Activation-induced Subcellular Redistribution of $G_{s\alpha}$

Philip B. Wedegaertner,^{*,†§} Henry R. Bourne,^{*,†||} and Mark von Zastrow^{*,†||#}

Departments of ^{*}Cellular and Molecular Pharmacology, ^{||}Medicine, and [¶]Psychiatry, [†]Cardiovascular Research Institute, and [#]Center for Neurobiology and Psychiatry, University of California at San Francisco, San Francisco, California

Submitted March 15, 1996; Accepted May 29, 1996
Monitoring Editor: Guido Guidotti

We have examined the subcellular distribution of α_s , the α subunit of the heterotrimeric G protein G_s , by using immunofluorescence microscopy. In transiently transfected HEK293 cells, wild-type α_s localizes to the plasma membrane. However, a mutationally activated α_s (α_{sR201C}) is diffusely distributed throughout the cytoplasm. Similarly, cholera toxin activation of α_s causes it to redistribute from the plasma membrane to cytoplasm in stably transfected cells. In HEK293 cells stably transfected with α_s and the β_2 -adrenergic receptor (β -AR), stimulation of the β -AR by the agonist isoproterenol also causes a translocation of α_s from the plasma membrane to cytoplasm. Replacing the agonist with antagonist allows α_s to return to the plasma membrane, demonstrating the reversibility of α_s translocation. Receptor-activated α_s does not colocalize with internalized β -AR at endosomes. Incubation of cells in hypertonic sucrose to inhibit clathrin-coated pit-mediated endocytosis of agonist-activated β -AR failed to block agonist-stimulated redistribution of α_s . These findings demonstrate that activated α_s reversibly undergoes a translocation from the plasma membrane to cytoplasm and begin to address the relationship between regulated trafficking of a seven-transmembrane receptor and its cognate G protein.

INTRODUCTION

Regulated and reversible translocation within the cell is a common paradigm for proteins involved in signal transduction pathways. Upon extracellular stimulation, many such proteins reversibly translocate from the plasma membrane to cytoplasm, from cytoplasm to the plasma membrane or nucleus, or between different membrane locations.

Heterotrimeric ($\alpha\beta\gamma$) G proteins act at the plasma membrane as molecular switches to couple activated seven-transmembrane receptors to appropriate effector proteins. Although this receptor–G protein–effector interaction at the intracellular surface of the plasma membrane is the best understood aspect of G-protein function, it has become increasingly clear that G proteins and G-protein–coupled receptors are not restricted to a homogeneous plasma membrane

environment. Some G proteins and receptors are concentrated in caveolae (Chang *et al.*, 1994; Chun *et al.*, 1994; Lisanti *et al.*, 1994), suggesting unique functions within distinct plasma membrane microdomains. G-protein–coupled receptors can undergo agonist-induced endocytosis upon activation and subsequent recycling to the cell surface (von Zastrow and Kobilka, 1992; von Zastrow *et al.*, 1993; Hoxie *et al.*, 1993; Grady *et al.*, 1995). Furthermore, numerous studies suggest important functions for G proteins at other cellular locations in addition to the plasma membrane. Certain G proteins have been detected at intracellular membranes, such as the Golgi complex (Stow *et al.*, 1991; Hermouet *et al.*, 1992; Wilson *et al.*, 1994), and have been implicated in intracellular vesicle trafficking (reviewed, Jones, 1994), whereas others associate with cytoskeletal structures, such as microtubules (Wang *et al.*, 1990) and actin (Ibarrondo *et al.*, 1995).

At least one G-protein α subunit may reversibly detach from the plasma membrane upon activation. A significant portion of cellular α_s , the G-protein α subunit responsible for stimulating adenylyl cyclase,

[†] Corresponding author.

[§] Present address: Department of Pharmacology, Kimmel Cancer Institute, Thomas Jefferson University, 233 S. 10th Street, Philadelphia, PA 19107.

changes from a membrane bound to soluble protein, as detected by cell fractionation after activation by receptor stimulation, GTPase inhibiting mutation, or cholera toxin treatment (Ransnäs *et al.*, 1989; Levis and Bourne, 1992; Negishi *et al.*, 1992). Increased solubility of α_s correlates with activation-induced depalmitoylation of α_s (Degtyarev *et al.*, 1993; Mumby *et al.*, 1994; Wedegaertner and Bourne, 1994). These results are consistent with a model in which removal of covalently attached palmitate, a modification required for membrane attachment of α_s (Wedegaertner *et al.*, 1993), allows reversible translocation of α_s from the plasma membrane to cytoplasm (Wedegaertner and Bourne, 1994).

Increased solubility of activated α_s may reflect either actual separation of the protein from the plasma membrane or merely decreased affinity for the plasma membrane (and/or membrane-bound $\beta\gamma$), which becomes evident upon cell disruption. In this report, immunofluorescence localization studies demonstrate that activated α_s indeed translocates reversibly from the plasma membrane to cytoplasm and provide new insight into the relationship between regulated trafficking of a seven-transmembrane receptor and its cognate G protein.

MATERIALS AND METHODS

Cell Culture and Transfection

Human embryonal kidney 293 (HEK293) cells (American Type Culture Collection, Rockville, MD) were propagated in Dulbecco's minimal essential media (UCSF Cell Culture Facility, San Francisco, CA) supplemented with 10% fetal bovine serum. Epitope-tagged α_s (Levis and Bourne, 1992) (cloned into pBC12 MI vector; Cullen, 1987) was cotransfected with pSVneo by calcium phosphate precipitation, and stable transformants were selected by using 250–500 $\mu\text{g}/\text{ml}$ G418 (Life Technologies, Gaithersburg, MD) as described (von Zastrow and Kobilka, 1992). To obtain cells cotransfected with both epitope-tagged α_s and human β_2 -adrenergic receptor, these cells were transfected with pWS290 vector containing β_2 -adrenergic receptor cDNA (Suryanarayana *et al.*, 1992), and stable transformants were selected by using 200 $\mu\text{g}/\text{ml}$ hygromycin (Invitrogen, San Diego, CA). Cell clones expressing ~700 fmol of β_2 -adrenergic receptor (β -AR) per milligram of cell protein (assayed by binding of ^3H -dihydroalprenolol) and epitope-tagged α_s at approximately threefold over endogenous levels of α_s (quantitated by immunoblotting with antisera recognizing both endogenous and epitope-tagged proteins) were used for further study. For studies of native α_s in receptor-expressing cells, a previously described clone of stably transfected cells expressing an epitope-tagged β -AR alone (~500 fmol/mg) was used (von Zastrow and Kobilka, 1992).

Immunofluorescence Localization

HEK293 cells were grown on glass coverslips and, after the appropriate treatment, fixed in 4% formaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized by incubation in a blocking buffer consisting of 5% nonfat milk and 1% Triton X-100 in PBS for 20 min. Fixation and permeabilization with methanol provided identical results. After fixation and permeabilization, cells were incubated with 12CA5 mouse monoclonal antibody (3 $\mu\text{g}/\text{ml}$; Berkeley Antibody) and β -AR-specific polyclonal rabbit antiserum (1:1000 dilution; von Zastrow and Kobilka, 1992) in blocking buffer

for 1 h. In some experiments, endogenous α_s was detected with RM/7 affinity-purified polyclonal rabbit antiserum (2 $\mu\text{g}/\text{ml}$; a generous gift of Dr. Allen Spiegel, National Institutes of Health, Bethesda, MD), and endogenous MEK was detected with a polyclonal rabbit antiserum that recognizes MEK1 and MEK2 (Transduction Laboratories, Lexington, KY). Coverslips were washed with blocking buffer, and appropriate secondary antibodies were added (donkey anti-mouse fluorescein isothiocyanate [FITC] conjugate and/or donkey anti-rabbit Texas Red conjugate; Jackson ImmunoResearch, West Grove, PA) at 1:100 dilution in blocking buffer for 30 min. Coverslips were washed in 1% Triton X-100 in PBS and mounted on glass slides with a drop of ProLong Antifade (Molecular Probes, Eugene, OR).

Immunofluorescence microscopy was performed with a Nikon Diaphot microscope equipped with Nikon 60X/NA1.4 objective and standard fluorescein/Texas Red filter sets. Images were captured with a Hamamatsu SIT camera and NIH Image software. Confocal fluorescence microscopy was performed with a custom-built laser scanning microscope (Dr. Stephen Smith, Stanford University, Stanford, CA) and the same objective as described (von Zastrow and Kobilka, 1994). For dual color colocalization experiments, negligible bleedthrough between FITC and Texas Red channels was confirmed by single-labeled specimens. Images were transferred to Adobe Photoshop, digitally processed, and printed on a Tektronix Phaser 440. In all cases, images presented are representative of at least three separate experiments.

The 12CA5 monoclonal antibody is specific for HA-tagged proteins in HEK293 cells. As shown previously, no specific staining is observed upon incubation of the 12CA5 antibody with HEK293 cells transfected with wild-type β -AR (not containing the HA epitope; von Zastrow and Kobilka, 1992). The RM/7 affinity-purified polyclonal antibody used to detect endogenous α_s has been used previously for immunocytochemical localization of α_s in rat pituitary cells (Wilson *et al.*, 1994). We have found that this antibody exhibits no specific staining of S49 *cyc*[−] cells, which are devoid of α_s ; however, the RM/7 antibody does stain the plasma membrane of α_s containing wild-type S49 cells (our unpublished results).

Cell Fractionation

HEK293 cells stably expressing β -AR and HA- α_s were incubated in the presence or absence of 10 μM isoproterenol for 20 min at 37°C. Plates were placed on ice and washed with PBS. For hypotonic lysis, cells were scraped off the plate in the presence of PBS and pelleted at low speed. The cell pellet was suspended in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8, 2.5 mM MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ aprotinin, and 10% glycerol), and cells were lysed by 10 passages through a 27-gauge needle. Nuclei were removed by microcentrifugation at 3000 rpm for 5 min. The resulting supernatant was centrifuged for 20 min at 150,000 $\times g$ in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA). The supernatant from this high-speed spin was designated the soluble fraction. The pellet was suspended in an equal volume of lysis buffer and designated the particulate fraction. For freeze-thaw lysis, PBS-washed plates were placed in a dry ice/ethanol bath for 2 min. Plates were transferred to a 37°C water bath, and cells were thawed in the presence of 1 ml of 0.5 M NaCl lysis buffer (20 mM HEPES, pH 7.2, 0.5 M NaCl, 2.5 mM MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 5 $\mu\text{g}/\text{ml}$ aprotinin). Broken cells were rapidly scraped and transferred to a centrifuge tube. This suspension was then centrifuged for 20 min at 150,000 $\times g$ in a TL-100 ultracentrifuge. As described above, the supernatant was designated the soluble fraction. The pellet was suspended in an equal volume of 0.5 M NaCl lysis buffer and designated the particulate fraction. Release of at least 90% of the cytosolic MEK protein into the soluble fraction confirmed that freeze-thaw lysis efficiently lysed these cells. Samples from either hypotonic or freeze-thaw lysis were subsequently analyzed by 10% SDS-PAGE and Western blotting with 12CA5 as described (Wedegaertner *et al.*, 1993).

RESULTS

Visualization of Epitope-tagged α_s Proteins by Immunofluorescence Microscopy

Immunofluorescence microscopy was used to examine the subcellular distribution of epitope-tagged α_s in its unactivated and activated states. All α_s proteins contained an internal hemagglutinin (HA) epitope tag (DVPDYA), detectable by monoclonal antibody 12CA5. The presence of this epitope allows efficient immunodetection of α_s (Levis and Bourne, 1992) and has no effect on the function and subcellular localization of α_s (Levis and Bourne, 1992; Wedegaertner and Bourne, 1994; also, see below).

The localization of HA-tagged α_s was first examined in transiently transfected HEK293 cells. α_s containing the HA epitope tag but no other mutation (hereafter referred to simply as wild-type α_s) was observed at the periphery of the cell and seemed to be associated with the plasma membrane (Figure 1A). The picture was quite different, however, for mutationally activated α_s (α_s R201C), in which an arginine-to-cysteine mutation at residue 201 decreases the α subunit's intrinsic GTP hydrolysis activity (Freissmuth and Gilman, 1989; Landis *et al.*, 1989). Instead of localizing at the plasma membrane, constitutively active α_s R201C was visualized in a diffuse distribution throughout the cytoplasm (Figure 1B). Because activation of α_s leads to its rapid depalmitoylation (Degtyarev *et al.*, 1993; Mumby *et al.*, 1994; Wedegaertner and Bourne, 1994), we also assayed the subcellular localization of a nonpalmitoylated mutant α_s , α_s C3S. This mutant protein is not constitutively active but is lacking the cysteine required for addition of palmitate. Previously, we ob-

served that this cysteine-to-serine mutant α_s resided predominantly in the soluble fraction of transiently transfected HEK293 cells, suggesting that palmitoylation is required for membrane binding (Wedegaertner *et al.*, 1993). Like α_s R201C, nonpalmitoylated α_s C3S distributed diffusely throughout the cytoplasm rather than localizing at the plasma membrane (Figure 1C).

We next examined the localization of epitope-tagged α_s in stably transfected HEK293 cells. As observed in transiently transfected cells, α_s was visualized at the plasma membrane of stably transfected cells (Figure 2A). Cholera toxin, which activates α_s by directly ADP ribosylating arginine 201 of α_s , caused a substantial redistribution of α_s . Cholera toxin-activated α_s was no longer observed at the plasma membrane, but instead it was detected in a diffuse distribution throughout the cytoplasm (Figure 2B). In addition, cholera toxin caused the redistribution of endogenous α_s in HEK293 cells not expressing HA-tagged α_s (our unpublished results). Thus, either mutation of arginine 201 or acute ADP ribosylation of the same arginine causes a redistribution of α_s . Furthermore, the cholera toxin experiment (Figure 2), which allows regulation of α_s activation, provides evidence that redistribution of α_s is a direct result of its activation rather than a cellular change caused by persistent expression of mutationally activated α_s .

Agonist Stimulation of the β -Adrenergic Receptor Causes α_s Redistribution

To examine this activation-induced redistribution of α_s in a more physiological context (i.e., receptor-me-

Figure 1. Subcellular localization of transiently transfected wild-type and mutant α_s . HEK293 cells were transiently transfected with HA epitope-tagged α_s (A), α_s R201C (B), or α_s C3S (C) cDNA in pcDNA-I. Cells were plated onto glass coverslips and fixed with 4% formaldehyde 48 h after transfection. Fixed cells were permeabilized and incubated with monoclonal antibody 12CA5. Bound antibody was detected by incubation with FITC-conjugated anti-mouse antibody. Images were visualized as described under MATERIALS AND METHODS. Bar, 10 μ m.

Figure 2. Cholera toxin-stimulated redistribution of HA- α_s . HEK293 cells stably expressing HA- α_s were fixed in 4% formaldehyde after no treatment (A) or incubation with 1 μ g/ml cholera toxin for 4 h (B). HA- α_s was detected by incubation with monoclonal antibody 12CA5 followed by FITC-conjugated anti-mouse antibody. Images were visualized as described under MATERIALS AND METHODS. Bar, 10 μ m.

diated G-protein activation), we turned to a HEK293 cell line stably expressing HA-tagged α_s and the β_2 -adrenergic receptor (β -AR). G_s is the cognate G protein for physiological signaling by β -AR; agonist binding to β -AR causes activation (GTP binding) of α_s . HEK293 cells normally contain very low levels of β -AR (almost undetectable by immunoblotting, immunofluorescence [von Zastrow and Kobilka, 1992], and cAMP-signaling assays). In these cotransfected HEK293 cells, we analyzed the acute receptor-mediated activation of α_s and compared the subcellular localization of unactivated and activated α_s and β -AR.

As in the case of mutational and cholera toxin-mediated activation, β -AR activation by the agonist isoproterenol caused a redistribution of α_s . As shown previously (von Zastrow and Kobilka, 1992), the β -AR displayed a dramatic, agonist-induced redistribution from the plasma membrane (Figure 3A) to endocytic vesicles (Figure 3C) visualized by brightly stained punctate accumulations in the cytoplasm. In the same cells, α_s redistributed from the plasma membrane to a diffuse distribution throughout the cytoplasm (Figure 3, B vs. D) similar to that observed with mutationally activated, nonpalmitoylated, and cholera toxin-activated α_s proteins.

The time course of α_s redistribution was quite similar to the previously shown time course of β -AR redistribution (von Zastrow and Kobilka, 1992). Redistribution of both proteins was clearly observed by fluorescence microscopy within 2 min after the addition of isoproterenol. This redistribution persisted for >1 h in the continued presence of agonist and was rapidly reversible. Removing the agonist and adding the antagonist propranolol to the medium to block receptor activation caused the return of both α_s and β -AR to a plasma membrane localization pattern in-

Figure 3. Isoproterenol-stimulated redistribution of HA- α_s and β -AR. HEK293 cells stably expressing β -AR and HA- α_s were fixed in 4% formaldehyde after no treatment (A and B), addition of 10 μ M isoproterenol for 20 min (C, D, G, and H), or 10 μ M isoproterenol for 20 min, followed by 10 μ M propranolol for 20 min (E and F). β -AR was detected by incubation with a polyclonal antiserum directed against the β -AR carboxyl terminus, followed by Texas Red-conjugated anti-rabbit antibodies (A, C, and E). HA- α_s was detected by incubation with monoclonal antibody 12CA5 followed by FITC-conjugated anti-mouse antibody (B, D, F, and G). MEK was detected by incubation with a polyclonal antibody specific for MEK, followed by Texas Red-conjugated anti-rabbit antibodies (H). Images were visualized as described under MATERIALS AND METHODS. Bar, 10 μ m.

distinguishable from that of unstimulated cells (Figure 3, E and F).

The localization pattern of receptor-activated α_s was similar to that of the endogenously expressed protein kinase MEK (Figure 3, G and H), which is cytosolic, on

the basis of subcellular fractionation in a number of cell types (Zheng and Guan, 1994), including HEK293 cells (our unpublished results). Confocal fluorescence microscopy confirmed that, whereas both α_s and β -AR were associated with the plasma membrane of unstimulated cells, these proteins were differentially distributed after activation. The β -AR was localized in endosomal vesicles of isoproterenol-stimulated cells. In contrast, receptor-activated α_s was localized diffusely throughout the cytoplasm as well as in a small number of concentrated accumulations that were smaller in size and clearly not associated with receptor-associated endosomes (Figure 4). This granular appearance of activated α_s was reproducibly observed in our experiments. We also observed punctate accumulations for the cytosolic protein kinase MEK (Figure 3H), indicating that this granular pattern of cytoplasmic localization is not unique to activated α_s .

These results show that, in the unactivated state, α_s and β -AR colocalize at the plasma membrane; however, after isoproterenol stimulation of the β -AR, α_s and β -AR do not show a similar pattern of redistribution (Figures 3 and 4). This difference is consistent with the idea that the two proteins redistribute via distinct mechanisms: endocytic vesicles for β -AR (von Zastrow and Kobilka, 1992) and a mechanism involving depalmitoylation and release from the plasma membrane for α_s (Wedegaertner and Bourne, 1994).

Figure 4. Confocal microscopy of isoproterenol-stimulated redistribution of HA- α_s and β -AR. HEK293 cells stably expressing β -AR and HA- α_s were fixed in 4% formaldehyde after no treatment (A and B) or after the addition of 10 μ M isoproterenol for 20 min (C and D). Cells were prepared for immunofluorescence as described for Figure 3, A–D. Confocal images were obtained as described under MATERIALS AND METHODS. Bar, 10 μ m.

Activation-induced Redistribution of Endogenous α_s

To confirm that the redistribution of α_s observed in transfected cells is a physiological process rather than an artifact of epitope tagging or overexpression, the subcellular localization of endogenously expressed α_s was examined by using an antibody recognizing its carboxyl terminus (Simonds *et al.*, 1989). Endogenous α_s , although detected weakly compared with epitope-tagged α_s in transfected cells, redistributed in an identical manner after receptor activation. Endogenously expressed α_s localized to the plasma membrane of unstimulated cells and distributed throughout the cytoplasm of isoproterenol-stimulated cells (Figure 5, B and D). This localization pattern, although distinguishable from that of internalized β -AR (Figure 5, A and C), was identical to that observed for the epitope-tagged α_s under the same conditions (Figure 3) as well as for a mutant nonpalmitoylated α_s (α_s C3S) not containing an epitope tag (Figure 5E).

Subcellular Fractionation of Activated α_s

Subcellular fractionation was used to examine further the distribution of β -AR-activated α_s . Cellular fractionation revealed an increase in solubility of mutationally activated and receptor-activated α_s in HEK293 cells, confirming results obtained previously for α_s in S49 *cyc*– cells (Ransnäs *et al.*, 1989; Levis and Bourne, 1992). At least 98% of HA- α_s resided in the particulate fractions of unstimulated HEK 293 cells (Figure 6A, lanes 1 and 2). In contrast, mutationally activated HA- α_s R201C (stably expressed in HEK293 cells) partitioned as a mostly (90%) soluble protein (Figure 6A, lanes 3 and 4). Isoproterenol activation of the β -AR also caused a shift of α_s from the particulate to the soluble fraction. Receptor activation resulted in an approximately threefold increase in soluble HA- α_s (Figure 6B, compare lanes 2 and 4). Although an increase in α_s solubility was detected on receptor stimulation, the amount of soluble α_s was small relative to total α_s . Even after activation, >90% of α_s remained in the particulate fraction (Figure 6B, compare lanes 1 and 3). Rapid freeze-thaw lysis in a 0.5 M NaCl buffer allowed detection of a greater amount of total soluble HA- α_s in the isoproterenol-stimulated cells (Figure 6C). Rapid lysis in high salt buffer revealed an increase in soluble HA- α_s from ~5% of the total in unstimulated cells to ~20% in isoproterenol-stimulated cells (Figure 6C, compare lanes 3 and 4); the relative increase was similar to that seen with lysis in hypotonic buffer.

Relationship between β -AR and α_s Redistribution

The similar kinetics of reversible redistribution of β -AR and α_s led us to examine the interdependence between these two protein-trafficking events. Cholera toxin, which activates α_s independently of receptor

Figure 5. Isoproterenol-stimulated redistribution of endogenous α_s . HEK293 cells stably expressing HA epitope-tagged β -AR were fixed in 4% formaldehyde after no treatment (A and B) or after the addition of 10 μ M isoproterenol for 20 min (C and D). β -AR was detected by incubation with monoclonal antibody 12CA5, followed by FITC-conjugated anti-mouse antibody (A and C). Endogenous α_s was detected by incubation with polyclonal antibody RM, followed by Texas Red-conjugated anti-rabbit antibody (B and D). HEK293 cells stably expressing nonepitope-tagged α_s C3S were fixed in 4% formaldehyde and processed for immunofluorescence by incubation with polyclonal antibody RM, followed by Texas Red-conjugated anti-rabbit antibody (E). Images were visualized as described under MATERIALS AND METHODS. Bar, 10 μ m.

activation by directly ADP ribosylating arginine 201 of α_s , causes a redistribution of α_s , as shown in Figure 2. To determine whether activation of α_s causes β -AR redistribution, we examined cholera toxin treatment of HEK293 cells stably expressing both β -AR and α_s . In HEK293 cells stably transfected with both β -AR and α_s , cholera toxin also caused redistribution of HA- α_s from the plasma membrane to the cytoplasm (Figure 7, B vs. D). However, immunofluorescence indicated that β -AR remained at the plasma membrane upon cholera toxin treatment (Figure 7, A vs. C). Thus, we can separate β -AR activation and endocytosis from α_s activation and redistribution; receptor activation is not

required for α_s translocation. Moreover, α_s activation is not sufficient for β -AR internalization.

Next, we blocked the ability of agonist-activated β -AR to internalize and analyzed its effect on β -AR-mediated redistribution of α_s . Incubation of cells in hypertonic (0.4 M) sucrose inhibits receptor-mediated endocytosis by inhibiting the formation of clathrin-coated pits (Daukas and Zigmond, 1985; Heuser and Anderson, 1989). Accordingly, we incubated HEK293 cells expressing β -AR and HA- α_s in hypertonic sucrose and stimulated β -AR by the addition of isoproterenol. As reported previously (Yu *et al.*, 1993; Moore *et al.*, 1995; Pippig *et al.*, 1995), β -AR remained at the plasma membrane in either the absence or presence of agonist activation (Figure 8, A vs. C). In contrast, α_s seemed to undergo full redistribution in response to isoproterenol (Figure 8, B vs. D). Neither cholera toxin-mediated (Figure 7) nor β -AR-mediated (Figure 8) redistribution of α_s requires endocytosis of β -AR. Clearly, α_s can be activated and redistributed in response to agonist stimulation of β -AR, although β -AR remains at the plasma membrane.

DISCUSSION

Our immunofluorescent localization results confirm and extend previous cell fractionation studies (Ransnäs *et al.*, 1989; Levis and Bourne, 1992; Negishi *et al.*, 1992) suggesting that activation induces redistribution of α_s from plasma membranes to cytoplasm. α_s dramatically shifts to the cytoplasm upon activation (i.e., GTP binding) caused by β -AR agonist, a GTPase-inhibiting mutation in α_s , or cholera toxin, indicating

Figure 7. Effect of cholera toxin on redistribution of both HA- α_s and β -AR. HEK293 cells stably expressing β -AR and HA- α_s were fixed in 4% formaldehyde after no treatment (A and B) or incubation with 1 μ g/ml cholera toxin for 4 h (C and D). β -AR was detected by incubation with a polyclonal antiserum directed against the β -AR carboxyl terminus, followed by Texas Red-conjugated anti-rabbit antibodies (A and C). HA- α_s was detected by incubation with monoclonal antibody 12CA5, followed by FITC-conjugated anti-mouse antibody (B and D). Images were visualized as described under MATERIALS AND METHODS. Bar, 10 μ m.

that activated α_s reversibly undergoes a genuine translocation off the plasma membrane. Our results provide the first immunocytochemical evidence for a change in a G-protein subunit's cellular location in response to changes in its activation state.

Additionally, we found that activation-induced redistribution of α_s , even β -AR-mediated activation, does not require the redistribution of the β -AR itself. Cholera toxin caused α_s redistribution in the absence of β -AR activation and internalization (Figure 7). α_s also redistributed in response to the β -AR agonist isoproterenol during incubation of cells in hypertonic sucrose, a condition that completely abrogates internalization of activated β -AR (Figure 8).

Dual localization of β -AR and α_s allowed a comparison of the two proteins' distribution. Both receptor and G-protein α subunit reside primarily at the plasma membrane in the unactivated cell. Upon addition of isoproterenol, both β -AR and α_s redistribute to distinct cellular locations. β -AR internalizes via endocytic vesicles, as shown previously (von Zastrow and Kobilka, 1992), whereas α_s appears diffusely distributed throughout the cytoplasm. Resolution by confocal microscopy indicates very little colocalization of activated β -AR and α_s . Although we cannot rule out that fixation of cells obscures colocalization of the two activated proteins, this seems unlikely on the basis of

Figure 8. Effect of hypertonic sucrose on redistribution of both HA- α_s and β -AR. HEK293 cells stably expressing β -AR and HA- α_s were incubated at 37°C for 30 min in PBS containing 0.4 M sucrose, followed by an additional incubation in 0.4 M sucrose for 20 min in the absence (A and B) or presence (C and D) of 10 μ M isoproterenol. Cells were fixed in 4% formaldehyde and permeabilized. β -AR was detected by incubation with a polyclonal antiserum directed against the β -AR carboxyl terminus, followed by Texas Red-conjugated anti-rabbit antibodies (A and C). HA- α_s was detected by incubation with monoclonal antibody 12CA5, followed by FITC-conjugated anti-mouse antibody (B and D). Images were visualized as described under MATERIALS AND METHODS. Bar, 10 μ m.

the lack of colocalization after fixation in either formaldehyde or methanol and the fact that receptor internalization is not required for α_s redistribution (Figures 7 and 8).

This different localization upon activation of β -AR and α_s is consistent with their different proposed mechanisms of protein trafficking. Previous results indicate that β -AR internalizes via clathrin coated-pits and endocytic membrane vesicles. On the other hand, rapid depalmitoylation of α_s is the simplest mechanism to explain its redistribution. Enzymatic removal of the thioester-linked palmitate attached to α_s would release α_s from the plasma membrane by removing its hydrophobic membrane anchor. Several lines of evidence are consistent with this model. First, isoproterenol-induced depalmitoylation of α_s (Wedegaertner and Bourne, 1994) and isoproterenol-induced redistribution of α_s (Figures 3–5), as assessed by immunofluorescence localization, are similarly rapid. Second, activation of α_s by other mechanisms, either a GTPase-inhibiting mutation (R201C) or cholera toxin-mediated ADP ribosylation, causes rapid depalmitoylation of α_s (Degtyarev *et al.*, 1993; Wedegaertner and Bourne,

1994) and redistribution of α_s into the cytoplasm (Figures 1 and 2). Third, a nonpalmitoylated mutant α_s (α_s C3S) displays a cytoplasmic localization (Figures 1 and 5) identical to that of activated α_s . Depalmitoylation of α_s is most likely responsible for its release from the plasma membrane.

Both β -AR and α_s return to the plasma membrane upon removal of agonist. For β -AR, endocytic vesicles seem to recycle and fuse to the plasma membrane returning the β -AR to the cell surface. For α_s , very little is known regarding the mechanism of its recycling from the intracellular milieu to the plasma membrane. Repalmitoylation by a plasma membrane-localized palmitoyl transferase is the simplest mechanism to explain the return of α_s to the plasma membrane. Indeed, the palmitoyl transferase responsible for α_s palmitoylation may reside at the plasma membrane (Dunphy *et al.*, 1996).

A seeming paradox involving receptor-activated α_s suggests that reversible translocation of α_s may be more complex than simple diffusion on and off the plasma membrane. Although immunofluorescence localization of receptor-activated α_s suggests that a substantial amount of the protein separates from the plasma membrane, we detect only a relatively small increase in the amount of soluble α_s by cell fractionation studies (Figure 6, B and C), with the majority remaining in the particulate fraction. This is in contrast to mutationally activated α_s R201C, which is predominantly cytosolic by cell fractionation (Figure 6A; Levis and Bourne, 1992). The most obvious difference between receptor-activated and mutationally activated α_s is in the lifespan of GTP-bound α_s . Most likely, receptor-activated and redistributed α_s rapidly hydrolyzes bound GTP and is thus converted to the GDP-bound form during cell lysis. However, α_s R201C probably exists in the cell predominantly in the GTP-bound form because of its greatly decreased (100-fold) rate of GTP hydrolysis (Freissmuth and Gilman, 1989). Although the GTP-bound and depalmitoylated form of α_s may be soluble and cytoplasmic, the GDP-bound form may rapidly attach to other proteins, the most obvious of which is $\beta\gamma$ and/or intracellular membranes, rendering it insoluble by cell fractionation experiments. The fact that we could detect more soluble receptor-activated α_s upon rapid lysis in high ionic-strength buffer (Figure 6C), which would disrupt protein-protein or charged lipid-protein ionic interactions, is consistent with this inference. Conversely, this difference between immunofluorescence and fractionation studies of receptor-activated α_s may not be biologically relevant; GDP-bound α_s may nonspecifically associate with membranes or proteins in the particulate fraction upon cell lysis (i.e., an artifact of cell fractionation), or we may be overestimating the degree of α_s redistribution as visualized by immunofluorescence.

The function of activation-induced depalmitoylation and redistribution of α_s is not clear. Rapid depalmitoylation and redistribution of α_s may serve as a mechanism of signal attenuation. Removal of α_s from the plasma membrane, where receptors and adenylyl cyclase are located, may damp a G_s -mediated signaling pathway (Wedegaertner and Bourne, 1994). This possibility remains to be tested. In addition, these observed cycles of palmitoylation/depalmitoylation and subcellular redistribution may allow α_s to move to other locations in the cell where its activity is required. For example, recent studies implicate α_s in the regulation of secretory and transcytosis pathways in certain cells (Bomsel and Mostov, 1993; Pimplikar and Simons, 1993). If α_s associates with cellular membranes, in addition to the plasma membrane, it will be interesting to determine the state of palmitoylation of α_s at other membranes.

Activation-induced depalmitoylation and redistribution probably represents a general mechanism for proteins to control their activity and cellular localization. For example, a myristoylated and palmitoylated form of nitric oxide synthase, endothelial nitric oxide synthase (ecNOS), undergoes depalmitoylation and redistribution from the plasma membrane to cytosol in response to the agonist bradykinin (Robinson *et al.*, 1995). Understanding the mechanism of this translocation mechanism will provide insight into the role of reversible palmitoylation and translocation of α_s and other signaling proteins.

ACKNOWLEDGMENTS

We thank Drs. C.S. Fishburn, T. Iiri, R.W. Mays, and P.T. Wilson for useful discussions and/or critical reading of this manuscript. This work was supported by American Cancer Society Fellowship PF-3776 (P.B.W.), and National Institutes of Health grants HL-07731 (P.B.W.), GM-27800 and CA-54427 (H.R.B.), and DA-00218 (M. von Z.).

REFERENCES

- Bomsel, M., and Mostov, K.E. (1993). Possible role of both the α and $\beta\gamma$ subunits of the heterotrimeric G protein, G_s , in transcytosis of the polymeric immunoglobulin receptor. *J. Biol. Chem.* 268, 25824–25835.
- Chang, W.-J., Ying, Y.-S., Rothberg, K.G., Hooper, N.M., Turner, A.J., Gambliel, H.A., De Gunzburg, J., Mumby, S.M., and Gilman, A.G. (1994). Purification and characterization of smooth muscle cell caveolae. *J. Cell Biol.* 126, 127–138.
- Chun, M., Liyange, U.K., Lisanti, M.P., and Lodish, H.J. (1994). Signal transduction of a G-protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc. Natl. Acad. Sci. USA* 91, 11728–11732.
- Cullen, B.R. (1987). Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* 152, 684–704.
- Daukas, G., and Zigmond, S.H. (1985). Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. *J. Cell Biol.* 101, 1673–1679.

- Degtyarev, M.Y., Spiegel, A.M., and Jones, T.L.Z. (1993). Increased palmitoylation of the G_s protein α subunit after activation by the β -adrenergic receptor or cholera toxin. *J. Biol. Chem.* 268, 23769–23772.
- Dunphy, J.T., Greentree, W.K., Manahan, C.L., and Linder, M.E. (1996). G-protein palmitoyltransferase activity is enriched in plasma membranes. *J. Biol. Chem.* 271, 7154–7159.
- Freissmuth, M., and Gilman, A.G. (1989). Mutations of G_{sa} designed to alter the reactivity of the protein with bacterial toxins. Substitutions at ARG¹⁸⁷ result in loss of GTPase activity. *J. Biol. Chem.* 264, 21907–21914.
- Grady, E.F., Garland, A.M., Gamp, P.D., Lovett, M., Payan, D.G., and Bunnett, N.W. (1995). Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. *Mol. Biol. Cell* 6, 509–524.
- Hermouet, S., de Mazancourt, P., Spiegel, A.M., Farquhar, M.G., and Wilson, B.S. (1992). High-level expression of transfected G-protein α 3 subunit is required for plasma membrane targeting and adenylyl cyclase inhibition in NIH 3T3 fibroblasts. *FEBS Lett.* 312, 223–228.
- Heuser, J.E., and Anderson, R.G.W. (1989). Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell Biol.* 108, 389–400.
- Hoxie, J.A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R., and Brass, L.F. (1993). Internalization and recycling of activated thrombin receptors. *J. Biol. Chem.* 268, 13756–13763.
- Ibarrondo, J., Joubert, D., Dufour, M.N., Cohen-Solal, A., Homburger, V., Jard, S., and Guillon, G. (1995). Close association of the α subunits of G_q and G_{11} G proteins with actin filaments in WRK₁ cells: relation to G-protein-mediated phospholipase C activation. *Proc. Natl. Acad. Sci. USA* 92, 8413–8417.
- Jones, T.L.Z. (1994). Posttranslational modifications and intracellular localization of α subunits. In: *G Proteins*, ed. A.M. Spiegel, Austin, TX: R.G. Landes, 49–65.
- Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R., and Vallar, L. (1989). GTPase-inhibiting mutations activate the α chain of G_s and stimulate adenylyl cyclase in human pituitary tumours. *Nature* 340, 692–696.
- Levis, M.J., and Bourne, H.R. (1992). Activation of the α subunit of G_s in intact cells alters its abundance, rate of degradation, and membrane avidity. *J. Cell Biol.* 119, 1297–1307.
- Lisanti, M.P., Scherer, P.E., Vidurigiene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.-H., Cook, R.F., and Sargiacomo, M. (1994). Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J. Cell Biol.* 126, 111–126.
- Moore, R.H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J., Carsud, N.D.V., Dickey, B.F., and Knoll, B.J. (1995). Ligand-stimulated β_2 -adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes. *J. Cell Sci.* 108, 2983–2991.
- Mumby, S.M., Kleuss, C., and Gilman, A.G. (1994). Receptor regulation of G-protein palmitoylation. *Proc. Natl. Acad. Sci. USA* 91, 2800–2804.
- Negishi, M., Hashimoto, H., and Ichikawa, A. (1992). Translocation of α subunits of stimulatory guanine nucleotide-binding proteins through stimulation of the prostacyclin receptor in mouse mastocytoma cells. *J. Biol. Chem.* 267, 2367–2369.
- Pimplikar, S.W., and Simons, K. (1993). Regulation of apical transport in epithelial cells by a G_s class of heterotrimeric G protein. *Nature* 362, 456–458.
- Pippig, S., Andexinger, S., and Lohse, M.J. (1995). Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* 47, 666–676.
- Ransnäs, L.A., Svoboda, P., Jasper, J.R., and Insel, P.A. (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.
- Robinson, L.J., Busconi, L., and Michel, T. (1995). Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J. Biol. Chem.* 270, 995–998.
- Simonds, W.F., Goldsmith, P.K., Woodard, C.J., Unson, C.G., and Spiegel, A.M. (1989). Receptor and effector interactions of G_s . Functional studies with antibodies to the α_s carboxyl-terminal decapeptide. *FEBS Lett.* 249, 189–194.
- Stow, J.L., de Almeida, J.B., Narula, N., Holtzman, E.J., Ercolani, L., and Ausiello, D.A. (1991). A heterotrimeric G protein, $G_{\alpha_{13}}$, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK₁ epithelial cells. *J. Cell Biol.* 114, 1113–1124.
- Suryanarayana, S., von Zastrow, M., and Kobilka, B.K. (1992). Identification of intramolecular interactions in adrenergic receptors. *J. Biol. Chem.* 267, 21991–21994.
- von Zastrow, M., and Kobilka, B.K. (1992). Ligand-regulated internalization and recycling of human β_2 -adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* 267, 3530–3538.
- von Zastrow, M., and Kobilka, B.K. (1994). Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J. Biol. Chem.* 269, 18448–18452.
- von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993). Subtype-specific differences in the intracellular sorting of G-protein-coupled receptors. *J. Biol. Chem.* 268, 763–766.
- Wang, N., Yan, K., and Rasenick, M.M. (1990). Tubulin binds specifically to the signal-transducing proteins, G_{sa} and G_{ia1} . *J. Biol. Chem.* 265, 1239–1242.
- Wedegaertner, P.B., and Bourne, H.R. (1994). Activation and depalmitoylation of G_{sa} . *Cell* 77, 1063–1070.
- Wedegaertner, P.B., Chu, D.H., Wilson, P.T., Levis, M.J., and Bourne, H.R. (1993). Palmitoylation is required for signaling functions and membrane attachment of $G_{q\alpha}$ and G_{sa} . *J. Biol. Chem.* 268, 25001–25008.
- Wilson, B.S., Komuro, M., and Farquhar, M.G. (1994). Cellular variations in heterotrimeric G-protein localization and expression in rat pituitary. *Endocrinology* 134, 233–243.
- Yu, S.S., Lefkowitz, R.J., and Hausdorff, W.P. (1993). β -adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J. Biol. Chem.* 268, 337–341.
- Zheng, C.-F., and Guan, K.-L. (1994). Cytoplasmic localization of mitogen-activated protein kinase activator MEK. *J. Biol. Chem.* 269, 19947–19952.