Many of the α subunits of heterotrimeric GTP-binding regulatory proteins (G proteins) are palmitoylated, a modification proposed to play a key role in the stable anchorage of the subunits to the plasma membrane. Palmitoylation of α subunits from the G_i family is preceded by N-myristoylation, which alone or together with βγ probably supports a reversible interaction of the α subunit with membrane as a prerequisite to the eventual incorporation of palmitate. Previous studies have not addressed, however, the question of whether membrane association alone, carried out through N-myristoylation, interaction with βγ, or other events, is sufficient for palmitoylation. We report here for α_q that it is not. We found that N-myristoylation is required for palmitoylation at least in part because it supports events subsequent to membrane attachment. Mutants of α_o designed to target the subunit to membrane without an N-myristoyl group are unable to be palmitoylated as evaluated by incorporation of [3H]palmitate. Mutants of α_o unable to interact normally with βγ yet still attach to membrane demonstrate that βγ, in contrast, is not required for palmitoylation. βγ becomes necessary, however, when the N-myristoyl group is absent. Our results suggest that N-myristoylation and βγ, while almost certainly relevant to the reversible interaction of α_o with membrane, also play at least partly overlapping, post-anchorage roles in palmitoylation.

Fatty acid acylation is one of the major covalent modifications of heterotrimeric GTP-binding regulatory protein (G protein) α subunits (1–4). For most α subunits of the G_i family, including α_i, α_o, and α_q, two distinct acylation events occur. N-Myristoylation takes place at the amino terminus and represents the attachment of a myristate through an amide bond to Gly^2 following cleavage of the initiator methionine (5, 6). Palmitoylation occurs at the adjacent Cys^o and represents the attachment of a palmitate (primarily) through a thioester bond (7, 8). α_o, α_i, and α_{12} family members are palmitoylated but not N-myristoylated (8–11), whereas α_i is N-myristoylated but not palmitoylated (12). The functional consequences of N-myristoylation and palmitoylation have been studied intensively. N-Myristoylation plays an important role in the attachment of G_i family α subunits to membrane (5, 6), targeting of the subunits to caveolin-enriched membrane domains (13), and interactions with the βγ heterodimer (14) and effectors such as adenylyl cyclase (15). Palmitoylation is relevant to the attachment of G protein α subunits to membrane (10, 16) and interaction of subunits with βγ (17) and RGS proteins (18).

The palmitoylation of G protein α subunits has drawn much attention recently, as this modification, unlike N-myristoylation, is reversible and subject to regulation. Several groups have demonstrated that the activation of G_s is accompanied by an increased rate of palmitate exchange on α_o, probably reflecting depalmitoylation and repalmitoylation of the subunit released from βγ (19–21). Whether the exchange coincides with a release of α_q from membrane is subject to debate (22, 23). Agonist-promoted palmitate exchange has also been reported for α_q (24) and α_i (25). The nature and reversibility of palmitoylation raise the expectation that it serves to regulate not only subunit location, but also activation/deactivation cycles and interactions of subunits with other proteins.

The basic requirements for palmitoylation remain obscure. For α subunits of the G_i family, three interrelated phenomena appear relevant: N-myristoylation, interaction with βγ, and proximity to membrane. A widely held hypothesis is that N-myristoylation imparts a small amount of hydrophobicity to the α subunit and/or facilitates interaction of the subunit with independently anchored βγ, therefore supporting a reversible interaction of the subunit with the plasma membrane; at the plasma membrane, the subunit is palmitoylated either enzymatically, i.e. by a membrane-localized palmitoyltransferase (26), or nonenzymatically (27). The relevance of the membrane is consistent with the observation that only membrane-associated subunits are found to be palmitoylated and that a mutant of α_i that is not N-myristoylated can nevertheless be palmitoylated when apparently brought to the membrane by overexpressed βγ (28).

Previous studies have not, however, addressed whether membrane association, carried out through N-myristoylation, interaction with βγ, or other events, is sufficient for palmitoylation. It is quite possible, for example, that N-myristoylation and/or βγ is required not only prior to, but following interaction of the subunit with membrane to support additional events culminating in palmitoylation. We report here, using α_q that this is in fact the case. We found that mutants of α_q that lack an N-myristoyl group but are still targeted to membrane are not palmitoylated. We also found that interactions with βγ are not normally required for palmitoylation, but, in the absence of N-myristoylation, become necessary. The requirement for βγ in lieu of N-myristoylation is not related to anchorage. We propose that N-myristoylation and βγ, while not doubt relevant to reversible interactions of subunits with membrane, also play redundant, post-anchorage roles in palmitoylation.
Experimental Procedures

**Plasmid Construction**—Rat α5 and human 5-hydroxytryptamine receptor α1 (5-HT1A) receptor (5-HT1A(r)) cDNAs (29, 30) in pcDNA3 and human β1 and bovine γ2 cDNAs (31, 32) in pCMV5 were used directly or as templates for subsequent mutagenesis. The mutations G2A, S6N, G204A, and Q205L, and the deletion of residues 8–11 for α5, were accomplished by the polymerase chain reaction using mismatched primers. The PCR products α5(5-HT1A)α1, α5G2A/α1, α5G2A/α1, 5-HT1A, α1, and 5-HT1A/α1, α5G2A were also constructed by polymerase chain reaction techniques in which overwinding ends were subsequently combined in a second round of polymerase chain reaction. The final constructs were cloned into the HindIII and XbaI restriction sites of pcDNA3. Sequences were verified directly.

**Transportation and Metabolic Labeling**—Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were cultured at 37 °C in a humidified atmosphere of 5% CO2 in minimal essential medium containing 10% (v/v) fetal calf serum and supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Transfections were carried out by calcium phosphate precipitation as described previously (33), usually with 10 μg of DNA/10 cm plate of just subconfluent cells. Cells were harvested 48–48 h thereafter. Biosynthetic labeling of proteins prior to harvesting was carried out as follows: (i) with [35S]methionine, in methionine-free Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (50 μCi/ml), usually initiated 8 h before harvesting; (ii) with [3H]myristic acid (American Radiolabeled Chemicals) in minimal essential medium supplemented with 150 mM NaCl, 2% deoxycholic acid, 2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (1 ml/10-cm plate); and (iii) with [3H]palmitic acid (American Radiolabeled Chemicals) in minimal essential medium supplemented with 5 mg/ml fatty acid-free bovine serum albumin (250 μCi/ml), usually initiated 1 h before harvesting.

**Cell Fractionation and Immunoprecipitation**—Cells were fractionated into membrane and cytosolic fractions were harvested in 20 mM HEPES, pH 8.0, 2 mM MgCl2, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenethylsulfonyl fluoride (0.5 mM/10-cm plate) and lysed at 0 °C by repeated passage through a 27-gauge needle. Subsequent procedures were carried out at 4 °C. Nuclei and unbroken cells were removed by centrifugation at 660 g for 5 min, and the cytosolic and membrane fractions were resolved subsequently by centrifugation at 100,000 × g for 1 h. The cytosolic fraction was used directly for immunoprecipitation or immunoblotting. The membrane fraction was extracted in the lysis buffer described above, but containing 1% sodium cholate, followed by clarification at 100,000 × g for 1 h. Immunoprecipitation of α1, and the various mutants from the cytosolic or extracted membrane fractions was accomplished by adding an equal volume of 100 mM sodium phosphate, pH 7.2, 2% deoxycholic acid, 2% Triton X-100, 200 mM NaCl, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 2 mM phenethylsulfonyl fluoride and then, following pre-clearance with nonimmune rabbit serum and protein A Sepharose, adding antiserum 9072 (1:50 to 1:100 final dilution) (34); antiserum 9072 was generated against the C-terminal 10 residues of α5. Immunoprecipitates were collected by centrifugation; washed three times in 50 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, and 2 mM EDTA; and resuspended in sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE). When fractionation was not required, cells were harvested directly in 50 mM sodium phosphate, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenethylsulfonyl fluoride (1 mM/10-cm plate). After 30 min on ice, the lysate was processed for immunoprecipitation as described above.

**Isolation of Caveolin-enriched Membrane Fractions**—The detergent-free approach of Song et al. (13) was used to prepare caveolin-enriched membrane fractions. HEK293 cells in 10-cm dishes were harvested in 2 ml of 500 mM sodium carbonate, pH 11; passed through a 27-gauge needle 25 times; and then processed in a Polytron tissue grinder with three 20-s bursts. The homogenate was adjusted to 45% sucrose by the addition of 35% sucrose in the same buffer. The homogenate was adjusted to 45% sucrose by the addition of 35% sucrose in the same buffer. Centrifugation was performed at 39,000 rpm for 16 h at 4 °C in an SW 41 rotor (Beckman Instruments). One-mL fractions were collected, and the protein within each fraction was precipitated by addition of trichloroacetic acid to 10% at 4 °C. The precipitates were collected, washed, and dissolved in sample buffer for analysis by Western blotting. Western blotting of protein samples was carried out using an antisera against α5, and caveolin was detected with an antiserum raised to the N terminus (Transduction Laboratories, Lexington, KY).

**Miscellaneous Methods**—Discontinuous SDS-PAGE (11% acrylamide) was carried out as described (34). Gels were stained with Coomassie Blue R-250, destained, and dried. For fluorography, the gels were cut into pieces, and the gels were immersed in Hyperfilm™ MP film (Amersham Pharmacia Biotech) for 30 min prior to drying. Dried gels were exposed to Hyperfilm™ MP film (Amersham Pharmacia Biotech) at −80 °C for 20–40 days (fluorography) or at room temperature for 4–10 days (autoradiography). Western blotting using chemiluminescence was accomplished following the directions provided by Amersham Pharmacia Biotech. GTP-S protection of the α subunit from tryptic digestion was performed according to Berlot and Bourne (35). Pertussis toxin (PTX)-catalyzed ADP-ribosylation was conducted by the method of Carty (36). The [35S]GTP-S-binding assay was carried out according to Windh et al. (37).

**RESULTS**

**Labeling of α5 with [3H]Palmitate Normally Requires N-myristoylation**—The observation that mutation of Gly2 (a residue that composes part of a consensus sequence for N-myristoylation) in α1 subunits abolishes palmitoylation as well as N-myristoylation (20, 38, 39) has led to the hypothesis that N-myristoylation, as a cotranslational modification, is a prerequisite to palmitoylation, a post-translational modification. To more firmly establish the relationship between N-myristoylation and palmitoylation, we initiated studies with another mutation, S6N, using α5 expressed in HEK293 cells. HEK293 cells lack α5 and therefore provide a suitable setting for the analysis of heterologously expressed α5 and mutants of α5. Ser6, like Gly2, is a consensus element for N-myristoylation (40, 41). HEK293 cells were transfected with plasmids encoding α5, α5G2A, and α5S6N and then incubated with [35S]methionine, [3H]myristate, or [3H]palmitate. The cells were subsequently fractionated into membrane and cytosol, and the subunits were immunoprecipitated from each fraction with an α5-directed antibody. As shown in Fig. 1 (upper panel), α5 cofractionated mostly with membrane, whereas α5G2A and α5S6N were found mostly in cytosol. As expected, α5 incorporated both [3H]myristate (middle panel) and [3H]palmitate (lower panel), whereas α5G2A incorporated neither; incorporation of [3H]palmitate by α5G2A was evident only in the membrane fraction. α5S6N, like α5G2A, did not incorporate either [3H]myristate or [3H]palmitate.

Mutational approaches are subject to the criticism that a mutation, while inhibiting N-myristoylation, might also have a direct bearing on nonenzymatic palmitoylation or substrate recognition by a palmitoyltransferase apart from N-myristoylation. We therefore studied the effects of a chemical inhibitor of N-myristoyltransferase, 2-hydroxymyristic acid (2-HMA) (42, 43). Treatment of α5-transfected cells with 1 mM 2-HMA for 16 h caused a redistribution of about half of α5 to cytosol (data not shown); correlated with this effect was an ~70% decrease in incorporation of [3H]myristate and [3H]palmitate. Although the suppression was not complete, the effects were consistent with the translocation data.

Effects of Preventing N-myristoylation Can Be Overcome by a Mutation That Enhances Interaction of α5 with βγ—In separate experiments, we began to address the role of α5 and conformational states of α5 in α5–GTP binding. We first evaluated [3H]palmitate labeling of α5G204A and α5G205L. α5G204A corresponds to α5G226A and α5G230A, which are refractory to activation and bind βγ with an especially high affinity (44, 45). α5G205L, like other Gln-to-Leu mutants, assumes a predominantly active conformation. The levels of expression of α5

1 The abbreviations used are: 5-HT1A, 5-hydroxytryptamine receptor; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; Mes, 4-morpholineethanesulfonic acid; GTP-S, guanosine 5′-3-O-(thio)triphosphate; PTX, pertussis toxin; 2-HMA, 2-hydroxymyristic acid.
Effects of βγ and N-Myristoylation on αγ Palmitoylation

αγG204A and αγQ205L in transfected HEK293 cells were similar (Fig. 2, upper and lowermost panels), and fractionation revealed little difference among these subunits in partitioning between membrane and cytosol (upper panel). Experiments with [3H]palmitate were performed at several times of incubation to confirm that steady-state levels of labeling had been attained. The labeling of αγG204A with [3H]palmitate, regardless of incubation time, was considerably stronger than that of αoγ, whereas that of αγQ205L was almost undetectable (middle panel). Because the G204A and Q205L mutants associate with membrane nearly equivalently, these data suggest that labeling with [3H]palmitate is sensitive to events beyond overt changes in interactions of subunit with membrane. Labeling with [3H]myristate, as a control, was the same for the three subunits (data not shown).

A small proportion of αγG2A and αoS6N usually cofractionates with membrane (see Fig. 1, for example). This can be the result of nonspecific association due to subunit denaturation; however, we found that GTPγS protects this population of subunit from degradation by trypsin, allowing only the 2-kDa (N-terminal) clip characteristic of a conformationally sound subunit (data not shown). Because αγG2A and αoS6N that cofractionate with the membrane do not incorporate [3H]palmitate, as noted previously, we hypothesized that the absence of N-myristoyl group and possibly a consequent decrease in affinity of the subunits for βγ might be the underlying defect. We therefore determined whether the G204A mutation, which would increase the affinity of the α subunit for βγ, would confer to the G2A and S6N forms of αoγ the capacity to be palmitoylated. We found that introduction of the G204A mutation did not change the distribution of αγG2A and αoS6N between membrane and cytosolic fractions (Fig. 3, upper panel). However, although αγG2A and αoS6N did not incorporate [3H]palmitate, αγG2A/G204A and αoS6N/G204A did (lower panel). The results lend support to the concept that events in addition to membrane attachment are required for [3H]palmitate labeling, and they specifically demonstrate that a non-myristoylated form of subunit can be palmitoylated if made to assume a conformation more refractory to activation and/or more conducive to interaction with βγ. The extent of labeling in the case of αoG2A/G204A and αoS6N/G204A was severalfold less than that achieved with wild-type αoγ (data not shown), due probably to the relatively greater amount of the latter associated with membrane.

We evaluated the capacity of αoγ and the various mutants to be [32P]ADP-ribosylated by PTX, a reaction that requires at least a transient interaction of the α subunit with βγ (46, 47). αγG204A, as expected based on its presumed high affinity for βγ, was a better substrate for ADP-ribosylation than αoγ (Fig.
Effects of \( \beta_\gamma \) and N-Myristoylation on \( \alpha_o \) Palmitoylation

FIG. 4. Pertussis toxin-catalyzed ADP-ribosylation of \( \alpha_o \) and various \( \alpha_o \) mutants. Lysates of HEK293 cells expressing the indicated forms of \( \alpha_o \) were incubated with PTX and \( ^{32}P \)NAD as described under “Experimental Procedures.” Subunit expression was analyzed by Western blotting, and \( ^{32}P \)ADP-ribose incorporation was evaluated by SDS-PAGE/autoradiography (overnight exposure) following immunoprecipitation.

4A). \( \alpha_o Q205L \) was also a good substrate, which was surprising, as an active form of the subunit would not be expected to interact well with \( \beta_\gamma \). The ADP-ribosylation of Glu-to-Leu mutants expressed in mammalian cells has not been evaluated previously. \( \alpha_o G2A \) and \( \alpha_o S6N \) were not substrates for ADP-ribosylation (Fig. 4B), a result consistent with the observation that the N-myristoyl group is relevant to interaction with \( \beta_\gamma \) (14). Introduction of the G204A mutation restored the capacity of \( \alpha_o G2A \) and \( \alpha_o S6N \) to be ADP-ribosylated, as it did the capacity of these subunits to be palmitoylated. The extent of ADP-ribosylation was less for \( \alpha_o S6N/G204A \) than for \( \alpha_o G2A/G204A \), which might be explained by a greater impact of the S6N mutation on interactions with \( \beta_\gamma \). The results with the G2A and S6N mutants imply that the loss of \(^{3}H\)palmitate labeling observed when the N-myristoyl group is absent can be reversed by G204A through an enhanced interaction with \( \beta_\gamma \).

Labeling of \( \alpha_o \) with \(^{3}H\)Palmitate Does Not Require Normal Interactions with \( \beta_\gamma \). Unless Myristate Is Absent—The above observations suggest a role for \( \beta_\gamma \) in palmitoylation. To begin addressing this role in more detail, we constructed a mutant of \( \alpha_o \) previously noted to associate only poorly with \( \beta_\gamma \) (48). This mutant, \( \alpha_o ^{D8\rightarrow11} \) (lacking Glu\(^{8}\)-Ala\(^{11}\)), was, as expected, a poor substrate for PTX-catalyzed ADP-ribosylation (Fig. 5A). The mutant behaved nearly identically to \( \alpha_o \) in terms of membrane/cytosol distribution and \(^{3}H\)myristate and \(^{3}H\)palmitate labeling (Fig. 5B). Therefore, whereas a mutation enhancing interaction of \( \alpha_o \) with \( \beta_\gamma \) enhanced \(^{3}H\)palmitate labeling (G204A; see above), a mutation attenuating interaction but maintaining N-myristoylation and membrane anchorage (\( \alpha_o ^{D8\rightarrow11} \)) had no effect. This result indicates that \(^{3}H\)palmitate labeling of \( \alpha_o \) does not require fully normal interactions of \( \alpha_o \) with \( \beta_\gamma \).

We wished to corroborate this result and therefore devised a mutant in which 10 lysine residues were inserted between Ser\(^{6}\) and Ala\(^{7}\) (\( \alpha_o K_{10} \)). We anticipated (and examined subsequently below) that the 10-lysine insertion, like \( \alpha_o ^{D8\rightarrow11} \), would disrupt interaction of the subunit with \( \beta_\gamma \). The 10-lysine insert was also chosen to permit a set of experiments with \( \alpha_o G2A \) to confirm the role of N-myristoylation in palmitoylation for even the membrane-attached subunit, as suggested by the G2A and S6N mutants. Polylysine domains support the attachment of various proteins to membrane, probably by virtue of electrostatic interactions with phospholipids (49–51); we felt that the 10-lysine insert might similarly support attachment of the largely cytosolic, non-myristoylated G2A form of subunit to membrane. The 10-lysine insert did not affect the folding of \( \alpha_o \) or \( \alpha_o G2A \) as evaluated by GTP\( \gamma \)S protection from tryptic digestion (data not shown). Consistent with previous experiments, 

\(~80\%\) of \( \alpha_o \) cofractionated with membranes, whereas most but not all of \( \alpha_o G2A \) were not associated with cytosol (Fig. 6A).

The 10-lysine insert increased the affinity of both \( \alpha_o \) and \( \alpha_o G2A \) for membrane, i.e. almost all of \( \alpha_o K_{10} \) and approximately one-half of \( \alpha_o G2A/K_{10} \) were cofractionated with membrane. Radiolabeling with fatty acids revealed that \( \alpha_o \) and \( \alpha_o K_{10} \) incorporated \(^{3}H\)myristate and \(^{3}H\)palmitate (Fig. 6B). Therefore, the 10-lysine insert, which is predicted to disrupt normal interactions of \( \alpha_o \) with \( \beta_\gamma \), was not deleterious to N-myristoylation or palmitoylation. This result suggests, again, that normal interactions with \( \beta_\gamma \) are not required for \( \alpha_o \) to be palmitoylated. In the case of the G2A mutant, however, the 10-lysine insert did not restore the capacity of the subunit (\( \alpha_o G2A/K_{10} \)) to be palmitoylated, despite the large fraction of subunit targeted to membrane. This result confirms a requirement for N-myristoylation in palmitoylation despite membrane attachment.

The failure of the 10-lysine insert to rescue palmitoylation of \( \alpha_o G2A \) might have resulted from incorrect targeting of the subunit. Caveolae are specialized plasma membrane domains that represent a major storage or reaction site for molecules involved in G protein-coupled signal transduction pathways, including G protein \( \alpha \) and \( \beta_\gamma \) subunits (13, 52–55). We therefore used cofractionation with caveolin-enriched domains as an
assay of appropriate targeting. The results of a typical sucrose step gradient centrifugation that separates caveolin-enriched domains from the bulk of cellular membranes and cytosolic proteins are shown in Fig. 7. Caveolin, a marker for caveolae, was localized predominantly to fractions 4 and 5. In agreement with a previous report (13), the bulk of αo cofractionated with caveolin. So, too, did αK10, αG2A was found with soluble protein (i.e. fractions 9–12), and αG2A/K10 partitioned between soluble protein and caveolin-enriched fractions. These data imply that αo, αK10, and most of αG2A/K10 that is not cytosolic target correctly.

We used an additional method of targeting αo to membrane without N-myristoylation to test further the apparent requirement of palmitoylation for N-myristoylation following attachment of subunit to membrane. The αo and αG2A subunits were appended to the C terminus of human 5-HT1AR, which possesses a seven-transmembrane domain motif and is targeted exclusively to membrane. Similar constructs of the α2a-adrenoceptor and α1 and the β2-adrenoceptor and α2, when expressed in intact cells, are sensitive to extracellular ligands (56, 57), and G protein-coupled receptors similarly fused to green fluorescent protein target predominantly to the plasma membrane (58, 59). The fusion precludes N-myristoylation of the α subunits. Cys417 and Cys420 of 5-HT1AR, which represent potential sites of palmitoylation (60), were changed to alamines. Fig. 8A shows the distribution of 5-HT1AR/αo and 5-HT1AR/αG2A. A prominent band at ~100 kDa, present in membrane only, was evident for both chimeric proteins. The mobility of the chimeras was less than predicted from cDNA (85 kDa), a difference that probably reflects glycosylation of the receptor.

The functional integrity of the appended αo and αG2A subunits was demonstrated in two ways. First, GTPγS protected the subunit from proteolysis by trypsin (i.e. a released ~37-kDa αo fragment was stabilized by GTPγS; data not shown). Second, R(+)-trans-8-hydroxy-2-[N-n-propyl-N-(3′-iodo-2′-propenyl)-amino]tetralin, a 5-HT1AR agonist, activated the appended subunit in each chimera as measured by [35S]GTPγS binding (Fig. 8B); the stimulated increase in binding was greater than that seen for 5-HT1AR and αo coexpressed as individual proteins (data not shown). Control experiments confirmed that no endogenous αo was responsible for the [35S]GTPγS binding. Despite targeting to membrane, however, neither chimera incorporated [3H]palmitate (Fig. 8C). These results show that, although functional and attached to membrane, chimeric, non-N-myristoylated αo is not palmitoylated.

Overexpressed βγ Rescues the Labeling of αo with [3H]Palmitate when the N-Myristoyl Group Is Absent—The results with αG2A/K10 and 5-HT1AR/αo support the assertion that an αo subunit that cannot be N-myristoylated is not palmitoylated, even though it is targeted to membrane. However, it might be argued that the proteins are not positioned correctly on the membrane, whether due to the absence of the N-myristoyl group or not. Because N-myristoylation is relevant to the interaction of αo with βγ, we evaluated PTX-catalyzed [32P]ADP-riboseylation of the two mutants, as we did the other mutants (see above). We also tested whether overexpression of βγ might promote [3H]palmitate labeling. The data in Fig. 9A imply that the variant subunits indeed interact poorly with βγ, i.e. insertion of 10 lysines or linkage of αo to 5-HT1AR resulted in a much diminished incorporation of [3H]ADP-ribose. αG2A/K10 and 5-HT1AR/αo alone did not incorporate [3H]palmitate (Fig. 9B), as noted previously. However, coexpression of exogenous βγ promoted labeling. The lack of an N-myristoyl group therefore normally precludes [3H]palmitate labeling, even though the subunit is associated with membrane, but the defect can be overcome by overexpression of βγ. The mutant/chimeric subunits are therefore not resistant to assuming a conformation or positioning on the membrane conducive to palmitoylation.

**DISCUSSION**

That N-myristoylation, interactions with βγ, and proximity to membrane are interrelated events has made it difficult to sort out the contributions of any single one of these phenomena to palmitoylation of αo family members. For the most part, proximity to the plasma membrane has been considered the key event, with palmitoylation occurring subsequently to establish a stable anchorage. Proximity can be achieved by N-myristoylation alone (5, 6), by an N-myristoylation-facilitated interaction of α with independently anchored βγ (14), or, in lieu of N-myristoylation, by overexpression of βγ (28). Studies re-
Effects of βγ and N-Myristoylation on αo Palmitoylation

The S6N mutation and 2-HMA were used to help address the concern that the effects of G2A on palmitoylation in previous studies were not so much due to inhibiting N-myristoylation (and consequently palmitoylation) as they were to a disruption of palmitoylation directly at the adjacent Cys.

The data here indicate that an association of αo with membrane, even perhaps a stable anchorage, is not sufficient for [3H]palmitate labeling. Small but easily distinguished populations of αo,G2A and αo,S6N, for example, often cofractionated with membrane, but did not incorporate [3H]palmitate. Moreover, αo,Q205L cofractionated entirely with membrane, but incorporated only a small amount of radiolabel. In the case of membrane-associated αo,G2A and αo,S6N, the underlying defect would appear to be the lack of an N-myristoyl group or the consequent diminished affinity for βγ. The defect is less clear for αo,Q205L, as N-myristoylation was unaffected by the mutation, and the interaction with βγ, at least as intimated by PTX-catalyzed [32P]ADP-ribosylation, appeared to be normal if not enhanced. We suspect that the active conformation itself assumed by αo,Q205L explains the diminished [3H]palmitate labeling and that this conformation overrides the positive inputs provided by N-myristoylation and/or βγ interaction. A GTPase-deficient form of αo (αo,R201C) has similarly been shown to label poorly with [3H]palmitate (19). Unlike αo,R201C, however, no net translocation of αo,Q205L to cytosol was observed to occur, consistent with observations for αo,Q204L (22).

The effects of G2A, S6N, and Q205L on αG2A and αS6N have been reported to occur, consistent with observations for αG204A (22). The effects of G2A, S6N, and Q205L on N-myristoylation, βγ interaction, or conformation may either abrogate palmitoylation directly or regulate depalmitoylation in such a manner as to inhibit radiolabel exchange or to cause a net decrease in palmitate associated with subunits.

In stark contrast to αo,Q205L, αo,G204A was characterized by an enhanced labeling with [3H]palmitate. αo,G204A, by reference to other Gly-to-Ala mutant α subunits (44, 45), resists entering into an active conformation and binds βγ particularly tightly. Thus, the inactive conformation per se or the heightened interaction with βγ enhances palmitoylation and/or protects the palmitoylated subunit from depalmitoylation; we assume that isotopic equilibrium has been reached and that the effects are not due to an enhanced turnover of label alone. The enhancement in [3H]palmitate labeling achieved with αo,G204A was not limited to wild-type αo, αo,G2A and αo,S6N, for which incorporation of [3H]palmitate was not normally evident, incorporated [3H]palmitate when mutated to additionally include G204A. Thus, we found that non-myristoylated subunits are capable of being palmitoylated. This has been shown previously for G2A mutants upon overexpression of βγ, wherein the amount of subunit associated with membrane increased (28), but our data demonstrate that it also occurs where changes in membrane association are not observed.

Effects of βγ and N-Myristoylation on αo Palmitoylation

The S6N mutation and 2-HMA were used to help address the concern that the effects of G2A on palmitoylation in previous studies were not so much due to inhibiting N-myristoylation (and consequently palmitoylation) as they were to a disruption of palmitoylation directly at the adjacent Cys.

The data here indicate that an association of αo with membrane, even perhaps a stable anchorage, is not sufficient for [3H]palmitate labeling. Small but easily distinguished populations of αo,G2A and αo,S6N, for example, often cofractionated with membrane, but did not incorporate [3H]palmitate. Moreover, αo,Q205L cofractionated entirely with membrane, but incorporated only a small amount of radiolabel. In the case of membrane-associated αo,G2A and αo,S6N, the underlying defect would appear to be the lack of an N-myristoyl group or the consequent diminished affinity for βγ. The defect is less clear for αo,Q205L, as N-myristoylation was unaffected by the mutation, and the interaction with βγ, at least as intimated by PTX-catalyzed [32P]ADP-ribosylation, appeared to be normal if not enhanced. We suspect that the active conformation itself assumed by αo,Q205L explains the diminished [3H]palmitate labeling and that this conformation overrides the positive inputs provided by N-myristoylation and/or βγ interaction. A GTPase-deficient form of αo (αo,R201C) has similarly been shown to label poorly with [3H]palmitate (19). Unlike αo,R201C, however, no net translocation of αo,Q205L to cytosol was observed to occur, consistent with observations for αo,Q204L (22).

The effects of G2A, S6N, and Q205L on N-myristoylation, βγ interaction, or conformation may either abrogate palmitoylation directly or regulate depalmitoylation in such a manner as to inhibit radiolabel exchange or to cause a net decrease in palmitate associated with subunits.

In stark contrast to αo,Q205L, αo,G204A was characterized by an enhanced labeling with [3H]palmitate. αo,G204A, by reference to other Gly-to-Ala mutant α subunits (44, 45), resists entering into an active conformation and binds βγ particularly tightly. Thus, the inactive conformation per se or the heightened interaction with βγ enhances palmitoylation and/or protects the palmitoylated subunit from depalmitoylation; we assume that isotopic equilibrium has been reached and that the effects are not due to an enhanced turnover of label alone. The enhancement in [3H]palmitate labeling achieved with αo,G204A was not limited to wild-type αo, αo,G2A and αo,S6N, for which incorporation of [3H]palmitate was not normally evident, incorporated [3H]palmitate when mutated to additionally include G204A. Thus, we found that non-myristoylated subunits are capable of being palmitoylated. This has been shown previously for G2A mutants upon overexpression of βγ, wherein the amount of subunit associated with membrane increased (28), but our data demonstrate that it also occurs where changes in membrane association are not observed.
Our results demonstrate that N-myristoylation is required for [3H]palmitic labeling of αo under normal circumstances and that mutations favoring inactive conformations and/or interactions with βγ can enhance palmitate incorporation into αo and rescue that of mutants devoid of the N-myristoyl group. Experiments with additional mutants were performed to test the notion that N-myristoylation and βγ exert roles in palmitoylation not only prior to, as usually acknowledged, but also following anchorage of subunit to membrane. The mutants that were utilized toward this end included αoΔ8–11, αoK10, and 5-HT1A/Rαo. These experiments together demonstrate that N-myristoylation or normal interactions with βγ, but not both, is required for [3H]palmitate labeling and that this requirement exists regardless of alterations in membrane attachment: αoΔ8–11 and αoK10, which are N-myristoylated but interact poorly with βγ, incorporate [3H]palmitate; αoG2A/G204A and αoS6N/G204A, which likely interact with βγ but are not N-myristoylated, also incorporate [3H]palmitate; and αoG2A, αoS6N, αoG2A/K10, and 5-HT1A/Rαo, which neither are N-myristoylated nor interact well with βγ, do not incorporate [3H]palmitate unless (in the case of at least αoG2A/K10 and 5-HT1A/Rαo) βγ is overexpressed. There were hardly any differences in the extent to which αo, αoΔ8–11, αoK10, and 5-HT1A/Rαo cofractionated with membrane, i.e. most if not all of these subunits were anchored. A substantial fraction of αoG2A/K10 also cofractionated with membrane. Issues of membrane identity notwithstanding, the requirements for N-myristoylation or βγ therefore appear to extend beyond those relating to proximity and anchorage.

The requirement for an N-myristoyl group in [3H]palmitate labeling of αo already attached to membrane is probably not related to its role in facilitating interaction of the subunit with βγ. αoΔ8–11 and αoK10, whose interactions with βγ are disrupted, incorporate [3H]palmitate to the same extent as αo itself. The role of N-myristoylation may instead involve targeting of the membrane-attached subunit to a relevant microdomain or the performance of some other post-attachment event. Fishburn et al. (62) have proposed a scheme in which the N-myristoyl group supports attachment of αo to internal membranes for expedited transfer to the plasma membrane and subsequent palmitoylation. Something of this nature may occur for αo. However, our data with αoG2A/K10 suggest appropriate plasma membrane targeting for at least a fraction of this non-myristoylated subunit, and there is good reason to believe that this is also the case with at least the 5-HT1A/Rαo chimeras based on the signaling and targeting properties of other receptor/protein fusion constructs in the intact cell (56–59). It is more likely that the N-myristoyl group is recognized by a palmitoyltransferase as part of the substrate or that it positions the N terminus of the α subunit in such a manner as to facilitate palmitoylation. This interpretation is consistent with the findings of Dunphy et al. (26) using bovine brain membrane extracts as a source of palmitoyltransferase activity and purified α11, wherein N-myristoylated α11 was found to be the preferred substrate. It is also conceivable that N-myristoylation protects αo against depalmitoylation, as suggested by Morales et al. with αa (63). However, in mutants of αa that lack the N-myristoyl group and that do not interact with βγ, we found no incorporation of [3H]palmitate whatsoever, implying that, if protection against an esterase is the case, the esterase is extremely active. That the non-myristoylated subunits might be fully palmitoylated but unable to exchange the lipid for [3H]palmitate is a formal possibility, in which case the N-myristoyl group might enhance labeling by actually increasing esterase activity. Regardless, it is quite clear that N-myristoylation serves a role in regulating palmitoylation that exceeds that of providing a loose association with membrane.

Although normal interactions with βγ appear not to be required for [3H]palmitate labeling under normal circumstances, these interactions become crucial when the N-myristoyl group is absent. This was first suggested by the observation that G204A rescues palmitoylation of αoG2A and αoS6N and later demonstrated by the fact that βγ rescues palmitoylation of αoG2A/K10 and 5-HT1A/RαoG2A. βγ has been proposed to help target α subunits to the plasma membrane (62). However, as discussed above, the mutant subunits are probably already targeted correctly. We therefore suspect that βγ performs essentially the same function as N-myristoylation: it enhances recognition of the α subunit by a palmitoyltransferase, prevents depalmitoylation by an unusually active esterase, or enhances turnover of palmitate. These actions may be direct or related to changes in α subunit conformation. Protection from an esterase is a commonly assumed role from studies with αa (19–21). However, Dunphy et al. (26) have shown an effect of βγ on
on palmitoylation of purified α1 in vitro. The requirement for βγ in our studies becomes evident only when the N-myristoyl group is absent.

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