Activation of the α Subunit of Gs 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 (αt) of Gs in genetically αt-deficient S49 cyc- cells. Addition of a hemagglutinin (HA) epitope did not alter the ability of wild type αt 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-αt) quantitatively from cell extracts. We activated the epitope-tagged αt in intact cells by: (a) exposure of cells to cholera toxin, which activates αt by covalent modification; (b) mutational replacement of arginine-201 in HA-αt by a cysteine residue, to create HA-αt-R201C; like the cholera toxin-catalyzed modification, this mutation activates αt by slowing its intrinsic GTPase activity; and (c) treatment of cells with the β-adrenoceptor agonist, isoproterenol, which promotes binding of GTP to αt, thereby activating adenylyl cyclase. Both cholera toxin and the R201C mutation accelerated the rate of degradation of αt (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-αt-R201C was found in the soluble fraction, as compared to 10% of wild type HA-αt. Isoproterenol rapidly (in <2 min) caused 20% of HA-αt 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-αt-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 αt in response to activation. Taken together, these findings show that activation of αt 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 βγ complex and can interact with the effector until its intrinsic GTPase converts it to α-GDP, allowing re-association with βγ. 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 (αt) of Gs, 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 αt-deficient cyc- S49 mouse lymphoma cell (3, 13) provides a null background for analyzing the behavior of recombinant αt, normal or mutant; the nucleotide-bound state of the recombinant αt can be readily altered by β-adrenoceptor (β-AR) stimulation and by cholera toxin-catalyzed ADP-ribosylation. Two αt mutations provide independent ways of manipulating the protein's activity: replacement by cysteine of the arginine residue at position 201 of αt 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 by cholera toxin-catalyzed ADP-ribosylation.

1. Abbreviations used in this paper: αt, α subunit of stimulatory G protein; βAR, β adrenergic receptor; CTX, cholera toxin; GTPyS, guanosine 5'-[γ-thio]triphosphate; HA, hemagglutinin; RIPA, radioimmunoprecipitation assay.
Materials and Methods

Materials

Cholera toxin was obtained from List Biologicals. [35S]methionine/cysteine (TransLabel) was obtained from ICN Radiochemicals (Irvine, CA). Radioactive ATP and cAMP were from DuPont NEN. 125I-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 αs 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 αs cDNA was ligated into the pTZ vector for production of phagemid. Single-stranded (coding) DNA was annealed to a 41-base primer, which contained a HindIII-EcoRI fragment from the HA-as cDNA to the 3' EcoRI-HindIII sequence. An identifying MluI restriction site was also included. The reaction product was used to transform E. coli MV1190, and the mutagenized cDNA (HA-αs) derived from an ampicillin-resistant colony was re-ligated back into pMV7 for transfection into S49 cells. To create HA-αs-R201C, a 5' SalI-EcoRI restriction fragment from HA-αs was ligated to an EcoRI-AflII fragment from a rat αs cDNA encoding the R201C substitution (provided by C. Landis; see reference 17). The resulting Sall-AflII fragment was re-ligated into pMV7. HA-αs-G226A was generated by ligating the 5' HindIII-EcoRI fragment from the HA-αs cDNA to the 3' EcoRI-HindIII fragment from an αs 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 NaHCO3, pH 8.3), then soaked in Amplify (Amersham Corp.) for 20 min, dried, 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-αs was excised and radioactivity was quantitated by liquid scintillation spectroscopy.

Cell Fractionation

For analysis of subcellular distribution of αs, cells were allowed to swell in hypotonic dounce buffer (20 mM Hepes, pH 7.4, 20 mM KCl, 2.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 8 μg/ml PMSE, 3 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 αs) 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 resuspended 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-αs was excised and radioactivity was quantitated by liquid scintillation spectroscopy.

Quantitation Standard

A recombinant fragment of HA-αs was used as an immunoblot standard in quantitation studies. For its preparation, the cDNA encoding residues 77-213 of HA-αs 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 αs cDNA by the method described above. Cells were maintained under 5% CO2 in DMEM supplemented with 10% heat-inactivated horse serum. For metabolic labeling, cells (at 107/ml) were starved for 3 h in methionine- and cysteine-free DMEM with 10% dialyzed horse serum. [35S]methionine/cysteine (TransLabel, 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 (DMEM/horse serum supplemented with 1 mM each cold methionine and cysteine). The pulse-labeled cells were divided into equal aliquots and incubated (3 x 106 cells/ml) in chase medium in T75 culture flasks at 37°C, 5% CO2. 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 x 106 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 MgCl2, 1 mM EDTA, 1 mM DTT, 8 μg/ml PMSE, 3 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 αs) 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 resuspended 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-αs was excised and radioactivity was quantitated by liquid scintillation spectroscopy.

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 homogenizing 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 14C-labeled molecular weight standards (Amersham 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 14C-labeled molecular weight standards (Amersham Corp.). Densitometry was performed using an Ultrascan XL (LKB Instruments Inc., Bromma, Sweden) on films that had been "preflashed" before exposure to the probing membrane.

**Results**

**Construction and Expression of Epitope-tagged α2**

Alternative splicing of α2 transcripts produces α2 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 α2 sequence generates a unique epitope “tag” in the α2 subunit (Fig. 1).

If HA-tagged α2 is to serve as a valid stand-in for untagged α2, 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 α2 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-α2 antiserum, shows that epitope-tagged α2 (lane 3) is expressed in cytoplasmic cells at a level comparable to that of the 52-kD form of α2 in wild type S49 cells (lane 1). The tagged α2 subunit migrates slower than its untagged counterpart, probably owing to the addition of a negative charge. Two mutant forms of α2, containing the R201C of G226A mutations, were also epitope-tagged and expressed in cytoplasmic cells (Fig. 2 A, lanes 4 and 5, respectively). The low expression of the α2-R201C protein in membranes has been observed previously, both in transfected cytoplasmic cells and in pituitary adenomas (L. Vallar, personal communication). Epitope-tagged α2 is hereafter referred to as HA-α2. Cytoplasmic cells expressing the different tagged α2 constructs are designated as HA cells (wild type α2), HARC cells (R201C), or HAGA (G226A) cells.

**Immunoprecipitation and Quantitation of Tagged α2**

We used immunoprecipitates from metabolically labeled cells to compare turnover of epitope-tagged normal and mut-
Figure 3. Adenylyl cyclase stimulated by normal and epitope-tagged αs. Adenylyl cyclase activities were measured in membranes prepared from 3E cells, generated (33) by transfecting cyc-cells with wild-type, non-epitope–tagged αs (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-αs. (A) 10⁷ 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 αs-specific anti-peptide polyclonal antibody, followed by 125I-labeled protein A. (B) Metabolic labeling. 2 x 10⁷ 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 35S-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).

Figure 5. Preparation of HA-αs from whole-cell membranes by nitrogen cavitation. The 12CA5 antibody did not co-immunoprecipitate G protein β subunit with HA-αs, 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 α7β complex before or during immunoprecipitation, varying the nature and concentration of ionic and non-ionic detergents failed to produce immunoprecipitates containing β7. It is unlikely that introduction of the epitope sequence into recombinant αs prevented its binding to β7, on two grounds: (a) The epitope does not detectably alter αs-mediated signal transduction or subcellular distribution of HA-αs, as shown above; and (b) the functional defect of the G226A mutant, which is thought to result from its tight attachment to β7 in membranes (4, 18), is perfectly preserved in HA-αs-G226A (Fig. 3, and see below). An alternative possibility is that the 12CA5 antibody itself interferes sterically with binding of β7 to HA-αs; in an overnight incubation, 12CA5-
tracts were subjected to immunoprecipitation and SDS-thionine and cysteine (chase). At appropriate times, cells were divided into fractions for immediate lysis (zero time) or continued culture in normal medium containing excess cold methionine/cysteine at position 201 instead of the arginine that is ADP-ribosylated in HA-αs. Both the covalently modified normal protein and the mutant protein hydrolyze GTP at reduced rates, relative to unmodified wild type αs (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 adenyl cyclase (isoproterenol, forskolin) or an inhibitor of cAMP degradation (isobutylmethylxanthine) or turnover of αs. These results are in keeping with the previously reported (5) failure of cAMP analogs or elevated cellular cAMP to lower αs in membranes of treated cells.

αs Content of Membrane vs. Soluble Fractions

Two observations led us to suspect that the R201C mutation may increase the relative amount of αs in the soluble fraction of cell extracts. First, the strong signal seen with radio-labeled HA-αs-R201C in pulse-chase studies, using whole cell extracts (Fig. 6 A), was not in keeping with the markedly reduced amount of HA-αs-R201C in membranes prepared from HARC cells, as compared to the amount of HA-αs in HA membranes (Fig. 2 A, lane 3 vs. lane 4); the amount of αs was 6.3-fold greater in HA than in HARC membranes, as determined by densitometry (Table I). Second, this marked decrease in membrane αs was not fully accounted for by the 3.7-fold greater fractional degradation rate of HA-αs-R201C compared to HA-αs (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-αs-R201C is in the soluble fraction. For each of the three cell lines (HA, HARC, and HAGA), 4 × 10⁶ cells were divided into two equal aliquots. One was detergent lysed and immunoprecipitated with 12CA5 beads to provide a measure of all the αs present in the cell. The other aliquot was subjected to immunoprecipitation with 12CA5 antibody, followed by SDS-PAGE, and immunoblot-
Figure 6. Degradation of normal and mutant HA-αs. Representative autoradiographs are shown in A, degradation curves in B and C. Cells (2 × 10⁵ per experiment) were starved in methionine/cysteine-free medium for 3 h, then pulse-labeled with 250 μCi/ml [³⁵S]methionine/cysteine for 20 min. The labeled cells were washed, divided into five equal aliquots, and incubated in chase medium (4 × 10⁵ 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-αs, HA-αs-R201C, and HA-αs-G226A. (C) Degradation curves for HA-αs and HA-αs-G226A, in the presence and absence of cholera toxin. Curves were constructed as follows: bands corresponding to normal or mutant HA-αs 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.

Figure 7. Distribution of normal and mutant HA-αs between soluble and particulate fractions. For each experiment, 4 × 10⁷ 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. The HA-αs 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 αs. Probes were visualized with ¹²⁵I-labeled protein A and autoradiography.

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

| Cell line | αs 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 αs molecules in whole cells or fractions, as quantitated with the anti-epitope antibody, using a purified recombinant epitope-labeled fragment of αs as a standard (see Materials and Methods). Numbers in parentheses represent the percentage of total αs in the soluble or pellet fractions.
Figure 8. Effect of cholera toxin on soluble HA-αs. Cells were pulse-labeled for 20 min with 250 μCi/ml 35S-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 αs from membranes (19, 24). Accordingly, we asked whether cholera toxin treatment of intact cells could elevate the amount of αs in the S150 fraction (Fig. 8). Cells were pulse labeled with [35S]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-αs was significantly increased in extracts of toxin-treated HA cells (Fig. 8, lanes 4 vs. 5 of row 1). As expected, HA-αs-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-αs-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 αs-G226A (4, 29) and accelerates its degradation (Fig. 6 C), the toxin does not induce re-entry of HA-αs-G226A into the soluble pool.

Figure 9. Effect of isoproterenol on soluble HA-αs and HA-αs-G226A. Cells (3 × 10⁷ 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-αs after treatment with isoproterenol or isoproterenol followed by propranolol. (A) HA cells (3 × 10⁷ 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-αs 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, 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, 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, antibody, revealed in wild type S49 cells a reproducible increase in soluble α, 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, protein. For this reason, we studied the phenomenon in HA cells, in which the pool of HA-α, 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, in the S150 fraction (Fig. 9); the isoproterenol-induced increase in soluble HA-α, 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, immediately following stimulation with isoproterenol, 30% of HA-α, was found in the S150 fraction—a threefold increase. In keeping with the functional defect of α,-G226A (4, 18, 30), isoproterenol failed to induce movement of HA-α,-G226A into the S150 fraction (Fig. 9).

Kinetic analysis showed that the isoproterenol-induced shift of HA-α, into the S150 fraction was rapid (maximally detectable immediately after addition of the agonist); the amount of soluble HA-α, 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, Because isoproterenol treatment elevates soluble HA-α, it should reciprocally reduce HA-α, 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, 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,.

To determine the rate of disappearance of HA-α, 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, 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, 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, amounting to ~20% of total α, and that propranolol shifted α, back to the membrane fraction.

**Discussion**

We have developed a useful model system for studying the behavior of α, in an intact cell. Addition of an epitope did not detectably alter the protein’s ability to mediate hormonal stimulation of adenyl 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, antibodies available to us. The epitope allowed us to examine the behavior of normal and mutant α, in intact cells. Activation of α,—by cholera toxin, mutation, or hormone—altered its rate of degradation, membrane avidity, and cellular content.

**Activation Accelerates α, Degradation**

Pulse-chase studies revealed that degradation of HA-α, is biphasic. Approximately 40% of the protein disappeared rapidly, within an hour or so; this newly synthesized HA-α, is located exclusively in the soluble fraction of the cell. After transfer to the particulate fraction, the remaining HA-α, decayed at a slow rate (0.03 h -1, half-life 22 h). The biphasic degradation curve suggests that newly synthesized HA-α, 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, from rapid degradation may result from association with another protein or proteins, such as the β, 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, than for endogenous α, because the promoter in the retroviral vector may induce relative overproduction of the recombinant α, polypeptide.

Another group has reported (12) that endogenous α, 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,. Does the substitution of five amino acids in HA-α, 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 Tran35Slab 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 Tran35Slab causes significant cell death and a decrease in subsequent doubling rate. Indeed, in the previous study (12) incorporation of radiolabel into α, 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 αa. Incomplete immunoprecipitation may have accounted for the weak αa 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-αa, 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 αa 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 αa gene (L. Vallar, personal communication). It is possible that the acceleration of αa degradation represents a physiologically relevant feedback response to activation of the Gs signalling pathway. In any case, activation of αa, whether by mutation or by toxin-catalyzed ADP-ribosylation, promotes transfer of αa into a soluble fraction and accelerates its degradation. It is appealing to infer that the soluble pool of αa represents the route of accelerated degradation taken by the activated protein. The inference is wrong, however, as shown by the behavior of HA-αa-G226A. The toxin accelerates turnover of HA-αa-G226A, but does not push it into the soluble fraction (Fig. 8).

**Activation Alters Membrane Avidity of αa**

It seems likely that a GTP-induced change in conformation of wild type HA-αa is responsible for both the increase in turnover rate and the looseness of its attachment to membranes. If so, how can the toxin accelerate degradation of HA-αa-G226A but fail to stimulate adenyl cyclase in membranes of cells carrying the G226A mutation (Fig. 3; and see references 4 and 30)? Indeed, the S49 clone containing the αa-G226A mutation was isolated by virtue of its inability to accumulate cAMP in response to choleragen (30). The apparent discrepancy can be accounted for by recognizing that the G226A mutation does not absolutely block GTP-induced activation of αa (18), but instead prevents GTP-induced dissociation of αa-G226A from the βγ subunit (18, 22). Thus, recombinant αa-G226A, produced in E. coli, binds and hydrolyzes GTP, and mediates GTP-induced stimulation of adenyl 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 βγ. Accordingly, the evidence is consistent with the idea that a change in the conformation of αa, 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 αa binds to the plasma membrane, isoproterenol, a β-AR agonist, rapidly (<2 min) induced a shift of approximately 20% of total HA-αa 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 αa 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-αa declined slowly (perhaps in association with β-AR desensitization); 10 min after addition of propranolol, a β-AR antagonist, soluble HA-αa declined to baseline. Because isoproterenol did not accelerate HA-αa degradation (result not shown), we assume that the soluble HA-αa 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-αa seen after exposure to isoproterenol reflects continuing cycling of HA-αa, between the soluble pool, which it enters after activation of Gβγ, and the membrane, to which it returns via a pathway not yet identified. The return pathway may require deactivation of αa (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 αa, GTPase reaction, for which the reported kcat is ~4 min⁻¹ (9, 10, 17).

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

A second finding with HA-αa-G226A, more difficult to explain, is that a larger fraction of HA-αa-G226A, as compared to HA-αa, 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 βγ, the G226A mutation partially impairs some (as yet unidentified) process required for attaching αa (or even αaβγ) to membranes.

**Quantitation of αa**

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-αa, which refines and extends earlier studies in S49 cells (1, 25, 26). In HA-αa-transfected S49 cyc⁻ cells, we found 16.6 ± 2.0 pmol HA-αa per mg membrane protein and 240,000 HA-αa molecules per cell. These values are in excellent agreement with earlier estimates (25) for endogenous αa in wild type S49 cells; this agreement reflects our choice to study an HA-αa—transfected clone in which the complement of HA-αa, detected with a polyclonal anti-αa antiserum, was similar to that of wild type S49 cells (Fig. 2). Previous studies indicate that wild type S49 cells contain, relative to αa, much smaller numbers of β-ARs (15) and adenyl cyclase molecules (1)-1,200 and 3,000 per cell, respectively. If these estimates are correct, an S49 cell contains 200 αa molecules for every β-AR and 80 αa molecules for every adenyl cyclase molecule. Although others have reported agonist-induced release of αa from membranes (24), the number of αa 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, 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, 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, molecules. (This is probably an underestimation, because some of the HA-α, molecules presumably recycled back to the membrane, as noted above.) The 48,000 HA-α, 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, molecules are in the GTP-bound active state, it need not be very large. Indeed, if the released HA-α, 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, molecules is probably much higher. In an in vitro study with purified proteins (10), the concentration of HA-α, molecules is probably much higher. In summary, our calculations indicate that the number of HA-α, molecules in HA-cyclase was only 3.4 nM ~0.5% of the minimal concentration of HA-α, 80 nM, released by isoproterenol.

In summary, our calculations indicate that the number of HA-α, molecules in HA-cyclase would be required for stoichiometric activation of adenylyl cyclase. This is in keeping with a likely proposition they would reach a concentration of 80 nM ~0.5% of the minimal concentration of HA-α, 80 nM, released by isoproterenol.

Altered state of the protein or a covalent modification; an unspecified change that makes it more easily detached from membranes. Our present efforts are directed toward this end.

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References

1. Alousi, A. A., J. R. Jasper, P. A. Insel, and H. J. Motulsky. 1991. Stoichiometry of receptor-G,-adenylyl cyclase interactions. FASEB (Fed. Am. Soc. Exp. Biol.) J. 5:2300-2303.
2. Birnbaum, L. 1990. G proteins in signal transduction. Annu. Rev. Pharmacol. Toxicol. 30:675-705.
3. Bourne, H. R., J. Coffino, and G. M. Tonkins. 1975. Selection of a variant lymphoma cell deficient in adenylyl cyclase. Science (Wash. DC). 187:750-752.
4. Bourne, H. R., D. Kaslow, H. R. Kaslow, M. R. Salomon, and V. Licko. 1981. Hormone-sensitive adenylyl cyclase: mutant phenotype with normally regulated β-adrenergic receptors uncoupled from catalytic adenylyl cyclase. Mol. Pharmacol. 20:435-441.
5. Chang, F.-H., and H. R. Bourne. 1989. Cholera toxin induces cAMP-independent degradation of G, J. Biol. Chem. 264:5352-5357.
6. Christgau, S., H. Schierbeck, H. I. Aamot, L. Aaragard, B. Kjeg, L. Kofod, K. Hjune, and S. Baekeksen. 1991. Pancretic β cells express two autotomic forms of glutamic acid decarboxylase, a 65-kDa hydrophilic form and a 64-kDa amiphilic form which can be both membrane-bound and soluble. J. Biol. Chem. 266:21257-21264.
7. Freissmuth, M., and A. G. Gilman. 1989. Mutations of Gδ designed to alter the reactivity of the protein with bacterial toxins. Substitutions at ARG35 result in loss of GTPase activity. J. Biol. Chem. 264:21907-21914.
8. Freissmuth, M., J. P. Casey, and A. G. Gilman. 1989. G proteins control diverse pathways of transmembrane signaling. FASEB (Fed. Am. Soc. Exp. Biol.) J. 3:2125-2131.
9. Graziano, M. P., and A. G. Gilman. Synthesis in Escherichia coli of GTPase-deficient mutants of Gδ, J. Biol. Chem. 264:15475-15482.
10. Graziano, M. P., M. Freissmuth, and A. G. Gilman. 1989. Expression of Go, in Escherichia coli. Purification and properties of two forms of the protein. J. Biol. Chem. 264:409-418.
11. Green, N., H. Alexander, A. J. Olson, S. Alexander, T. M. Shinnick, J. G. Suncliffe, and R. A. Lerner. 1982. Immunogenic structure of the influenza virus hemagglutinin. Cell. 23:477-487.
12. Haddock, J. R., M. Ross, C. Watkins, and C. C. Malbon. 1990. Cross-regulation between G-protein-mediated pathways. Stimulation of adenylyl cyclase increases expression of the inhibitory G protein, Gi, J. Biol. Chem. 265:14784-14790.
13. Harris, B. A., J. D. Robishaw, S. M. Mumba, and A. G. Gilman. 1985. Molecular cloning of complementary DNA for the alpha subunit of the G protein that stimulates adenylyl cyclase. Science (Wash. DC). 229:1274-1277.
14. Houghton, R. A. 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc. Natl. Acad. Sci. USA. 82:5131-5135.
15. Insel, P. A., L. C. Mahan, H. J. Motulsky, L. M. Stoolman, and A. M. Koachman. 1983. Time-dependent decreases in binding affinity of agonists for β-adrenergic receptors of intact 293 lymphoma cells. J. Biol. Chem. 258:13977-13980.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.
17. Landis, C. A., S. B. Masters, A. Spada, A. M. Pace, H. R. Bourne, and L. Valler. 1989. GTPase inhibiting mutations activate the α chain of Gi 219 and stimulate adenylyl cyclase in human pituitary tumours. Nature (Lond.). 340:692-696.
18. Lee, E., R. Tausig, and A. G. Gilman. 1992. The G226A mutant of Gαs highlights the requirement for dissociation of G protein subunits. J. Biol. Chem. 267:1212-1218.
19. Lynch, C. J., L. Morbach, F. P. Blackmore, and J. H. Etxon. 1986. α-subunits of N are released from the plasma membrane following cholera toxin activation. FEBS (Fed. Eur. Biochem. Soc.) Lett. 200:333-336.
20. Magee, A. I., L. Gutierrez, I. A. McKay, C. J. Marshall, and A. Hall. 1987. Dynamic fatty acylation of p21α (EMBO (Eur. Mol. Biol. Organ.) J. 6:3351-3357.
21. McKenzie, F. R., and G. Milligan. 1990. Prostaglandin E1-mediated, cyclic AMP-independent, down-regulation of Gαs in neuroblastoma x glioma hybrid cells. J. Biol. Chem. 265:17084-17093.
22. Miller, R. T., S. B. Masters, K. A. Sullivan, B. Beiderman, and H. R. Bourne. 1988. A mutation that prevents GTP-dependent activation of the α chain of Gi, Nature (Lond.). 334:712-715.
23. Moon, R. T., and E. Lazarides. 1984. Biogenesis of the avian erythroid membrane skeleton: receptor-mediated assembly and stabilization of ankyrin (golbin) and spectrin. J. Cell Biol. 98:1899-1904.
24. Negishi, M., H. Hashimoto, and A. Iiokawa. 1992. Translocation of α subunits of stimulatory guanine nucleotide-binding proteins through stimulation of the protocathelcin receptor in mouse mastoyctoma cells. J. Biol. Chem. 276:2367-2369.
25. Rainski, L. A., and P. A. Insel. 1988. Quantitation of the guanine nucleo-
tide binding regulatory protein G, in S49 cell membranes using antipeptide antibodies to α. *J. Biol. Chem.* 263:9482-9485.

26. Ransnäs, L. A., P. Svoboda, J. R. Jasper, and P. A. Insel. 1989. Stimulation of β-adrenergic receptors of S49 lymphoma cells redistributes the α subunit of the stimulatory G protein between cytosol and membranes. *Proc. Natl. Acad. Sci. USA.* 86:7900-7903.

27. Robishaw, J. D., M. D. Smigel, and A. G. Gilman. 1986. Molecular basis for two forms of the G protein that stimulates adenylyl cyclase. *J. Biol. Chem.* 261:9587-9590.

28. Ross, E. M., M. E. Maguire, T. W. Sturgill, R. L. Biltonen, and A. G. Gilman. 1977. Relationship between the β-adrenergic receptor and adenyl cyclase. Studies of ligand binding and enzyme activity in purified membranes of S49 lymphoma cells. *J. Biol. Chem.* 252:5762-5775.

29. Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylyl cyclase assay. *Anal. Biochem.* 58:541-548.

30. Salomon, M. R., and H. R. Bourne. 1981. Novel S49 lymphoma variants with aberrant cyclic AMP metabolism. *Mol. Pharmacol.* 19:109-116.

31. Sternweis, P. C. 1986. The purified α subunits of G, and G, from bovine brain require 87 for association with phospholipid vesicles. *J. Biol. Chem.* 261:631-637.

32. Stryer, L., and H. R. Bourne. 1986. G proteins: a family of signal transducers. *Ann. Rev. Cell Biol.* 2:391-419.

33. Sullivan, K. A., R. T. Miller, S. B. Masters, B. Beiderman, W. Heideman, and H. R. Bourne. 1987. Identification of receptor contact site involved in receptor-G protein coupling. *Nature (Lond.)* 330:758-760.

34. Van Dop, C., M. Tsukokawa, H. R. Bourne, and J. Ramachandran. 1984. Amino acid sequence of retinal transducin at the site ADP-ribosylated by cholera toxin. *J. Biol. Chem.* 259:696-698.