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

1. Abbreviations used in this paper: αs, 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 from mediating stimulation of cAMP synthesis by cholera toxin and β-AR agonists (4, 22, 30), apparently because αs-G226A cannot readily dissociate from βγ (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-αs 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 αs with a peptide epitope, derived from the hemagglutinin (HA) protein of influenza virus (11), which is the target of a high-affinity mAAb.

The resulting experimental model, using epitope-tagged αs, expressed in the S49 cyc- cell, reveals that activating mutations, like cholera toxin-catalyzed ADP-ribosylation, produce two changes in the intracellular behavior of αs: 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. [35S]Metionine/cysteine (Trans35SLabel) 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 Mutagen kit according to the manufacturer’s instructions (Bio-Rad Laboratories, Cambridge, MA). Briefly, the αs cDNA was ligated into the pT2 vector for production of phagemid. Single-stranded (coding) DNA was annealed to a 41-base oligomer containing the information (noncoding strand, 5’ CCATCGCTG- of phagemid. Single-stranded (coding) DNA was annealed to a 41-base

Antibodies

The mAb 12CA5 (generously provided by Dr. Ian Wilson) was raised against the hemagglutinin (HA) protein of influenza virus (11). The antibody was purified from ascites using a Bio-Rad Affi-gel column. The column eluate was dialyzed against several

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 MgCl2, 1 mM EDTA, 1 mM DTT, 5 μg/mg PMSF, 8 μg/ml each leupeptin and aprotinin) on ice for 45 min. The mixture was then centrifuged at 150,000 g in a TL-100 tabletop ultracentrifuge (Beckman Instruments Inc., Westbury, NY) for 1 h. 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 mouse normal antibodies. 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 αs-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 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, plus protease inhibitors) for 10 min on ice. Samples were then dounce-homogenized using a Wheaton 1-ml tissue grinder (90°C cell disruption by light microscopy), and centrifuged for 30 min at 150,000 g in a TL-100 tabletop ultracentrifuge (Beckman Instruments). The supernatant (S100) fraction was 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 αs-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 douncing in the hypotonic buffer described above, then centrifuged two times 41°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 αs-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 DME supplemented with 10% heat-inactivated horse serum. For metabolic labeling, cells (at 10^6/ml) were starved for 3 h in methionine- and cysteine-free DME with 10% dialyzed horse serum. [35S]Metionine/cysteine (Trans35SLabel, 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 75 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.
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 125I-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 recorded membrane.

**Results**

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

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

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

**Immunoprecipitation and Quantitation of Tagged αs**

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

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

**Figure 2.** Western blot analysis of S49 clones. (A) Wild type S49 (24.3.2) and cys- (94.15.1) cells are compared to clones generated by transfecting cys- cells with cDNAs encoding HA-αs, HA-αs-R201C, and HA-αs-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 αs. The antibody probe was visualized with 125I-labeled protein A and autoradiography (24-h exposure). (B) Membrane-bound vs. soluble αs. 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.

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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 αs 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 αs 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 αs.
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 × 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).

The 12CA5 antibody did not co-immunoprecipitate G protein β subunit with HA-αs, as assessed by probing immunoprecipitates with an anti-β antisera; 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 αβγ complex before or during immunoprecipitation, varying the nature and concentration of ionic and non-ionic detergents failed to produce immunoprecipitates containing βγ. It is unlikely that introduction of the epitope sequence into recombinant αs prevented its binding to βγ, 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 βγ 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 βγ 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 medium.

Figure 6 A shows representative autoradiographs for each cell line tested, and Figs. 6 B and C depict the time courses of α, degradation, as quantitated by densitometry (Table I). Second, this marked decrease in membrane α, was not fully accounted for by the 3.7-fold greater fractional degradation rate of HA-αcR201C compared to HA-α (0.11 vs. 0.03 h⁻¹, respectively).

For turnover studies, cells were labeled for 20 min by incubation with [³²P]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 autographs for each cell line tested, and Figs. 6 B and C depict the time courses of α, degradation, as quantitated by liquid scintillation spectroscopy of excised bands. All cell lines tested showed biphasic decay of α, as decreased by 40–45%; the kinetics of this first phase may account, at least in part, for the lower amount of α, detected in membrane preparations from HARC cells, as compared to α, in membranes of HA and HAGA cells.

Two observations led us to suspect that the R201C mutation may increase the relative amount of α, in the soluble fraction of cell extracts. First, the strong signal seen with radiolabeled HA-αcR201C in pulse-chase studies, using whole cell extracts (Fig. 6 A), was not in keeping with the markedly reduced amount of HA-αcR201C in membranes prepared from HARC cells, as compared to the amount of HA-α, in HA membranes (Fig. 2 A, lane 3 vs. lane 4); the amount of α, was 6.3-fold greater in HA than in HARC membranes, as determined by densitometry (Table I). Second, this marked decrease in membrane α, was not fully accounted for by the 3.7-fold greater fractional degradation rate of HA-αcR201C compared to HA-α (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-αcR201C is in the soluble fraction. For each of the three cell lines (HA, HARC, and HAGA), 4 × 10⁷ cells were divided into two equal aliquots. One was detergent lysed and immunoprecipitated with 12CA5 beads to provide a measure of all the α, 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-αs. Representative autoradiographs are shown in A, degradation curves in B and C. Cells (2 × 10^6 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 × 10^6 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-G226A, and HA-αs-R201C. (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 × 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-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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>αs per cell*</th>
<th>Whole cell</th>
<th>P150</th>
<th>S150</th>
<th>Degradation rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>240,000</td>
<td>216,000(90)</td>
<td>24,000(10)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>HARC</td>
<td>168,000</td>
<td>34,000(20)</td>
<td>134,000(80)</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>HAGA</td>
<td>144,000</td>
<td>108,000(75)</td>
<td>36,000(25)</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

* 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 I). 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 assayed 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-\(\alpha\), in the S150 Fraction

If an increase in the amount of GTP-bound \(\alpha\) subunit is the mechanism by which cholera toxin induces HA-\(\alpha\), to shift into the S150 fraction, then isoproterenol, a \(\beta\)-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-\(\alpha\), antibody, revealed in wild type S49 cells a reproducible increase in soluble \(\alpha\), 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 \(\alpha\), protein. For this reason, we studied the phenomenon in HA cells, in which the pool of HA-\(\alpha\), subunits in the S150 fraction is small but readily immunoprecipitated and quantitated (see Fig. 7, lane 3 of row 7).

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

Kinetic analysis showed that the isoproterenol-induced shift of HA-\(\alpha\), into the S150 fraction was rapid (maximally detectable immediately after addition of the agonist); the amount of soluble HA-\(\alpha\), 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-\(\alpha\). Because isoproterenol treatment elevates soluble HA-\(\alpha\), it should reciprocally reduce HA-\(\alpha\), 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-\(\alpha\), after a 2-h lag (Fig. 8); the lag is presumably required for the toxin to penetrate the cells and catalyze ADP-ribosylation of \(\alpha\). To determine the rate of disappearance of HA-\(\alpha\), from the soluble fraction after cessation of \(\beta\)-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 \(\beta\)-AR stimulation very rapidly (i.e., in seconds), soluble HA-\(\alpha\), 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 \(\alpha\), 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 \(\alpha\), amounting to ~20% of total \(\alpha\), and that propranolol shifted \(\alpha\), back to the membrane fraction.

Discussion

We have developed a useful model system for studying the behavior of \(\alpha\), 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-\(\alpha\), antibodies available to us. The epitope allowed us to examine the behavior of normal and mutant \(\alpha\), in intact cells. Activation of \(\alpha\) by cholera toxin, mutation, or hormone—altered its rate of degradation, membrane avidity, and cellular content.

Activation Accelerates \(\alpha\), Degradation

Pulse-chase studies revealed that degradation of HA-\(\alpha\), is biphasic. Approximately 40% of the protein disappeared rapidly, within an hour or so; this newly synthesized HA-\(\alpha\), is located exclusively in the soluble fraction of the cell. After transfer to the particulate fraction, the remaining HA-\(\alpha\), decayed at a slow rate (0.03 h \(^{-1}\), half-life 22 h). The biphasic degradation curve suggests that newly synthesized HA-\(\alpha\), 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 \(\alpha\)-spectrin (23) are two such examples. Protection of \(\alpha\), from rapid degradation may result from association with another protein or proteins, such as the \(\beta\) 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-\(\alpha\), than for endogenous \(\alpha\), because the promoter in the retroviral vector may induce relative overproduction of the recombinant \(\alpha\), polypeptide.

Another group has reported (12) that endogenous \(\alpha\), 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-\(\alpha\). Does the substitution of five amino acids in HA-\(\alpha\), 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 Tran35Slabel 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 Tran35Slabel causes significant cell death and a decrease in subsequent doubling rate. Indeed, in the previous study (12) incorporation of radiolabel into \(\alpha\), 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 $\alpha$. Incomplete immunoprecipitation may have accounted for the weak $\alpha$ 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 R20IC mutation increased degradation of HA-$\alpha$, 3.5–to 5-fold and caused the $\alpha$ subunit to move from the particulate to the soluble fraction. These observations explain the greatly reduced amounts of $\alpha$ 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 R20IC, in the $\alpha$ gene (L. Vallar, personal communication). It is possible that the acceleration of $\alpha$ degradation represents a physiologically relevant feedback response to activation of the G, signalling pathway. In any case, activation of $\alpha$, whether by mutation or by toxin-catalyzed ADP-ribosylation, promotes transfer of $\alpha$, into a soluble fraction and accelerates its degradation. It is appealing to infer that the soluble pool of $\alpha$, represents the route of accelerated degradation taken by the activated protein. The inference is wrong, however, as shown by the behavior of HA-$\alpha$-G226A. The toxin accelerates turnover of HA-$\alpha$-G226A, but does not push it into the soluble fraction (Fig. 8).

**Activation Alters Membrane Avidity of $\alpha$.**

It seems likely that a GTP-induced change in conformation of wild type HA-$\alpha$, 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-$\alpha$-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 $\alpha$-G226A mutation was isolated by virtue of its inability to accumulate cAMP in response to cholera toxin (30). The apparent discrepancy can be accounted for by recognizing that the G226A mutation does not absolutely block GTP-induced activation of $\alpha$, (18), but instead prevents GTP-induced dissociation of $\alpha$-G226A from the $\beta_{7}$ subunit (18, 22). Thus, recombinant $\alpha$-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 $\beta_{y}$. Accordingly, the evidence is consistent with the idea that a change in the conformation of $\alpha$, induced by binding GTP and prolonged by the R20IC mutation or by toxin-catalyzed ADP-ribosylation, suffices to accelerate degradation.

In keeping with the idea that the activated conformation reduces avidity with which $\alpha$, binds to the plasma membrane, isoproterenol, a $\beta$-AR agonist, rapidly (<2 min) induced a shift of approximately 20% of total HA-$\alpha$, 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 $\alpha$, 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-$\alpha$, declined slowly (perhaps in association with $\beta$-AR desensitization); 10 min after addition of propranolol, a $\beta$-AR antagonist, soluble HA-$\alpha$, declined to baseline. Because isoproterenol did not accelerate HA-$\alpha$, degradation (result not shown), we assume that the soluble HA-$\alpha$, 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-$\alpha$, seen after exposure to isoproterenol reflects continuing cycling of HA-$\alpha$, between the soluble pool, which it enters after activation of Gs by $\beta$-ARs, and the membrane, to which it returns via a pathway not yet identified. The return pathway may require deactivation of $\alpha$, (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 $\alpha$, GTPase activity, for which the reported $k_{\text{cat}}$ is $\sim$4 min$^{-1}$ (9, 10, 17).

Why should activation of $\alpha$,—whether induced by isoproterenol, ADP-ribosylation, or mutation—reduce its apparent affinity for membranes? The most obvious explanation is that the $\beta_{7}$ subunits of G proteins anchor inactive (GDP-bound) $\alpha$, subunits to the plasma membrane (31), and that GTP-induced activation simply reduces the affinity of $\alpha$, for $\beta_{7}$. In accord with this explanation, neither isoproterenol (Fig. 9) nor ADP-ribosylation (Fig. 8) released HA-$\alpha$-G226A from the particulate fraction, probably because the G226A mutant is unable to dissociate from $\beta_{7}$ (4, 18). We have not yet tested this explanation, however, because the 12CA5 antibody does not co-immunoprecipitate $\beta_{7}$ with HA-$\alpha$, as described in the Results section.

A second finding with HA-$\alpha$-G226A, more difficult to explain, is that a larger fraction of HA-$\alpha$-G226A, as compared to HA-$\alpha$, 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 $\beta_{7}$, the G226A mutation partially impairs some (as yet unidentified) process required for attaching $\alpha$, (or even $\alpha$,)$\beta_{7}$ to membranes.

**Quantitation of $\alpha$.**

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-$\alpha$, which refines and extends earlier studies in S49 cells (1, 25, 26). In HA-$\alpha$-transfected S49 cycl$^{+}$ cells, we found 16.6 ± 2.0 pmol HA-$\alpha$, per mg membrane protein and 240,000 HA-$\alpha$, molecules per cell. These values are in excellent agreement with earlier estimates (25) for endogenous $\alpha$, in wild type S49 cells; this agreement reflects our choice to study an HA-$\alpha$-transfected clone in which the complement of HA-$\alpha$, detected with a polyclonal anti-$\alpha$, antiserum, was similar to that of wild type S49 cells (Fig. 2). Previous studies indicate that wild type S49 cells contain, relative to $\alpha$, much smaller numbers of $\beta$-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 $\alpha$, molecules for every $\beta$-AR and 80 $\alpha$, molecules for every adenyl cyclase molecule.

Although others have reported agonist-induced release of $\alpha$, from attachment to membranes (24), the number of $\alpha$, molecules released from membranes of intact cells has not previously been precisely quantitated. Our quantitative estimate shows that hormone treatment activates many 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 ~45000 HA-α, molecules (that is, 20% of 240000 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 48000 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 active α, required for half-maximal stimulation of adenylyl cyclase was only 3.4 nM~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-c~s is probably much higher. In an in vitro study with purified proteins (10), the concentration of active α, required for half-maximal stimulation of adenylyl cyclase was only 3.4 nM~5% of the minimal concentration of HA-α, 80 nM, released by isoproterenol.

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The epitope approach, which has proved a sensitive and precise tool for assessing metabolism and subcellular distribution of α, 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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