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Reciprocal regulation of $G_{s\alpha}$ by palmitate and the $\beta\gamma$ subunit

(trimeric G proteins/lipid modification/membrane localization/protein–protein interaction)

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ABSTRACT **Hormonal activation of G_s, the stimulatory regulator of adenylyl cyclase, promotes dissociation of α_s from $G\beta\gamma$, accelerates removal of covalently attached palmitate from the $G\alpha$ subunit, and triggers release of a fraction of α_s from the plasma membrane into the cytosol. To elucidate relations among these three events, we assessed biochemical effects *in vitro* of attached palmitate on recombinant α_s prepared from Sf9 cells. In comparison to the unpalmitoylated protein (obtained from cytosol of Sf9 cells, treated with a palmitoyl esterase, or expressed as a mutant protein lacking the site for palmitoylation), palmitoylated α_s (from Sf9 membranes, 50% palmitoylated) was more hydrophobic, as indicated by partitioning into TX-114, and bound $\beta\gamma$ with 5-fold higher affinity. $\beta\gamma$ protected GDP-bound α_s, but not α_s·GTP[γS], from depalmitoylation by a recombinant esterase. We conclude that $\beta\gamma$ binding and palmitoylation reciprocally potentiate each other in promoting membrane attachment of α_s and that dissociation of α_s·GTP from $\beta\gamma$ is likely to mediate receptor-induced α_s depalmitoylation and translocation of the protein to cytosol in intact cells.**

Heterotrimeric ($\alpha\beta\gamma$) G proteins act as molecular switches to relay information from activated receptors to appropriate effector proteins (1, 2). Receptor-catalyzed replacement of bound GDP by GTP and GTP hydrolysis by the $G\alpha$ subunit respectively turn on and turn off G protein-mediated signals. $G\alpha$·GDP binds to the $\beta\gamma$ subunit and is inactive, while $G\alpha$·GTP dissociates from $G\beta\gamma$, allowing both subunits to regulate effectors.

Efficient signaling requires location of G protein subunits at the cytoplasmic face of the plasma membrane, where they can interact with receptors and effectors. Three classes of lipid modification mediate and regulate locations of G protein subunits at the plasma membrane (3–5). G protein α subunits are covalently modified at or near their N termini by the fatty acids myristate and/or palmitate, while γ subunits are prenylated at their C termini. While myristoylation and isoprenylation are irreversible modifications, palmitoylation is reversible and subject to dynamic regulation in intact cells (3). The α subunit (α_s) of G_s, the stimulatory regulator of adenylyl cyclase, is palmitoylated but not myristoylated (6). We have identified an α_s palmitoylation cycle in intact cells (3, 7), in which hormone receptors appear to regulate membrane vs. cytosolic localization of α_s by controlling its depalmitoylation by cellular esterase(s). Two sets of observations support this interpretation. An α_s mutation (α_s-C3S) replacing the amino acid attachment site of palmitate prevented membrane localization of α_s and abrogated its ability to mediate hormonal regulation of adenylyl cyclase (8). In addition, G_s activation by stimulation of β-adrenoreceptors promoted depalmitoylation of normal α_s (7, 9) and effected transfer of a fraction of α_s molecules into cytosol (10–12).

Recent evidence from several laboratories (5, 7, 9, 13), leads us to propose the following biochemical mechanism to explain the receptor-triggered palmitoylation cycle of α_s: In this mechanism palmitate plays a dual role, increasing both the hydrophobicity of α_s and its affinity for binding $\beta\gamma$. GTP-induced dissociation of $\beta\gamma$ increases susceptibility of the α subunit to depalmitoylation; the resulting loss of palmitate decreases intrinsic avidity of α_s for the lipid bilayer, promoting its transfer to cytosol. Studies of other G proteins are in keeping with this hypothesis. In all α subunits of the α_i family (except α_t), covalently attached palmitate and myristate together play this dual role, although one or the other lipid may be selectively responsible for membrane attachment (9, 14–16) or for $\alpha/\beta\gamma$ interaction (17–19). Prenylation of the γ polypeptide similarly increases both the hydrophobicity and the affinity of $\beta\gamma$ for binding $G\alpha$ (20, 21). Biochemical and genetic experiments point to a key role in $\alpha/\beta\gamma$ interaction for the N termini of $G\alpha$ subunits (22, 23), to which myristate and/or palmitate are attached. Moreover, crystal structures of G protein trimers show $\beta\gamma$ associated with two $G\alpha$ surfaces, of which one is the N terminal α-helix of $G\alpha$ (24, 25). The likely proximity of myristate and/or palmitate on the $G\alpha$ N terminus to the prenyl group at the $G\gamma$ C terminus and the proximity of both to the proposed location of the plasma membrane (24, 25) are striking. Together, these findings point to N termini of α subunits as potential physical interaction sites for the proposed cooperation between $\beta\gamma$ and lipids, both in attaching $G\alpha$ to the plasma membrane and also in $\beta\gamma$-mediated prevention of α_s from depalmitoylation in intact cells.

To explore the biochemical basis of a mechanism linking membrane attachment, $\beta\gamma$ affinity, and the palmitoylation cycle of α_s, we compared hydrophobicity and $\beta\gamma$ binding affinity of palmitoylated vs. unpalmitoylated recombinant α_s and assessed the effect of $\beta\gamma$ on depalmitoylation of α_s. Our findings provide a clearer picture of mechanism of the palmitoylation cycle and pose questions regarding the potential role(s) of palmitate attached to other subunits.

MATERIALS AND METHODS

Purification of [^{3}H]Palmitoylated α_s and $\beta_1\gamma_2$. Purification of α_s was performed using hexahistidine (H_6)-tagged $\beta\gamma$ subunit as described (13) with modifications. Sf9 cells (1.5×10^6 cells per ml), maintained in Sf-900II medium at 28°C, were infected with baculoviruses encoding α_s, β_1, and H_6-γ_2 (1 plaque-forming unit per cell for each virus) and propagated for

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Abbreviations: WT, wild type; GTP[γS], guanosine 5′[γ-thio]triphosphate.

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48 h at 28°C. To produce [^{3}H]palmitoylated α_s, infected cells were incubated at 10^7 cells per ml for an additional 2 h in medium containing 5 mM sodium pyruvate and 0.25 mCi/ml [9,10-^{3}H]palmitic acid. Cells were lysed by nitrogen cavitation; membrane fractions were prepared (26). After membrane fractions were extracted with 0.6% (wt/vol) $C_{12}E_{10}$ (polyoxyethylene 10-lauryl ether, the principal component of Lubrol), supernatant fractions were applied to a column of HiTrap Chelating (Pharmacia; 5 ml bed volume) charged with Ni^{2+} and equilibrated with buffer A [20 mM Tris·HCl, pH 7.8/100 mM NaCl/1 mM $MgCl_2$/10 μM GDP/1 mM 2-mercaptoethanol/10 mM imidazole/protease inhibitors (27)/0.5% $C_{12}E_{10}$]. The column was washed with 100 ml of buffer A containing 300 mM NaCl, warmed to room temperature, washed with 15 ml buffer A containing 100 mM NaCl, and then eluted with 30 ml of buffer A containing 1% sodium cholate, 50 mM $MgCl_2$, 30 μM $AlCl_3$, and 10 mM NaF. Finally, for elution of $\beta\gamma$ subunits, the column was washed with 20 ml of buffer A containing 300 mM imidazole and 0.7% 3-[(3-cholamindopropyl)dimethylammonio]-1-propanesulfonate. Peak fractions containing α_s [AlF_4^- eluent for α_s-wild type (WT) and first 33 ml of high salt wash for α_s-C3S] were further chromatographed on a column of Mono Q (Pharmacia; 1-ml bed volume) (28). Fractions were subjected to SDS/PAGE and visualized either by Western blot analysis with anti-α_s antibody (10) or by fluorography (7). $\beta_1\gamma_2$ was purified using H_6-α_{i1}, as described (13).

Purification of Cytosolic α_s. To purify α_s from cytosol, Sf9 cells were infected with baculovirus encoding α_s without β and γ subunit. After cell lysis the supernatant fraction was sequentially chromatographed on columns of HiTrap Q (Pharmacia; 5-ml bed volume × 2) (27), Econo-Pac HTP (Bio-Rad; 5-ml bed volume) with potassium phosphate gradient (29), and Mono Q (1-ml bed volume) (28) in the absence of detergents.

Separation of Palmitoylated and Unpalmitoylated α_s by HPLC. The samples were applied to a C_4 reverse-phase column, Synchropak RP-4 (250 × 4.6 mm i.d., Synchrom, Lafayette, IN), equilibrated with 0.1% trifluoroacetic acid and 45% CH_3CN at 30°C. The column was eluted at a flow rate of 1.0 ml/min with a linear gradient of CH_3CN (45–60%) in 30 min. Fractions (0.5 ml) were assayed for radioactivity and subjected to SDS/PAGE and silver staining.

Triton X-114 Phase Partitioning. Partitioning of protein samples between aqueous and detergent-rich phases using TX-114 was performed as described (30, 31). Equal volumes of normalized fractions of either water phase (W) or detergent phase (D) were subjected to SDS/PAGE and visualized by Western blot analysis with anti-α_s or anti β antibody or by fluorography.

Guanosine 5′-[γ-thio]Triphosphate (GTP[γS]) Binding and GTPase Assays. GTP[γS] binding or GTPase was quantitated as described (27, 28). Apparent on rates (k_{app}) of GTP[γS] binding were determined as described (29).

Depalmitoylation Assay. Depalmitoylation assays were performed as described (32) with modifications. Samples were incubated at 22°C with palmitoyl esterase (a generous gift from Sandra L. Hofmann, University of Texas Southwestern Medical Center, Dallas) in buffer B (50 mM Tris·HCl, pH 7.8/50 mM NaCl/5 mM $MgCl_2$/1 mM EDTA/1 mM 2-mercaptoethanol/10 μM GDP) in the presence of 0.44% octyl glucoside (32). When the rate of depalmitoylation was determined, the reaction was done in the presence of 0.025% $C_{12}E_{10}$. The [^{3}H]palmitate released into the supernatant was quantitated (32). [^{3}H]Palmitate covalently bound to proteins was analyzed by SDS/PAGE and fluorography.

RESULTS

Purification of Palmitoylated α_s. We infected Sf9 cells with baculoviruses expressing α_s-WT or a palmitoylation site mutant (α_s-C3S) (8), with or without viruses expressing β_1 and a hexahistidine-tagged γ_2 subunit (H_6-γ_2) (13). In the absence of coexpressed $\beta\gamma$, ≈50% of the α_s-WT or α_s-C3S was found α_s-WT in the supernatant fraction (cytosolic α_s). Although the cytosolic α_s in such extracts was not aggregated, α_s in the particulate fraction of cells without coexpressed $\beta\gamma$ was probably denatured and aggregated, as indicated by resistance to extraction with 1% sodium cholate or 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. In contrast, coexpression of $\beta\gamma$ targeted functional α_s-WT and α_s-C3S (easily solubilized in detergent and capable of binding radioactive GTP[γS]) to membranes. Indeed, both α_s-WT and α_s-C3S were located predominantly in the particulate fraction when coexpressed with $\beta\gamma$ (results not shown). [In situations where recombinant Gα subunits are overexpressed, as in Sf9 cells and in transiently transfected mammalian cells, it is important to determine whether such proteins in particulate fractions are functionally intact, because particulate fractions from such cells often contain nonfunctional G protein subunits that are denatured and aggregated. In our experiments, particulate α_s was functionally intact when we coexpressed $\beta\gamma$ with α_s.] Hereafter termed membrane-derived α_s, these proteins were easily solubilized with detergents and served as sources

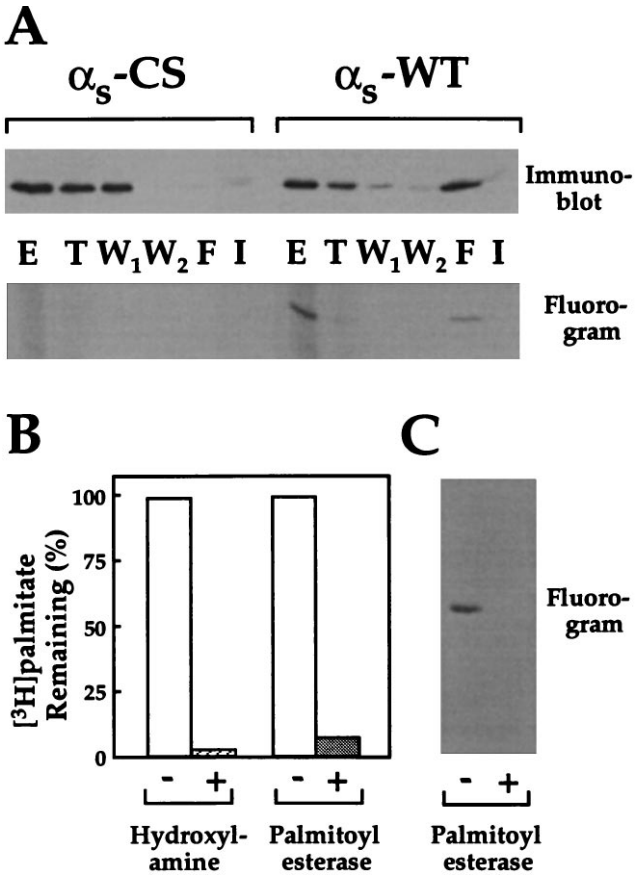

FIG. 1. Purification of palmitoylated α_s and removal of palmitate. (*A*) Sf9 cells infected with baculoviruses encoding β_1 and H_6-γ_2 plus either α_s-WT or α_s-C3S were incubated with [^{3}H]palmitate and lysed. Detergent extracts (in the nonionic detergent $C_{12}E_{10}$) from particulate fractions were chromatographed on a Ni^{2+} column as described. Aliquots of the initial detergent extract (E), flow through (T), washes with high and low salt (W_1, first 33 ml, and W_2), AlF_4^- eluent (F), and imidazole eluent (I) were subjected to SDS/PAGE and immunoblotted with anti-α_s antibody or visualized by fluorography. (*B* and *C*) Purified [^{3}H]palmitoylated α_s-WT (100 nM, 210 cpm/pmol) was incubated at 25°C for 7 h with 1 M Tris·HCl (pH 7.0) or 1 M hydroxylamine (pH 7.0) or was treated with palmitoyl esterase (0.7 μg/ml) for 50 min. Released radioactivity was determined, and the remaining protein-bound radioactivity was calculated (*B*) or visualized by SDS/PAGE followed by fluorography (*C*).

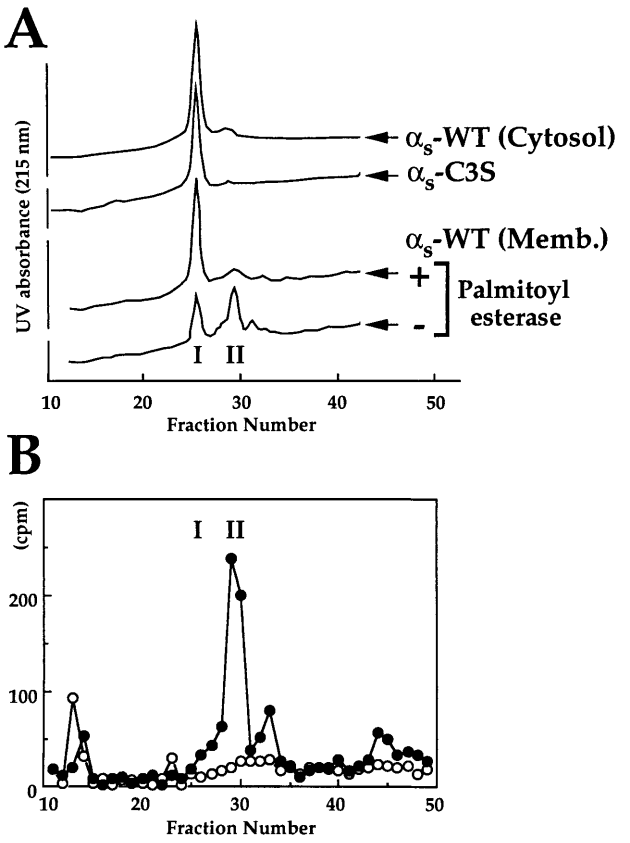

FIG. 2. Separation of palmitoylated vs. unpalmitoylated α_s by reverse-phase chromatography. (*A*) Ten picomoles of cytosolic α_s-WT, α_s-C3S, and membrane-derived α_s-WT (purified [^{3}H]palmitoylated α_s) were chromatographed on a C_4 HPLC reverse-phase column. (*B*) Radioactivity in column fractions after chromatography (as in *A*) of purified [^{3}H]palmitoylated α_s-WT incubated with (○, 10 pmol of total protein) or without (●, 5 pmol of total protein) palmitoyl esterase.

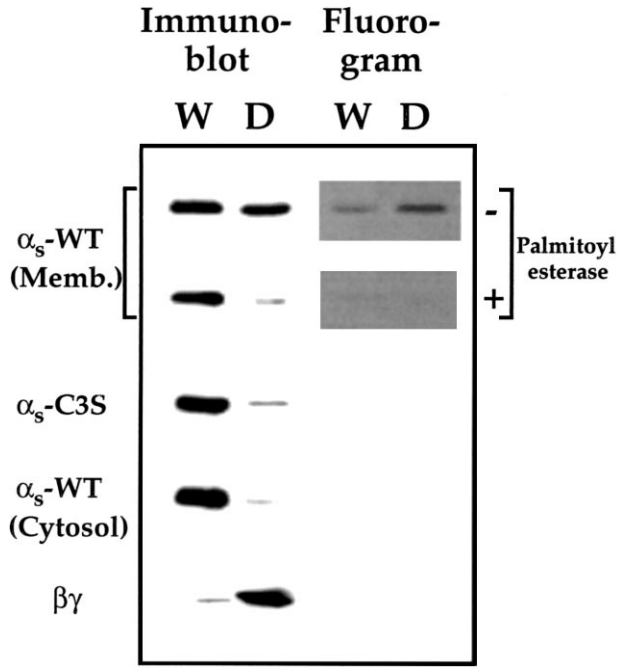

FIG. 3. Triton X-114 phase partitioning to assess the hydrophobicity of palmitoylated and unpalmitoylated α_s. Membrane-derived palmitoylated α_s-WT incubated without or with palmitoyl esterase, α_s-C3S, cytosol-derived α_s-WT, or $\beta\gamma$ subunit (each at 50 nM) were subjected to TX-114 phase partitioning as described. Equal volumes of normalized fractions of either water phase (W) or detergent phase (D) were subjected to SDS/PAGE and visualized either by immunoblotting with anti-α_s or anti-β antibody or by fluorography.

for purified α_s-C3S and palmitoylated α_s-WT proteins studied in this paper.

Purification of α_s from Sf9 cells coexpressing α_s, β_1, and H_6-γ_2 provided a strong indication that palmitoylated α_s binds more tightly to the $\beta\gamma$ subunit than does unpalmitoylated α_s (Fig. 1). $\alpha_s\beta\gamma$ heterotrimers in detergent extracts of membranes were first applied to a Ni^{2+} column. [^{3}H]palmitoylated α_s-WT (from Sf9 cells incubated with [^{3}H]palmitate; see *Materials and Methods*) was selectively retained on the column and could be eluted with AlF_4^-. α_s-C3S [or unpalmitoylated α_s-WT molecules, possibly depalmitoylated during preparation by contaminating palmitoyl esterases (5)] were retained less tightly on the Ni^{2+} column, apparently because of their lower affinity for $\beta\gamma$ (see below); these were eluted in the first washing step (Fig. 1*A*). Radioactivity incorporated into the protein was shown to be linked to α_s-WT by a thioester linkage, because it was quantitatively removed by incubation with either hydroxylamine or palmitoyl esterase (Fig. 1 *B* and *C*).

α_s-WT from the AlF_4^- elution and αs-C3S in the first wash fraction were further purified by passage over a Mono-Q column, as described in *Materials and Methods*.

Separation of Palmitoylated vs. Unpalmitoylated α_s. Palmitoylated and unpalmitoylated α_s migrated separately on a reverse phase C_4 HPLC column (Fig. 2), in a fashion consistent with their presumed difference in hydrophobicity. α_s-WT eluted from the column in two reproducible peaks (Fig. 2*A*, peaks I and II). [^{3}H]Palmitate-labeled α_s-WT eluted in peak II (at a higher concentration of CH_3CN), while unpalmitoylated α_s-WT (lacking [^{3}H]palmitate), α_s-WT derived from Sf9 cytosol, and α_s-C3S all migrated in peak I (Fig. 2 *A* and *B*). Relative sizes of peaks I and II from α_s-WT preparations indicated that ≈50% of α_s-WT derived from Sf9 membranes is linked to palmitate. (Note: SDS/PAGE of peaks I and II revealed, in silver-stained gels, one protein at the size expected for α_s.)

Incubation of membrane-derived α_s-WT with palmitoyl esterase caused disappearance of the [^{3}H]palmitoylated protein in peak II (Fig. 2*B*, closed vs. open symbols) and a corresponding increase in (unpalmitoylated) protein migrating in peak I (Fig. 2*A*).

Palmitoylation Increases Hydrophobicity of α_s and Its Affinity for $\beta\gamma$. The idea that palmitoylation enhances hydrophobicity of α_s is in accord with localization of α_s-C3S in cytosol of intact cells and the migration of α_s-WT from membrane to cytosol that accompanies activation-induced depalmitoylation (7, 8, 12). Selective partitioning of membrane-derived α_s-WT in TX-114 confirms this suggestion (Fig. 3). Most (77%) of the [^{3}H]palmitate linked to α_s-WT partitioned into the detergent phase, while α_s-C3S, cytosol-derived α_s-WT, and palmitoyl esterase-treated α_s-WT partitioned almost exclusively into the water phase. In agreement with the 50% stoichiometry of palmitoylation in membrane-derived α_s-WT (Fig. 2), total α_s-WT protein partitioned 60% into water and 40% into detergent, as assessed by densitometry of immunoblotted protein (Fig. 3). In keeping with its hydrophobicity, prenylated $\beta\gamma$ partitioned exclusively into detergent (Fig. 3), while a water-soluble control protein, bovine serum albumin, partitioned exclusively into water (not shown).

Because our purification procedure suggested that palmitoylated α_s binds $\beta\gamma$ more tightly than does unpalmitoylated α_s (Fig. 1), we directly assessed the effect of palmitoylation on the interaction of α_s with $\beta\gamma$. $\beta\gamma$ slows binding of GTP to α_s, apparently by inhibiting dissociation of bound GDP (29). We therefore measured the apparent rate of association of GTP[γ^{35}S] with α_s in the presence of different concentrations

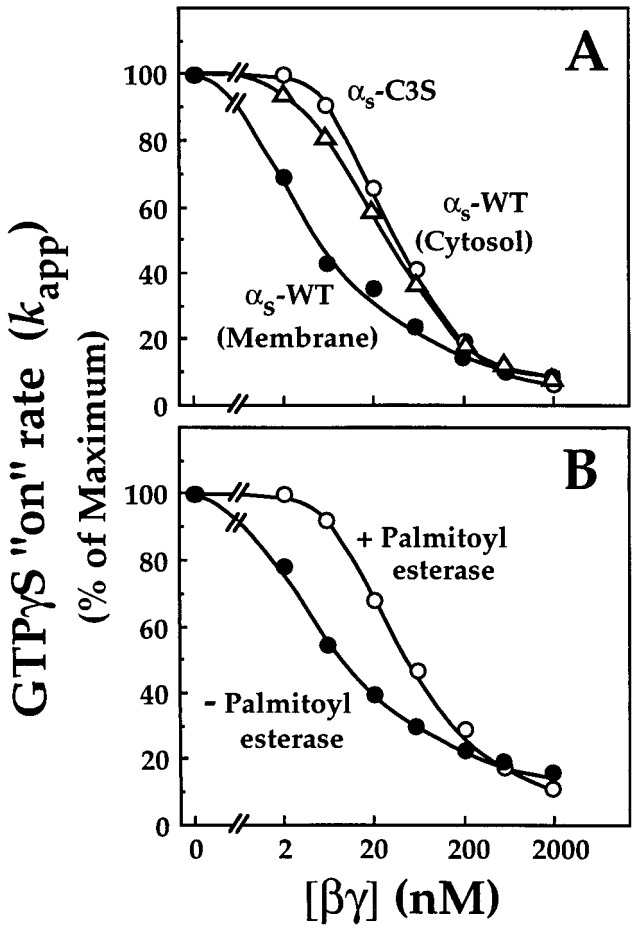

FIG. 4. Effect of $\beta\gamma$ subunit on rate of association of GTP[γS] with palmitoylated and unpalmitoylated α_s. Recombinant $\beta\gamma$ at the indicated concentrations was incubated with (*A*) membrane-derived (palmitoylated) α_s-WT (●), α_s-C3S (○), or cytosol-derived α_s-WT (△) or (*B*) with membrane-derived (palmitoylated) α_s previously incubated in the absence (●) or presence (○) of palmitoyl esterase; in each case the concentration of α_s was 10 nM. Each α_s preparation was incubated at 22°C with 1 μM GTP[γ^{35}S] (10^5 cpm/pmol) and apparent on-rates of GTP[γS] binding (k_{app}) were calculated as described. Boiled $\beta\gamma$ (2 μM) did not produce any inhibition (not shown).

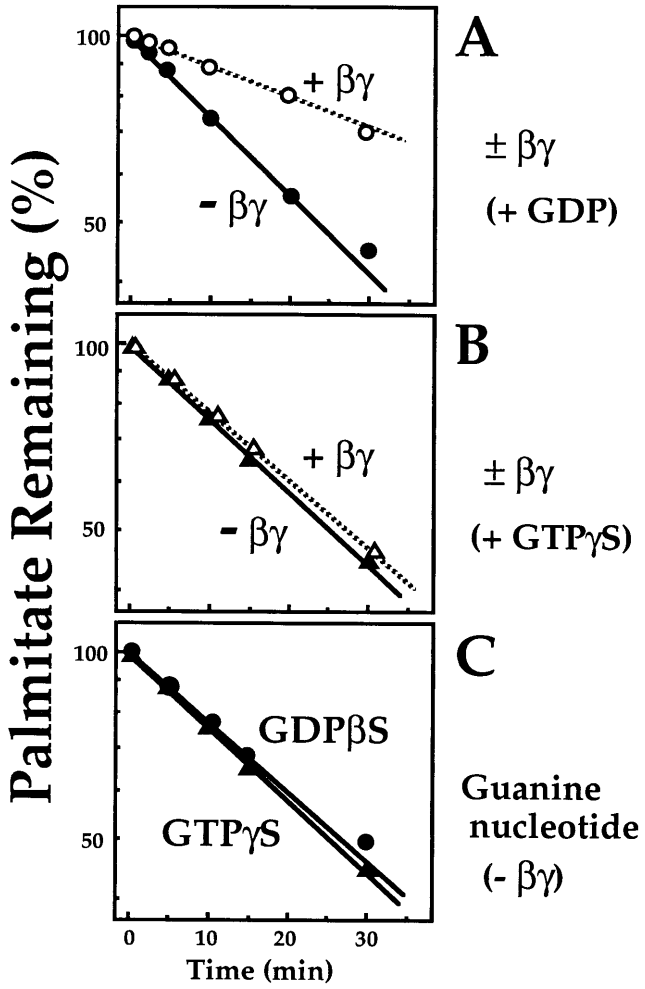

FIG. 5. Effect of $\beta\gamma$ and guanine nucleotides on depalmitoylation of α_s by palmitoyl esterase. [^{3}H]Palmitoylated membrane-derived α_s-WT (100 nM) was incubated at 22°C with palmitoyl esterase (0.14 μg/ml) in buffer B in the presence of 0.025% $C_{12}E_{10}$. At the times indicated, reactions were terminated, released [^{3}H]palmitate was quantitated, and remaining α_s-bound palmitate was calculated. (*A*) GDP-bound [^{3}H]palmitoylated α_s-WT was incubated in the absence of $MgCl_2$ and in the presence of recombinant $\beta\gamma$ (○) or boiled $\beta\gamma$ (●) (500 nM each). (*B*) [^{3}H]Palmitoylated α_s-WT was incubated with palmitoyl esterase with recombinant $\beta\gamma$ (○) or boiled $\beta\gamma$ (●) (500 nM each). Before the assay, the [^{3}H]palmitoylated protein was incubated with 15 μM GTP[γS] in the presence of 15 mM $MgCl_2$ at 22°C for 50 min. (*C*) [^{3}H]Palmitoylated α_s-WT was incubated with palmitoyl esterase in the absence of $\beta\gamma$. Before the assay, the [^{3}H]palmitoylated protein was incubated either with 15 μM GDP[βS] (●) or with 15 μM GTP[γS] (▲) in the presence of 15 mM $MgCl_2$ at 22°C for 50 min.

of $\beta\gamma$ (Fig. 4). As expected, the IC_{50} of this inhibitory effect was consistently ≈5-fold higher with unpalmitoylated α_s (α_s-C3S, cytosol-derived α_s-WT, or α_s-WT treated with palmitoyl esterase) than with membrane-derived α_s-WT, in which half the α_s molecules are palmitoylated. Thus palmitoylation increases the apparent affinity of α_s for $\beta\gamma$. In the absence of $\beta\gamma$, GTP[γS] bound to α_s-WT and α_s-C3S at similar rates (0.19 vs. 0.22 min^{-1}). Parallel experiments (not shown) testing the IC_{50} of $\beta\gamma$ for inhibition of steady state GTP hydrolysis by α_s revealed quantitatively similar differences between palmitoylated and unpalmitoylated α_s.

***βγ* Slows Depalmitoylation of α_s.** Activation of α_s accelerates its depalmitoylation in intact cells (7). Hypothetically, increased susceptibility of activated α_s to action of cellular palmitoyl esterases could result from GTP-induced conformational change; alternatively, if $\beta\gamma$ protects against these esterases, accelerated depalmitoylation could be caused by activation-induced dissociation of α_s from $\beta\gamma$. The results shown in Fig. 5 indicate that the latter hypothesis is correct. $\beta\gamma$ substantially slowed depalmitoylation of α_s·GDP by a purified recombinant palmitoyl esterase (Fig. 5*A*). $\beta\gamma$ had no effect on α_s depalmitoylation in the presence of GTP[γS] (Fig. 5*B*), presumably because binding of the GTP analog caused α_s to dissociate from $\beta\gamma$. In the absence of $\beta\gamma$, however, α_s·GTP[γS] and α_s·GDP were depalmitoylated at the same rate (Fig. 5*C*).

DISCUSSION

Our observations demonstrate reciprocal relations between $\beta\gamma$ binding to α_s and palmitoylation of this Gα subunit. As compared with the unpalmitoylated protein, palmitoylated α_s associates more tightly with $\beta\gamma$ during purification (Fig. 1) and has a higher apparent affinity for $\beta\gamma$ *in vitro*, as indicated by the decreased IC_{50} for $\beta\gamma$ inhibition of GTP[γS] binding (Fig. 4). Conversely, $\beta\gamma$ protects α_s from depalmitoylation by palmitoyl esterase (Fig. 5). In addition, our observations are the first to confirm directly the inference that the presence or absence of attached palmitate determines the location of α_s in membranes vs. cytosol (Figs. 2–4). Here we discuss biochemical mecha-

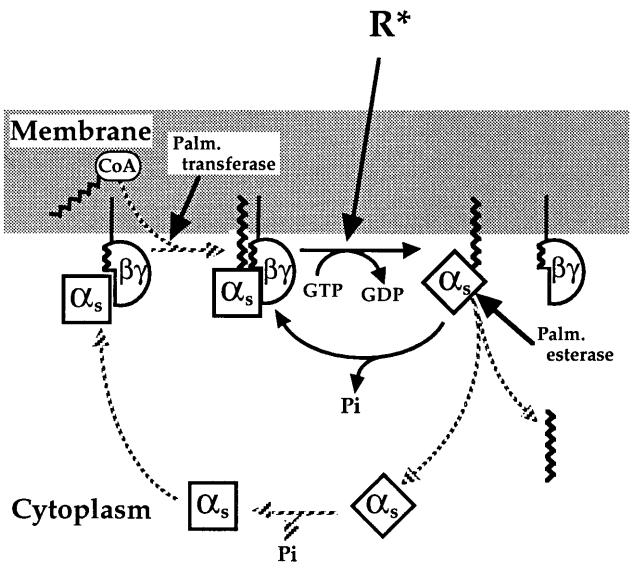

FIG. 6. Model of the α_s palmitoylation cycle. In the resting state, α_s is held at the membrane by the hydrophobic interaction of its attached palmitate with the bilayer and by association with $\beta\gamma$, which is itself attached to the membrane. In addition, palmitoylation enhances affinity of α_s for $\beta\gamma$ and $\beta\gamma$ reciprocally promotes the palmitoylated state by protecting α_s from attack of palmitoyl esterase and by enhancing palmitoylation catalyzed by palmitoyl transferase. Receptor-triggered activation of α_s induces its translocation to cytoplasm by causing subunit dissociation and rapid depalmitoylation. After converting bound GTP to GDP, cytosolic α_s returns to the membrane by binding $\beta\gamma$, followed by repalmitoylation by the transferase. In comparison to our previous model (3), the proposed cycle stresses reciprocal regulation by palmitate and $\beta\gamma$ and specifies the order of steps required for reattachment of cytosolic α_s to the membrane.

nisms underlying this reciprocal interaction, its implications for the activation-regulated palmitoylation cycle of α_s (Fig. 6) and membrane localization of the G_s heterotrimer, and comparisons of α_s to other Gα subunits.

Biochemical Mechanism of Reciprocal Relations Between $\beta\gamma$ and Palmitate. Three-dimensional structures of transducin (25) and $G_i\alpha$ (24) show that the N terminal α helix of Gα provides an important binding interface with $\beta\gamma$. This surface is very close to the likely sites of Gα lipid modifications at or near the protein's extreme N terminus [although these modifications are absent in available crystals (24, 25)]. Thus the most obvious explanation for reciprocity between $\beta\gamma$ and palmitate on α_s is that the hydrophobic lipid attached to α_s directly contributes binding energy to the $\alpha_s/\beta\gamma$ interaction, and that, in turn, $\beta\gamma$ shields the lipid from attack by esterase.

An additional possibility, which does not exclude the first, is that the increased hydrophobicity of palmitoylated α_s (Fig. 3) acts indirectly: Palmitate would enhance avidity of α_s for membrane bilayers or detergent micelles, potentiating the $\alpha_s/\beta\gamma$ interaction by increasing α_s concentration in physical proximity to membrane-bound or detergent-solubilized $\beta\gamma$. $\beta\gamma$ would reciprocally enhance association of palmitoylated α_s with membrane or detergent, and proximity of the α_s N terminus to the lipid bilayer could decrease its accessibility to action of the esterase. To our knowledge such indirect effects of lipid attachment have not been ruled out for any G$\alpha/\beta\gamma$ interaction; all reported studies of such interactions (summarized in table 1 of ref. 3) have been carried out with proteins attached to cell membranes or associated with detergent micelles.

Finally, reciprocity could result from palmitate- and $\beta\gamma$-dependent changes in the conformation of α_s. Indeed, association with $\beta\gamma$ stabilizes the N-terminal α-helix of Gα subunits in crystals (24, 25). It is also possible that attached palmitate similarly stabilizes a Gα conformation with higher affinity for $\beta\gamma$—an untested possibility, in that none of the crystals so far reported contains palmitoylated Gα.

Our data are consistent with all three mechanisms. Although to us the first two appear more likely, all three could combine to produce the reciprocal relations of $\beta\gamma$ and palmitate with respect to α_s.

The Palmitoylation Cycle and Membrane Localization of α_s. Our *in vitro* findings, combined with evidence from *in vivo* experiments, suggest a biochemical basis for the palmitoylation cycle of α_s (Fig. 6): In the resting state, palmitoylated α_s is held at the plasma membrane by a combination of increased hydrophobicity due to the lipid modification and enhanced affinity for binding membrane-bound $\beta\gamma$. The GTP-triggered conformational change in α_s that accompanies G_s activation induces subunit dissociation, releasing α_s from the protective embrace of $\beta\gamma$ and making α_s accessible to palmitoyl esterases. Resulting depalmitoylation decreases avidity of α_s for the membrane bilayer, allowing translocation of the relatively hydrophilic α_s molecule from membrane to cytosol. After GTP hydrolysis α_s·GDP returns to the membrane by binding $\beta\gamma$. Indeed, $\beta\gamma$ reportedly (33, 34) enhances susceptibility of Gα proteins to the forward reaction, palmitoylation. Thus localization of α_s in membranes depends upon reciprocal potentiation of its membrane avidity by $\beta\gamma$ and attached palmitate, and loss of either part of this dual regulation promotes translocation of the protein from membrane to cytosol—and vice versa.

In a possible addition to this scenario, attached palmitate could play a key role in the well-established (35) selective association of α_s with the plasma membrane in preference to membranes of intracellular organelles. In keeping with this notion, palmitoyl transferase activity is much more abundant in the plasma membrane fraction of liver homogenates (33). Thus even if $\beta\gamma$ were not selectively located in the plasma membrane (relative to other cellular membranes), palmitoylation occurring exclusively or predominantly at that site would retain α_s close to receptors and effectors at the cell surface by stabilizing its association with $\beta\gamma$ and the lipid bilayer.

Palmitate on Other α Subunits. Although α_q, like α_s, is palmitoylated but not myristoylated, the palmitate on α_s appears to play a more important role in determining hydrophobicity, $\alpha/\beta\gamma$ interaction, and membrane avidity. In the case of α_q, other N-terminal residues contribute significantly to hydrophobicity, over and above the effect of palmitates attached to cysteines at positions 9 and 10 (31). Even in its unpalmitoylated state α_q is sufficiently hydrophobic to partition almost equally into the detergent and water phases of TX-114; enzymatic removal of the of N-terminal 34 residues of α_q, however, cause it to partition completely into the water phase (31). Although the C9,10S mutation impaired its activation of phospholipase C (8, 31), treatment of palmitoylated α_q with palmitoyl esterase failed to alter interactions of α_q with receptor, $\beta\gamma$, and phospholipase C (31). For this reason, the effects of the mutation were attributed to replacement of cysteines by serines, rather than to absence of palmitate (31). This negative result may be partly explained by the relatively hydrophobic nature of α_q even in the absence of palmitoylation; in addition, an apparently low stoichiometry of α_q palmitoylation (20–40%, ref. 31) may have obscured a real functional effect of palmitate.

In the α_i family Gα subunits are irreversibly attached to myristate at a glycine residue just upstream of the cysteine to which palmitate is linked. Although both lipid modifications are likely to contribute to $\beta\gamma$ affinity and avidity for membranes, their relative and/or specific roles are just beginning to be explored (3, 5, 16). It should be noted in this connection that hydrophobicity contributed by myristate is only marginally sufficient for assured association with cellular membranes, in contrast to palmitate, which is longer and more hydrophobic (36). The dual mechanisms of membrane attachment by

structures near the N termini of α_q and α_i resemble those mediated by structures at or near the C termini of Ras proteins: a polybasic domain (Ki-ras) or palmitoylation (Ha- and N-ras) is required in addition to isoprenylation for localization to the plasma membrane (37).

We are just beginning to explore how different lipid modifications and peptide sequences regulate membrane avidity and localization of Gα proteins. Success in this task will eventually help to elucidate mechanisms responsible for localizing a much larger array of proteins at the cytoplasmic surface of cell membranes.

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