-maximal stimulation of adenylyl cyclase was only 3.4 nM—~5% of the minimal concentration of HA-α_s, 80 nM, released by isoproterenol.

In summary, our calculations indicate that the number of HA-α_s molecules in HA cells—and, by inference, the number of α_s in wild type S49 cells—considerably exceeds the minimal number that would be required for stoichiometric activation of adenylyl cyclase. This is in keeping with a previous study (5), in which cholera toxin-treated cells maintained maximally elevated cellular cAMP at a time when they had lost ~90% of their complement of α_s. Although the proportion (20%) of total cellular HA-α_s redistributed following isoproterenol treatment is relatively small, the number of α_s redistributed per receptor is large, and their concentration adjacent to the membrane is probably quite high. The signaling role of these "solubilized" HA-α_s molecules is presently unknown. Specifically, we do not know whether the HA-α_s molecules that stimulate adenylyl cyclase belong to the soluble or the particulate-bound pool. In addition, the soluble α_s isolated by our cell fractionation procedures do not necessarily represent HA-α_s in the cytosol; instead, the "solubility" of HA-α_s may simply reflect some change that makes it more easily detached from membranes during cell lysis. This change could reflect altered conformation of the protein or a covalent modification; an example of the latter is depalmitoylation of $p21^{ras}$, which reduces its avidity for binding to membranes (20). Further studies will resolve these issues.

The epitope approach, which has proved a sensitive and precise tool for assessing metabolism and subcellular distribution of α_s, can readily be applied to similar studies of other G proteins. Epitopes in α, β, or γ subunits should eventually provide tools to tackle an even more difficult set of problems, by facilitating detection of protein–protein interactions responsible for G protein–mediated signaling in intact cells. Our present efforts are directed toward this end.

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