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Persistent membrane association of activated and depalmitoylated G protein α subunits

(signal transduction/thioesterase/localization/immunofluorescence)

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ABSTRACT **Heterotrimeric signal-transducing G proteins are organized at the inner surface of the plasma membrane, where they are positioned to interact with membrane-spanning receptors and appropriate effectors. G proteins are activated when they bind GTP and inactivated when they hydrolyze the nucleotide to GDP. However, the topological fate of activated G protein α subunits is disputed. One model declares that depalmitoylation of α, which accompanies activation by a receptor, promotes release of the protein into the cytoplasm. Our data suggest that activation of G protein α subunits causes them to concentrate in subdomains of the plasma membrane but not to be released from the membrane. Furthermore, α subunits remained bound to the membrane when they were activated with guanosine 5′-(3-*O*-thio)triphosphate and depalmitoylated with an acyl protein thioesterase. Limitation of α subunits to the plasma membrane obviously restricts their mobility and may contribute to the efficiency and specificity of signaling.**

A family of heterotrimeric guanine nucleotide-binding proteins (G proteins) transduces chemical and sensory signals across the plasma membrane by sequential interactions with receptors and second messenger-generating effectors (e.g., enzymes and ion channels). These interactions result from nucleotide-driven conformational changes of G protein α subunits (1). Agonist-bound receptors catalyze the exchange of GDP for GTP on the α subunits of their cognate G proteins, and the ensuing change in the conformation of α promotes its dissociation from a high-affinity complex of β and γ subunits. These dissociated subunits are competent to modulate the activity of effectors. The intrinsic GTPase activity of α serves as a molecular clock, returning the protein to the GDP-bound state and allowing reformation of inactive heterotrimer. Members of the newly appreciated family of regulators of G protein signaling recently have been shown to stimulate the GTPase activity of certain α subunits, thus speeding termination of the signaling process (2).

G proteins are bound at the inner face of the plasma membrane, where they are positioned strategically to interact with membrane-spanning receptors and appropriate effectors. Individual cells express an imposing array of proteins that participate in G protein-mediated signaling, including a large number of receptors; a substantial variety of G protein subunits; many distinct effectors that exist in multiple isoforms; and regulators such as receptor kinases, arrestin, and regulators of G protein signaling. Organization of these signaling components at the plasma membrane may restrict access between them, on the one hand, or enhance the efficiency and speed of signal transduction on the other (3). One of the more thoroughly characterized examples of G protein-mediated signal transduction is carried out by the hormone-sensitive adenylyl cyclase system. Relevant receptors communicate with homologous G proteins, one of which (G_s) activates adenylyl cyclase, whereas the others (G_i) act as inhibitors (1). We have shown that the components of the hormone-sensitive adenylyl cyclase system are localized in subdomains of the plasma membrane (4).

The molecular basis for the interaction of G proteins with membranes is understood incompletely. All of the subunits of these proteins lack hydrophobic domains that could promote interactions with the phospholipid bilayer. However, G proteins contain several covalent lipid modifications that affect both protein–membrane and protein–protein interactions (5, 6). G protein γ subunits are prenylated (7, 8), and members of the G_i subfamily of α subunits are myristoylated (9–11). In addition, nearly all α subunits are acylated on cysteine residues near their amino termini by formation of thioesters, usually with palmitate (12–14). Prenylation and myristoylation are stable lipid modifications; the lipids remain covalently bound throughout the lifetime of a protein. By contrast, palmitoylation is reversible. Furthermore, activation of G_s by the β-adrenergic receptor is accompanied by an increase in the turnover of palmitate on the α subunit of only this G protein (15–17). Similar enhancement in palmitate turnover has been observed for the α subunit of G_q (regulator of phospholipase C-β) after stimulation of receptors for gonadotropin-releasing hormone (18). A simple hypothesis is that the activated, dissociated α subunit is a better substrate for an acyl protein thioesterase (APT1), and there is evidence for such *in vitro* (19). Activation is thus thought to cause at least a transient decrease in the stoichiometry of palmitoylation of α, although this decrease has yet to be established (20). Although there is general agreement that activation of G proteins causes an increased turnover of palmitate on α, the topological fate of these proteins is not settled. Wedegaertner and Bourne (6, 15) propose that activation and depalmitoylation of α promote its release from the plasma membrane. Our data support a different conclusion: membrane association of α persists whether the protein is activated and/or depalmitoylated.

MATERIALS AND METHODS

Nucleotides were purchased from Boehringer Mannheim. Guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) was purified by a published procedure (21), modified by replacement of DEAE with Mono Q HR 10/10 chromatography (Pharmacia). Reagents for cell culture were obtained from GIBCO/BRL.

Culture and Fractionation of Cells. MA104 cells were derived from rhesus monkey kidney (22) and used for examination of endogenous α_i by immunofluorescence, immunogold electron microscopy, and subcellular fractionation.

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Abbreviations: APT1, acyl protein thioesterase 1; GDPβS, guanosine 5′-(2-*O*-thio)diphosphate; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; HA, hemagglutinin; HEK, human embryonic kidney.

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MA104, simian COS-m6, and human embryonic kidney (HEK) 293 cells were used for transient transfections. Cells were cultured at 37°C in an atmosphere of 10% CO_2 in high glucose DMEM supplemented with 10% fetal calf serum, 5 units/ml of penicillin, and 5 μg/ml of streptomycin. Cells were fractionated as described (10), except that 250 mM sucrose was added to the homogenization buffer. Protein concentrations were determined by using the Bradford assay with BSA as the standard (23).

Antibodies, Western Blotting, and Immunoprecipitation. Affinity-purified (24) polyclonal antibodies, designated B087, were generated in rabbits against a synthetic peptide corresponding to the carboxyl-terminal 10-aa residues of α_{i1} and α_{i2} (13). The reactivity of B087 antibodies with G protein α subunits is $\alpha_{i1} = \alpha_{i2} \gg \alpha_{i3}$, α_o (4). We have established the utility of these antibodies for detection of α_i by immunocytochemical and Western blotting techniques (4). Antiserum 584 is specific for α_s (all isoforms; ref. 25). P960 antibodies are broadly reactive with a number of G protein α subunits, including α_i and α_s (25, 26). Polyclonal antibodies against the amino terminal half of human caveolin were purchased from Transduction Laboratories (Lexington, KY).

Immunoblotting of proteins, transferred from SDS/PAGE gels (27) to nitrocellulose (28), was performed with enhanced chemiluminescence reagents from Amersham; for quantification, blots were processed (24) with ^{125}I-labeled goat anti-rabbit IgG F(ab′)$_2$ from NEN. Immunoprecipitation of hemagglutinin (HA) epitope-tagged α_s (19) and endogenous α_i (29) from cells incubated with [^{3}H]palmitic acid (1 mCi/ml for 1 h) were performed as described (30). The immunoprecipitates were resolved by SDS/PAGE and transferred to nitrocellulose. Quantification of radioactive blots was performed with a phosphorimager (Fujifilm BAS-1500).

Immunocytochemistry. To obtain plasma membranes for analysis by immunofluorescence, MA104 cells adherent to coverslips coated with poly-L-lysine were sonicated with a Vibra Cell sonicator (Sonics & Materials, Danbury, CT; refs. 4, 31). In some instances, membranes were incubated for 30 min at 37°C with nucleotides in a solution of 25 mM K Hepes, 125 mM KCl, 5 mM NaCl, 5 mM magnesium acetate, 1 mM DTT, 1 μg/ml each leupeptin and lima bean trypsin inhibitor, and 1 mg/ml BSA (pH 7.5 at 20°C). Membranes were fixed with 4 or 10% paraformaldehyde with similar results. Coverslips were processed for immunofluorescence by using secondary antibodies labeled with Texas Red (Zymed) or Oregon Green (Molecular Probes, Eugene, OR; ref. 31). Membranes were viewed and photographed with a Zeiss epifluorescence photomicroscope III RS with a 100-W dc mercury lamp. For whole cell immunofluorescence, cultures of MA104 cells were fixed with 4% paraformaldehyde in PBS for 20 min and made permeable with 0.1% saponin for 5 min on ice. Whole cells were viewed and photographed with an Olympus Provis microscope (Lake Success, NY).

For immunogold electron microscopy, MA104 cell plasma membranes were isolated from the upper surface of cells by the method of Sanan and Anderson (32) with modifications as described (4). Membranes were either fixed immediately or incubated with guanine nucleotides as described above. Grids were viewed and photographed with a JEOL JEM-100CX electron microscope. Immunogold labeling with α_i antibodies yields clusters of gold particles that usually are associated with negatively stained structures on the inner surface of the plasma membrane (4). For quantification, a rectangle was drawn on each photographic print to cover the largest possible area of clearly visible plasma membrane. The total number of gold particles within the rectangle and the number of particles per cluster were counted without knowledge of the identity of the micrograph.

Expression Vectors and Transfections. Cytomegalovirus promoter-based plasmids (pCMV) for the expression of wild-type α_s or α_{i1} have been described (10). Mutant proteins are designated by the α subunit name, the wild-type amino acid residue (single letter code), the position of this residue, and the residue used for replacement. DNA encoding α_sR187C [amino acid numbering of the short isoform α_s (33) equivalent to α_sR201C of the long isoform] was excised from NpT7–5 with *Nco*I and end-filled with the Klenow fragment of DNA polymerase I before a second digestion of the vector with *Hin*dIII and gel purification. To subclone the α_sR187C DNA fragment into pCMV5, this vector was digested with *Eco*RI, end-filled, and digested with *Hin*dIII. cDNAs encoding α_{i1}Q204L and α_sQ213L in pCMV5 were supplied by Hiroshi Itoh (Tokyo Institute of Technology; ref. 34), and a cDNA encoding HA epitope-tagged wild-type α_s in pcDNAI (35) was provided by Henry Bourne (University of California, San Francisco).

Transient transfections were performed by using 1 μg/ml DNA and 10 μg/ml Lipofectamine according to the manufacturer's instructions (GIBCO/BRL). Cells were transfected when they were 60–90% confluent, and the next day they were harvested and fractionated or seeded onto coverslips (for immunofluorescence to be performed the next day). HEK 293 cells were grown on fibronectin-coated culture dishes to maintain adherence during transfection.

Depalmitoylation of G_α Proteins. Polyhistidine-tagged APT1 was purified from a 5-liter culture of *Escherichia coli* harboring the plasmid pQE60–6HAPT1 (19). Cells were harvested by centrifugation, and the pellet was frozen with liquid nitrogen. Further manipulations were performed at 4°C. The cell pellet was thawed in 250 ml of 50 mM Tris·HCl, pH 8.0, supplemented with 32 μg/ml each of L-1-tosylamido-2-phenylethyl chloromethyl ketone and 1-chloro-3-tosylamido-7-amino-2-heptanone. Lysozyme (50 mg) and DNase (50 μg) were added sequentially to the stirring suspension. After 30 min, the suspension was centrifuged at 100,000 × *g* for 30 min. APT1 was purified from the supernatant fraction by nickel nitrilotriacetic acid affinity chromatography (Qiagen, Santa Clarita, CA) on a 10-ml column. The resin was washed with 100 ml of 50 mM Tris·HCl, pH 8/10 mM imidazole/100 mM NaCl and eluted by addition of 75 ml of 50 mM Tris·HCl, pH 8/100 mM imidazole. The eluted protein was concentrated to 20 ml in a Centriprep30 (Amicon) and dialyzed twice against 2 liters of 20 mM Na Hepes, pH 8/2 mM $MgCl_2$/1 mM EDTA. The yield of essentially homogeneous APT1 was 600 mg. The activity of the recombinant enzyme was not distinguishable from that of APT1 purified from rat liver (19).

Membranes were prepared from HEK 293 cells that had been transfected to express HA-tagged α_s and incubated for 1 h in [^{3}H]palmitic acid (1 mCi/ml) to radiolabel palmitoylated proteins. Aliquots of membrane protein (100 μg suspended in a solution of 20 mM Na Hepes, pH 8/2 mM $MgCl_2$/1 mM EDTA/1 mg/ml BSA) were incubated with the indicated guanine nucleotide (100 μM) and purified APT1 for 30 min at 30°C (1 mg/ml membrane protein). To quantify depalmitoylation of α subunit substrates, a portion of each reaction mixture (65%) was solubilized and immunoprecipitated with the antibody to the HA epitope tag of α_s. The amount of tritium associated with HA-tagged α_s was quantified by phosphorimager analysis. To separate membrane-bound and soluble α subunits, the remaining portion of each reaction mixture was centrifuged at 200,000 × *g*; duplicate supernatant and pellet fractions were analyzed by Western immunoblotting.

RESULTS

Cells adherent to coverslips were sonicated to prepare fragments of plasma membrane with their inner surface exposed. These membranes are convenient for monitoring the pattern of distribution of G proteins by immunofluorescence. To compare the distribution of endogenous α_i in the basal and activated state, plasma membranes from MA104 cells were

incubated at 37°C in the presence of various nucleotides. The punctate pattern of immunofluorescence for inactive α_i was similar for membranes incubated with GDPβS (an analog of GDP that cannot be converted to GTP; Fig. 1*B*) and control membranes (fixed without 37°C incubation; Fig. 1*A*): variably sized, closely juxtaposed dots. In contrast, the immunofluorescent dots on membranes incubated with the activating nucleotide GTPγS were consistently larger, brighter, and more distant from each other. Most of the results of numerous control experiments are summarized here without data. The same activation-dependent change in the pattern of immunofluorescence was detected with two antibodies directed against different sites on α_i (polyclonal antibodies B087 and monoclonal antibody R4; ref. 36) and with plasma membranes prepared from human skin fibroblasts. Another hydrolysis-resistant analog of GTP, 5′-guanylyl imidodiphosphate, caused the same changes as did GTPγS; ATP (or hydrolysis-resistant analogs thereof), GDP, or GTP (with or without the receptor mimic mastoparan) did not. Presumably, GTP is hydrolyzed too quickly for a change in the immunofluorescent pattern of α_i to occur or be sustained. Incubation of membranes with GDP and aluminum fluoride (which also activates G protein α subunits) usually yielded a pattern similar to that caused by GTPγS. The distributions of other membrane proteins were examined to determine the specificity of the effect of GTPγS. Caveolin was chosen, because this protein occupies subdomains of the plasma membrane that are similar to those described for G proteins (in buoyant density and resistance to detergent solubilization; refs. 4, 37–39). The pattern of immunofluorescent staining of caveolin was unaltered by incubation of plasma membranes with GDPβS or GTPγS (Fig. 1 *D–F*). Similarly, we observed no nucleotide-dependent change in the characteristic distribution of clathrin (not shown). We conclude that exposure of plasma-membrane fragments to GTPγS did not cause extensive, nonspecific changes in membrane structure.

To address the possibility that GTPγS induced release of α from membranes, adherent plasma membranes were prepared by sonication but were harvested in SDS/PAGE sample buffer for analysis by Western immunoblotting. In duplicate samples, the amount of α_i recovered from membranes was not altered consistently and differentially by incubation with GDPβS or GTPγS (Fig. 1, *Upper*, lanes 1–6).

Immunogold electron microscopy was performed to determine whether exposure of isolated plasma membranes to GDPβS or GTPγS altered the ultrastructural distribution of α_i. In control experiments (MA104 cell plasma membranes were kept on ice before fixation), immunogold particles were grouped on irregularly sized and shaped structures, similar to our previous observations with fibroblasts (4); these structures remain unidentified. The groups of gold particles are presumably analogous to the punctate pattern of immunofluorescence. There was little association of α_i with either coated pits or morphologically identifiable, invaginated caveolae. Our examination of dozens of micrographs of isolated plasma membranes did not find any consistent nucleotide-dependent alteration in the morphology or positioning of immunogold-labeled structures. The number of gold particles per unit area

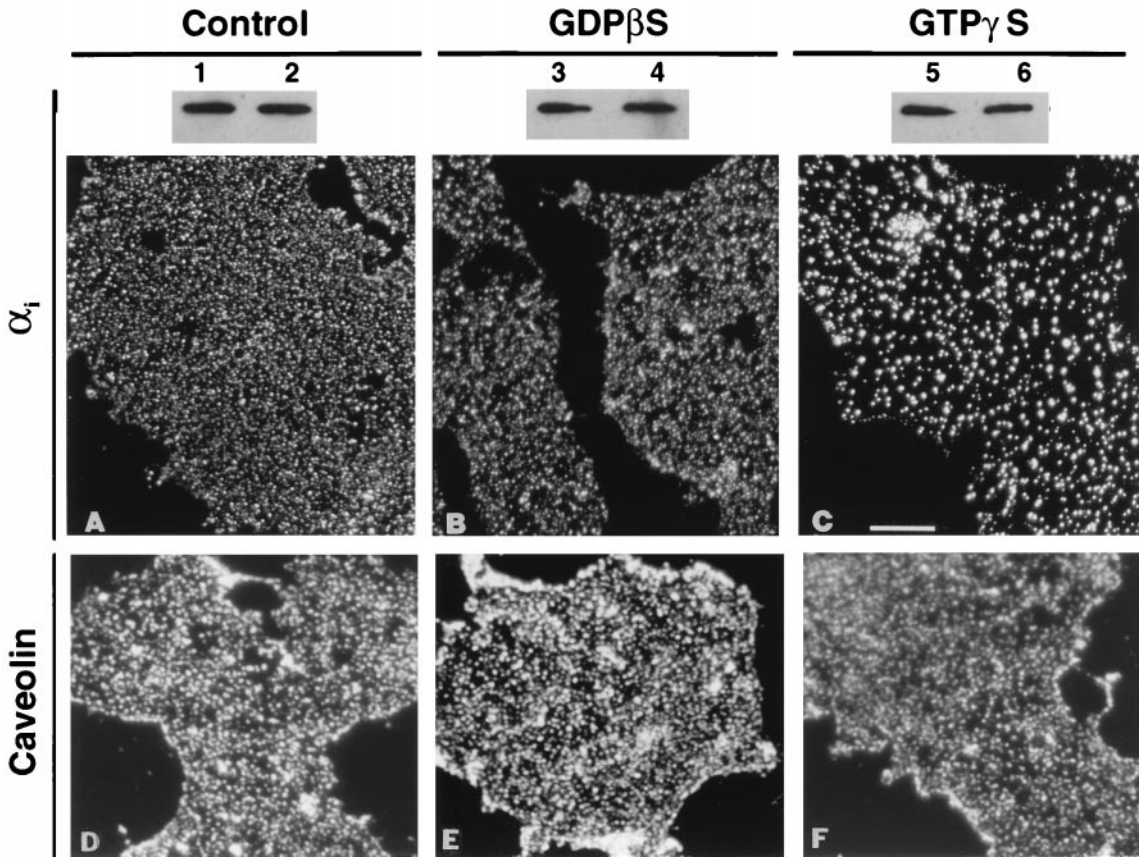

FIG. 1. GTPγS alters the immunofluorescence pattern for α_i but does not release the protein from the plasma membrane. MA104 cells were grown on coverslips and then sonicated to isolate fragments of adherent plasma membrane with their inner surface exposed. Membranes were processed for immunofluorescence (*A–F*), or duplicate samples were solubilized in SDS/PAGE sample buffer and processed for Western immunoblotting (lanes 1–6). Membranes, viewed *en face* in *A–F*, were either processed immediately (control, *A* and *D*, lanes 1 and 2) or incubated at 37°C for 30 min in the presence of 10 μM guanosine 5′-(2-*O*-thio)diphosphate (GDPβS; *B* and *E*, lanes 3 and 4) or GTPγS (*C* and *F*, lanes 5 and 6) before processing. Affinity-purified B087 antibodies (specific for α_i) were used at a concentration of 10 μg/ml for immunofluorescence (*A–C*) and 50 ng/ml for immunoblotting (lanes 1–6). Antibodies against caveolin were diluted to 1 μg/ml (*D–F*). Texas Red conjugated to goat anti-rabbit IgG (20 μg/ml) was used as the secondary antibody in *A–F*. (Bar = 4 μm.)

of plasma membrane was about 40% higher for membranes incubated with GTPγS, suggesting that the antibodies react somewhat better with α_i fixed in the activated state *in situ* (Table 1). On average, there were about twice as many gold particles per group on membranes incubated with GTPγS as there were on those incubated with GDPβS, suggesting that α_i concentrated into fewer sites (Table 1). These differences in the number of gold particles per group and per μm^2 (GDPβS vs. GTPγS) were significant, as analyzed by Tukey's test ($P < 0.05$). The increases in the amount and concentration of immunogold labeling of GTPγS-treated membranes are consistent with the altered pattern of immunofluorescence (Fig. 1).

In additional experiments, we isolated plasma membranes on Percoll gradients, incubated them with guanine nucleotide, and fractionated them on Optiprep gradients to determine whether GDPβS- and GTPγS-bound α_i (or α_s) occupied different subdomains that could be resolved by density gradient centrifugation (39); they could not. The data are not shown, because they are similar to those we reported for Madin–Darby canine kidney cells (4). Any rearrangement of endogenous α_i, caused by GTPγS, seems to be a modest concentration of the protein into fewer sites that are similar in morphology and buoyant density to those occupied by inactive α_i. We found no evidence for a substantial GTPγS-induced release of α_i or α_s from plasma membranes of MA104 cells.

As an alternative approach, cells were transfected to express wild-type α subunits or mutant proteins that are constitutively active because of loss of GTPase activity (α_{i1}Q204L, α_sQ213L, or α_sR187C). Postnuclear supernatant fractions from transfected COS cells were fractionated into membrane-containing pellet and cytosolic fractions by centrifugation at 200,000 × *g*. The amount of constitutively active α_i or α_s in the cytosolic fraction was similar to (or less than) the amount of wild-type protein in this fraction (Fig. 2*A*). The small amount of wild-type α in the supernatant fraction of COS cells is caused by overexpression (endogenous α subunits in untransfected cells are completely membrane-associated). To rule out misfolding and aggregation as a basis for insolubility of constitutively active α subunits, we attempted to extract the wild-type and mutant proteins from the pellet fractions with sodium cholate. Wild-type and constitutively active proteins were solubilized to a similar extent (≥50%, not shown). Localization of constitutively active mutant proteins was examined further by immunofluorescence of COS (not shown) and MA104 cells. The cells overexpressing wild-type or constitutively active α_i displayed similar immunofluorescence patterns similar to one another (Fig. 2 *B* and *C*) and to the less intense pattern for endogenous α_i (not shown). The predominant pattern of immunofluorescence consisted of closely juxtaposed dots and brighter staining of the cell periphery, consistent with labeling

Table 1. Quantification of immunogold-labeling of α_i on plasma membranes

Sample*	Number of micrographs†	Gold particles per group‡	Gold particles per μm^2
Control	19	3.4 ± 0.4	50 ± 7.1
GDPβS	22	4.7 ± 0.5	61 ± 8.1
GTPγS	24	8.7 ± 0.9	87 ± 7.1

*Samples are plasma membranes torn from the upper surface of MA104 cells. Controls were fixed immediately, whereas GDPβS and GTPγS samples were incubated with 10 μM nucleotide at 37°C for 30 min before fixation and processing for immunogold labeling with affinity-purified B087 antibodies.

†Micrographs were from three independent experiments; each included all three samples.

‡Gold particles were grouped on stained structures of variable shape and size (largest dimension, 20–600 nm) on the inner surface of plasma membranes; see ref. 4.

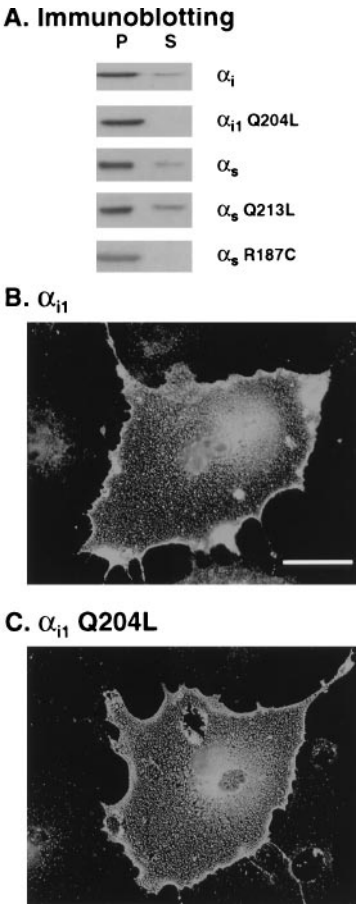

FIG. 2. Constitutively active α subunits are associated with membranes. Cells were transiently transfected to express wild-type or mutant α subunits, as indicated. (*A*) Approximately 20% of the membrane pellet (20 μg, P) and soluble cytosolic (10 μg, S) fractions from transfected COS cells were analyzed by Western immunoblotting with B087 antiserum (1:10,000 dilution) to detect α_i or with affinity-purified 584 antibodies (250 ng/ml) to detect α_s. (*B* and *C*) Transfected MA104 cells were processed for immunofluorescence with affinity-purified B087 antibodies (10 μg/ml) and Oregon Green-conjugated goat anti-rabbit IgG (15 μg/ml). (Bar = 5 μm.)

of plasma membrane. The intensity and resolution of dots varied from cell to cell, apparently caused by variable levels of expression. This variability may explain why we failed to discern any consistent difference in the pattern of dots between wild-type and constitutively active proteins, as we had anticipated from the results shown in Fig. 1 *B* and *C*. The hazy staining near the nucleus represents (out of focus) labeling of what appear to be intracellular membranes. These patterns of immunofluorescence for whole cells are not consistent with a cytosolic localization of activated α_{i1}. The patterns of immunofluorescence for wild-type and α_s Q213L were also indistinguishable from each other and were similar to that for α_i (not shown).

Based on studies of mutant forms of HA-tagged α_s, Wedegaertner and Bourne (6, 15) proposed that depalmitoylation of α_s, which accompanies activation, permits dissociation of this protein from the plasma membrane. A drawback of the mutational approach to studying palmitoylation is that observed changes may result from the loss of the cysteine residue, the fatty acid, or both. As an alternative, we employed purified APT1 to remove palmitate from HA-tagged α_s expressed in 293 cell membranes. APT1 removed up to 50% of [^{3}H]palmitate from HA-tagged α_s (in the presence of GDPβS or GTPγS, Fig. 3*A*), but exposure to the enzyme did not alter the extent of association of HA-tagged α_s or endogenous α_i with mem-

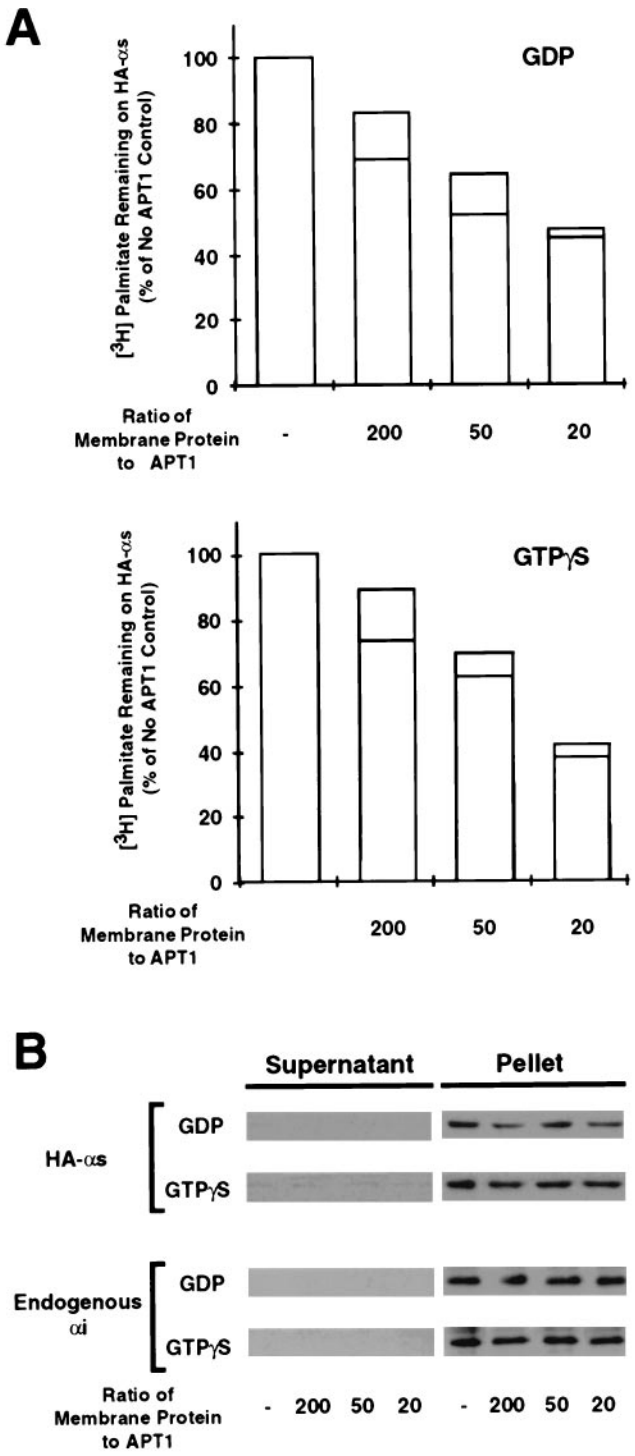

FIG. 3. G protein α subunits remain membrane-associated after depalmitoylation by APT1. HEK 293 cells were transfected to express HA-tagged α_s, incubated with [^{3}H]palmitic acid to radiolabel palmitoylated proteins, and fractionated to isolate membranes. Aliquots of membranes were incubated with the indicated guanine nucleotide and purified APT1 for 30 min at 30°C. Samples from each incubation were divided for analysis of depalmitoylation or release of α subunits from the membranes. (*A*) To detect depalmitoylation by APT1, a portion of each sample was solubilized and subjected to immunoprecipitation with the antibody to the HA tag of α_s. The amount of tritium associated with HA-tagged α_s (HA-α_s) after exposure to APT1 is calculated relative to the control sample that was not exposed to APT1. The upper bar graph represents data from samples containing GDP, and the lower bar graph represents those containing GTPγS. The horizontal lines of the bars represent the individual results of single immunoprecipitations divided in two and run on separate gels. (*B*) Membrane-bound and released α subunits (from a portion of each sample) were separated by centrifugation at 200,000 × *g*. The supernatant and pellet fractions were analyzed by Western immunoblotting for HA-tagged α_s and endogenous α_i with affinity-purified P960 antibodies (0.8 μg/ml). Detection of endogenous α_i required the blot to be exposed to film longer than was necessary for detection of overexpressed HA-tagged α_s.

branes (Fig. 3*B*). Incomplete depalmitoylation may be explained (at least in part) by access of APT1 to only those α subunits exposed on the outer surface of membrane vesicles. More than 85% of the tritium label was removed from HA-tagged α_s when detergent was included in the reaction (data not shown).

DISCUSSION

Current data suggest the need for nonrandom, functional organization of signaling components at the plasma membrane to achieve the requisite speed, magnitude of response, functional specificity, and regulation that characterize G protein-mediated signaling systems (3, 40). We have shown that the components of the hormone-sensitive adenylyl cyclase system are localized in subdomains of the plasma membrane and have suggested that this localization is a reflection of the functional organization of the system (4). Although contrary to some reports, the data presented herein indicate a persistent, membrane-delimited localization of α_s and α_i throughout the cycle of G protein activation, depalmitoylation, and return to the heterotrimeric basal state. Sustained association with the membrane limits the range of translocation of G proteins, and we anticipate that this limitation facilitates preservation of the organization necessary for fidelity and efficiency of signaling.

We have been unable to observe substantial release of G protein α subunits from membranes exposed to GTPγS, 5′-guanylyl imidodiphosphate, or receptor agonist [despite great effort to replicate published experimental conditions (41, 42) precisely with the same cell types, data not shown]. Ransnas and Insel (41) reported that incubation of S49 cell membranes in the presence or absence of GDP resulted in release of a remarkable 30% of the total (inactive) α_s into the supernatant fraction. They found that incubation of these membranes with either GTPγS or a β-adrenergic receptor agonist, isoproterenol (to activate G_s), increased the release of α_s 3-fold. We did not detect release of α_s from S49 cell membranes incubated with any of the various nucleotides or isoproterenol, although we could have detected release of as little as 10% of α_s, had it occurred. We are confident that G_s is activated under these conditions, because we routinely detect GTPγS- or isoproterenol-stimulated adenylyl cyclase activity in membrane preparations from S49 cells (4, 43). In agreement with our results, Jones *et al.* (20) observed sustained membrane association of α_s in S49 cells exposed to isoproterenol. In a complementary approach, we compared the subcellular distribution of constitutively active mutant forms of α_i and α_s with their wild-type counterparts (Fig. 2). We found no obvious differences in fractionation or whole cell immunofluorescence. Membrane association of constitutively active α subunits has been confirmed by several groups of investigators (44–49), consistent with our failure to observe substantial release of α subunits after exposure to GTPγS. The Bourne group (15, 50), on the other hand, described a cytosolic residence for a single constitutively active mutant form of α_s.

There is general agreement that depalmitoylation of α_s is regulated by receptors (15–18). Bourne and colleagues (6, 15) proposed that depalmitoylation triggers release of α_s from the plasma membrane. This hypothesis stems, in part, from their observation that mutation of palmitoylated cysteine residues of α_s and α_q caused localization of these α subunits in the soluble fraction of transfected cells (35). In contrast, Degt-

yarev *et al.* (44), Hepler *et al.* (51), Edgerton *et al.* (52), and we (17) found little, if any, difference in the subcellular distribution of palmitoylation site mutants of α_s or α_q compared with wild-type proteins when they were expressed in HEK 293, COS, or Sf9 cells. Moreover, when we depalmitoylated HA-tagged α_s with APT1, the protein was not released from membranes, even when GTPγS was also present (Fig. 3). Although removal of palmitate may decrease the avidity of α subunits for the membrane somewhat, the bulk of the available data does not support the notion of substantial release of unpalmitoylated α_s or α_q from the membrane.

Immunofluorescence studies indicate that nonpalmitoylated cysteine (Cys-3) mutants of α_i subfamily members (ref. 53 and unpublished data) and the Src family tyrosine kinase $p59^{fyn}$ (54) are found predominantly on intracellular membranes. It is not known whether the lack of palmitate or alteration of the cysteine residue prevents these proteins from attaining or maintaining their proper association with the plasma membrane. The capacity of α_q to stimulate phospholipase C-β is an example of the importance of amino-terminal cysteine residues themselves, rather than their acylation by palmitate (51). When α subunits are depalmitoylated, as they are by APT1, our data suggest that they are retained at the membrane by interactions that do not require palmitate. Rather than dictating plasma membrane association, palmitoylation may serve to regulate interactions of α with other proteins, perhaps by promoting interactions with $\beta\gamma$ (55), decreasing affinity for regulators of G protein signaling (56), and modulating other interactions that are yet to be appreciated.

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