The Importance of N-terminal Polycysteine and Polybasic Sequences for G\textsubscript{14}α and G\textsubscript{16}α Palmitoylation, Plasma Membrane Localization, and Signaling Function*

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Plasma membrane targeting of G protein α (Gα) subunits is essential for competent receptor-to-G protein signaling. Many Gα are tethered to the plasma membrane by covalent lipid modifications at their N terminus. Additionally, it is hypothesized that G\textsubscript{q} family members (G\textsubscript{q}, G\textsubscript{11}, G\textsubscript{14}, and G\textsubscript{16}) in particular utilize a polybasic sequence of amino acids in their N terminus to promote membrane attachment and protein palmitoylation. However, this hypothesis has not been tested, and nothing is known about other mechanisms that control subcellular localization and signaling properties of G\textsubscript{14}α and G\textsubscript{16}α. Here we report critical biochemical factors that mediate membrane attachment and signaling function of G\textsubscript{14}α and G\textsubscript{16}α. We find that G\textsubscript{14}α and G\textsubscript{16}α are palmitoylated at distinct polycysteine sequences in their N termini and that the polycysteine sequence along with the adjacent polybasic region are both important for G\textsubscript{16}α-mediated signaling at the plasma membrane. Surprisingly, the isolated N termini of G\textsubscript{14}α and G\textsubscript{16}α expressed as peptides fused to enhanced green fluorescent protein each exhibit differential requirements for palmitoylation and membrane targeting; individual cysteine residues, but not the polybasic regions, determine lipid modification and subcellular localization. However, full-length G\textsubscript{16}α, more so than G\textsubscript{14}α, displays a functional dependence on single cysteines for membrane localization and activity, and its full signaling potential depends on the integrity of the polybasic sequence. Together, these findings indicate that G\textsubscript{14}α and G\textsubscript{16}α are palmitoylated at distinct polycysteine sequences, and that the adjacent polybasic domain is not required for G\textsubscript{α} palmitoylation but is important for localization and functional activity of heterotrimeric G proteins.

Heterotrimeric G protein localization at the plasma membrane is critical for transmembrane signaling from activated receptors to linked effector molecules. Both the Gα and Gγ subunits of the heterotrimer bind the lipid bilayer to position the G protein for activation by cognate G protein-coupled receptors (1, 2). In response to receptor-induced activation with GTP, the G protein subunits redistribute and selectively stimulate membrane-bound effector molecules (reviewed in Refs. 3, 4). The four Gα subunits of the G\textsubscript{q} family, G\textsubscript{q}, G\textsubscript{11}, G\textsubscript{14}, and G\textsubscript{16}, transfer receptor-generated signals to phospholipase C-β isozymes 1–4 (PLC-β1–4) (reviewed in Ref. 5) and other protein binding partners (reviewed in Ref. 6). Activated Gα subunits stimulate the enzymatic activity of PLC-β, triggering the breakdown of the membrane lipid phosphatidylinositol (4,5)-biphosphate into the second messenger molecules inositol (1,4,5) trisphosphate and diacylglycerol. G\textsubscript{α} and G\textsubscript{11} that are not associated with the membrane display impaired or abolished capacity to become activated by receptor or to activate PLC-β (7–13). Whether this also is the case for G\textsubscript{14}α and G\textsubscript{16}α is unknown because relatively few studies to date have focused on the signaling properties of these poorly understood G\textsubscript{q} family members.

Unlike the membrane-spanning receptors that directly activate G proteins, the soluble heterotrimeric G protein complex relies on extrinsic forces to mediate localization at the plasma membrane. Long chain lipid modifications of both the Gα and Gγ subunits directly interact with lipid bilayers and stabilize the protein complex at the plasma membrane. In general, Gα subunits are targeted for lipid modification at their N terminus, but the profiles of lipid incorporation vary among Gα subtypes with regard to the fatty acid moiety and the sites of attachment (1). G\textsubscript{q} and G\textsubscript{11} are both incorporated palmitate at two N-terminal cysteine residues (Cys-9 and Cys-10) (7–14, 16). Palmitate is a 16-carbon saturated fatty acid that is bound post-translationally to regulate localization and function of target proteins (reviewed in Refs. 17, 18). Unpalmitoylated G\textsubscript{α} and G\textsubscript{11} (C/A or C/S site mutants) are cytotoxic and cannot mediate receptor-to-effector signaling compared with wild-type G\textsubscript{q/11} (7–9, 12, 13, 16, 19).

Nothing is known about the lipidation states of G\textsubscript{14}α and G\textsubscript{16}α or of other biochemical mechanisms that may regulate G\textsubscript{14}α and G\textsubscript{16}α plasma membrane localization and signaling function. Within the region of the N-terminal α-helix, the

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2 The abbreviations used are: PLC-β, phospholipase C-β; GFP, green fluorescent protein; EGFP, enhanced GFP; HEK 293, human embryonic kidney 293; DMEM, Dulbecco’s modified Eagle’s medium; DJBM, n-dodecyl-β-D-maltoside; C/A, cysteine-to-alanine mutation; C/S, cysteine-to-serine mutation; EE and Glu-Glu, glutamate-glutamate epitope tag sequence; R/Q, arginine-to-glutamine mutation; K/Q, lysine-to-glutamine mutation; NH2OH, hydroxylamine; Q/L, glutamine-to-lysine mutation; PIPES, 1,4-piperazineethanesulfonic acid.
sequences of G14α and G16α are notably different from Gα and G11α (Fig. 1A). Although Gqα and G11α are 83% identical in their first 40 amino acids, G14α and G16α share only 65 and 35% identity, respectively, with Gα across the same region. Furthermore, G14α and G16α are unique among Gα subunits in that they each have three cysteine residues in their N-terminal domain that are putative sites for palmitoylation. Consequently, the N termini of G14α and G16α may be differentially involved in the biochemical regulation of membrane localization and signaling function of these particular Gα proteins.

Current evidence supports a dual requirement for Gα palmitoylation and heterotrimer formation to drive plasma membrane localization of Gα and G11α (11, 12, 19–21). Recent models also suggest that Gα family members in particular may utilize multiple positively charged basic residues in the Gα N terminus as a third signal for membrane targeting and attachment. Three-dimensional molecular modeling of G14α, G11α, G14α, and G16α predicts a cluster of basic amino acids in the N terminus of each Gα to fold in such a way so as to form a positively charged patch on the protein surface (22). These residues are expected to align on one face of the Gα N-terminal α-helix in a position favorable for ionic interactions with anionic phospholipids in the plasma membrane. It also has been postulated that this polybasic region is a necessary signal for Gα palmitoylation (22). Palmitoylation occurs at cellular membranes, and palmitoyltransferase activity appears to be enriched in the plasma membrane specifically (17, 23). Therefore, Gα localization at the plasma membrane mediated by the electrostatic potential of the polybasic region may be a prerequisite for protein palmitoylation. These ideas, however, have never been tested experimentally.

In this study, we identify critical Gα-specific N-terminal amino acids and biochemical factors that mediate membrane localization and signaling function of G14α and G16α. We show that G14α and G16α are palmitoylated at distinct polycysteine sequences in their N termini and that the G proteins utilize these sequences for plasma membrane association and effector activation. Furthermore, G16α exhibits a functional dependence on the N-terminal polybasic domain in addition to the polycysteine sequence to maintain membrane localization and biological activity. With a comprehensive comparison of these properties in the isolated Gα N termini and in the full-length heterotrimeric G proteins, we report the unexpected finding that the full-length Gα proteins exhibit differential dependence on the polycysteine and polybasic sequences for localization and function than do the N termini when expressed alone as EGFP fusion proteins. Importantly, we show for the first time that the polybasic domain itself is not involved in palmitoylation of a proximal substrate like the adjacent polycysteine sequences in the Gα N terminus, but instead plays a key role in localization and function of the whole heterotrimeric G protein.

EXPERIMENTAL PROCEDURES

Plasmids and Materials—Human G14α and G16α cDNA expression plasmids with internal Glu-Glu (EE) epitope tags (G14α, G16α-Q205L (G14α-Q/L), G16α, and G16α-Q212L (G16α-Q/L)) were purchased from University of Missouri-Rolla cDNA Resource Center, as were the Gβ3 and Gγ2 expression plasmids. pCDNA3.1 was from Invitrogen, and pEGFP-N1 was from Clontech. KpnI, Agel, NotI, and SacII restriction enzymes were purchased from New England Biolabs. Primers were synthesized by Sigma Genosys and Operon Biotechnologies, Inc. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. HEK 293 and HeLa cells were from ATCC. Lipofectamine™ 2000 transfection reagent was purchased from Invitrogen. Anti-FLAG M2 affinity gel and anti-FLAG M2 monoclonal antibody-peroxidase conjugate both were from Sigma. BD Living Colors™ Full-Length A.v. polyclonal antibody (anti-GFP) was from Clontech, and Glu-Glu monoclonal antibody (anti-EE) was from Covance. B861 (anti-G16α), a polyclonal antibody generated against a peptide fragment of G15/16 and Z811, an anti-Gq/11/14/16 polyclonal antibody, which recognizes each of the Gα subunits of the Gqα family and was generated against a C-terminal peptide sequence shared by Gqα and G14α, were generous gifts from Dr. Paul Sternweis (University of Texas Southwestern Medical Center, Dallas). Protein A-Sepharose® 4 Fast Flow was from GE Healthcare. Peroxidase-conjugated goat anti-mouse IgG antiserum was purchased from Rockland, Inc., and peroxidase-conjugated goat anti-rabbit antibody was from Bio-Rad. [9,10-3H]Palmitic acid (31 Ci/mmole) and En3Hance™ autoradiography enhancer were purchased from PerkinElmer Life Sciences, and myo-[2-3H]inositol (20 Ci/mmole) was purchased from American Radiolabeled Chemicals, Inc.

cDNA Constructs and Mutagenesis—PCR amplification was used to isolate the N-terminal domains of G14α and G16α from the full-length Gα plasmids. Specifically, primers were designed to amplify cDNA sequences G14α (1-102) and G16α (1-123). Gα-specific oligomers included an N-terminal KpnI restriction site (sense oligomer) or an Agel C-terminal site (antisense oligomers). For amplifying the N terminus of G14α (NTG14α), the primers were as follows: 5′-AAGGTACCGCC-ACCATGGCCGGC-CTGCT, CTGC-3′ (sense) and 5′-CGAC-CCGTTCCACGCGGCGCTCCTTCTT-3′ (antisense). For amplifying the N terminus of G16α (NTG16α), the primers were as follows: 5′-AAGGTACCACATGGCCGGCCGGC-CTGCT, CTGC-3′ (sense) and 5′-CGAC-CCGTTCCACGCGGCGCTCCTTCTTCT-3′ (antisense). The amplified Gα sequences were digested with KpnI and Agel restriction enzymes and subcloned into pEGFP-N1 upstream and in-frame with the EGFP coding region.

A FLAG epitope tag was added to the 3′ end of the NGα-EGFP coding region by PCR amplification of a KpnI-NotI fragment that included the FLAG sequence. An antisense primer was designed to eliminate the stop codon of the EGFP sequence and introduce the FLAG sequence with a new 3′ stop codon. The antisense primer was 5′-ATCGATCGATGCGGCCGCT- TTACTTGTGCTGGCTCTTGGATGCTTCTTACAG-CTCGTCAATGC-3′ and was used in combination with either the NTG14α or NTG16α sense primer listed previously to generate a KpnI-NotI fragment from the existing NTGα-EGFP sequence for subcloning into pEGFP-N1.

To introduce site-specific mutations into the NTGα-EGFP-FLAG and full-length Gα-EE constructs, the QuikChange mutagenesis kit was used according to the manufacturer’s sug-
gestions. The R/Q and K/Q mutations in the NTG14α-9Q and NTG14α-5Q constructs were generated by multiple rounds of site-directed mutagenesis with different primer sets. The specific mutations were introduced in the following order for NTG14α-EGFP-FLAG: 3Q (R23Q, K29Q, K30Q), 5Q (3Q plus K12Q, R16Q), 7Q (5Q plus R26Q, R27Q), and 9Q (7Q plus R33Q, R34Q). The specific mutations were introduced in the following order for NTG14α-EGFP-FLAG: 2Q (K36Q, K37Q), 3Q (2Q plus R30Q, 5Q (3Q plus K19Q, R23Q).

The NTG14α-5Q sequence was substituted into G16α and G16α-Q212L using PCR amplification and subcloning techniques. G16α-(1–123) was cut from G16α and G16α-Q212L at sites KpnI and SacII. The same region was amplified from NTG16α-5Q using the NTG16α sense primer listed previously and the following antisense primer: 5′-GATGCCCAGGGCTCTGCTGCTGCTGCTCCAAG-3′. The amplified fragment was subcloned into the open G16α plasmids at sites KpnI and SacII.

**Cell Culture and Transient Transfection**—HEK 293 cells and HeLa cells were cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; CellGro) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and penicillin/streptomycin (CellGro). For transient transfections, LipofectamineTM 2000 transfection reagent was used according to the manufacturer’s suggested protocol. Transfections were carried out for 24 h after transfection.

**Fractionation and Serial Extraction**—Cells were transiently transfected at a density of 7.125 × 10^6 cells/plate in 10-cm² plates with 24 μg of DNA/plate. The following day, cells were lysed in hypotonic lysis buffer (50 mM HEPES, pH 8.0, 1 mM EDTA, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride) with a Teflon Dounce homogenizer (50 strokes), and broken cells were normalized to isotonic conditions. The R/Q and K/Q mutations in the NTG14α-5Q sequence was substituted into G16α-(1–123) was cut from G16α and G16α-Q212L at sites KpnI and SacII. The same region was amplified from NTG16α-5Q using the NTG16α sense primer listed previously and the following antisense primer: 5′-GATGCCCAGGGCTCTGCTGCTGCTGCTCCAAG-3′. The amplified fragment was subcloned into the open G16α plasmids at sites KpnI and SacII.

**Immunoblot Analysis**—Nitrocellulose membranes were treated as described previously (26). For immunoblotting, membranes were incubated in a primary antibody dilution for 1 h at room temperature or overnight at 4 °C. The following dilutions were used for primary antibodies: anti-GFP 1:1,000, anti-EE 1:2,000, Z811 1:2,000, and anti-G16α 1:10,000 in blocking buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5% milk, 0.5% Tween 20, 0.02% sodium azide), and anti-FLAG 1:2,000 in Tris-buffered saline + 0.1% Tween 20 (TBST, 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Membranes were washed three times with TBST and then probed with the appropriate horseradish peroxidase-conjugated secondary antisera for 1 h at room temperature. The following dilutions were used for secondary antibodies: goat anti-rabbit IgG 1:30,000 in TBST and goat anti-mouse IgG 1:20,000 in TBST. The protein bands were visualized using chemiluminescence and exposed to film.

Where indicated, protein expression levels were quantified by densitometry analysis using Scion Image freeware software. Briefly, protein bands were selected within identical rectangles, and the integrated density was calculated for each sample. Background density measured in an identical rectangle on a section of film separate from the protein band of interest was subtracted from each measurement.

**Metabolic Labeling and Immunoprecipitation**—Cells were transfected at a density of 7.125 × 10^6 cells/plate in 10-cm² plates with a total of 24 μg of DNA/plate. For experiments with the NTGα-EGFP-FLAG fusion proteins, 24 μg of the fusion protein of interest was transfected per plate. For experiments with full-length Gα, cells were transfected with 16.8 μg of EE-tagged Gα ± C/S, 4.8 μg of GBγ1, and 2.4 μg of GBγ2. Twenty four hours after transfection, cells were collected and immunoprecipitated according to a method described in detail elsewhere with minor modifications (27, 28). Specifically, cells for autoradiography were metabolically labeled with 1 mCi/ml [3H]palmitic acid for 2 h at 37 °C in palmitate labeling media (DMEM, 2.5% dialyzed fetal bovine serum, 1× sodium pyruvate, and 1× non-
essential amino acids (CellGro). Because G\(_{14}\alpha\) has been recalcitrant to metabolic labeling with \([^{3}H]\)palmitate in these studies for unknown reasons, three 10-cm\(^2\) plates of G\(_{14}\alpha\) ± C/S-expressing cells were used for each labeling experiment and each immunoblotting experiment, whereas one 10-cm\(^2\) plate of transfected cells was used for each of the other experiments. All cells were lysed and fractionated as described. Cytosol fractions were saved for immunoprecipitation.

For NTG\(_{0}\)-EGFP-FLAG-expressing cells, the membrane pellet was resuspended in 500 \(\mu\)l of Low Salt Buffer and Dounce-homogenized as before (50 strokes). The broken membranes were diluted with an additional 1.5 ml of Low Salt Buffer to wash away nonspecific proteins in the membrane fraction, and ultracentrifugation was repeated. The membrane pellet was resuspended in 500 \(\mu\)l of Tris Buffer (50 mM Tris, pH 7.4, 5 mM MgCl\(_2\), 1 mM EGTA, 150 mM NaCl, 1 mM EDTA, 2 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupeptin, 1 \(\mu\)M phenylmethysulfonyl fluoride), and Dounce-homogenized as before. n-Dodecyl-\(\beta\)-maltoside (D\(_{2}BM\); Calbiochem) was added to a final concentration of 2%. Membrane proteins were extracted with 2% D\(_{2}BM\) for 3 h, rotating end-over-end at 4 °C, and debris was pelleted by ultracentrifugation (100,000 \(\times\) g, 4 °C, 30 min). Membrane extracts and cytosol were incubated overnight at 4 °C with anti-FLAG M2 affinity gel, rotating end-over-end. The following day, the anti-FLAG resin was pelleted and washed three times with Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl). The resin then was resuspended in 2\(\times\) Laemmli Sample Buffer (100 mM Tris, pH 6.8, 0.5% SDS, 20% glycerol, 0.5% \(\beta\)-mercaptoethanol, 0.004% bromophenol blue).

For \(\Gamma\alpha\) ± C/S-expressing cells, membrane pellets were immediately resuspended in Tris Buffer without an additional wash step, and D\(_{2}BM\) was added to both membrane and cytosol fractions to a final concentration of 2%. G\(_{14}\alpha\) ± C/S and G\(_{16}\alpha\) ± C/S were extracted from membranes and cytosol with a 3-h incubation in 2% D\(_{2}BM\) at 4 °C with end-over-end rotation. Cell debris was pelleted by ultracentrifugation (75,000 rpm, 4 °C, 30 min). Prior to immunoprecipitation, soluble extracts were blocked for 2 h with 0.7 \(\mu\)g/\(\mu\)l bovine serum albumin (Sigma) and 50 \(\mu\)l of protein A-Sepharose. Anti-EE monoclonal antibody was diluted 1:150 in each membrane and cytosol sample for overnight immunoprecipitation of EE-tagged G\(_{14}\alpha\) and G\(_{16}\alpha\) proteins at 4 °C. The following day, Sepharose beads were pelleted at 200 \(\times\) g, 4 °C for 1 min. Beads were washed three times with Tris Buffer + 0.01% D\(_{2}BM\), then resuspended in 2\(\times\) Laemmli sample buffer.

All samples were heated to 95 °C for 1 min and spun down at 14,000 rpm, and the entire supernatants were loaded onto 11% polyacrylamide gels (\(\Gamma\alpha\) ± C/S samples) or 15% polyacrylamide gels (NTGa-EGFP-FLAG samples) for SDS-PAGE separation. Samples for immunoblot analysis were transferred to nitrocellulose membranes by Western transfer, and immunoblotting was carried out as described. Gels for autoradiography were prepared according to a method described previously (28). Fixed proteins were treated with En\(^{3}\)Hance\textsuperscript{TM} solution for autoradiography amplification according to manufacturer’s instructions and were exposed to film at −80 °C for indicated times. Hydroxylamine treatment and \([^{35}S]\)methionine labeling were carried out as described previously (28).

Confocal Fluorescence Microscopy—HeLa cells at a density of 1.75 \(\times\) 10\(^5\) cells/well were transfected directly on sterile coverslips in 24-well plates with 0.8 \(\mu\)g of DNA/well. Twenty four hours post-transfection, cells were fixed using an adaptation of methods described previously (29). In brief, cells were washed one time in phosphate-buffered saline and then incubated in fixation buffer (20 mM PIPES, pH 7.0, 1 mM MgCl\(_2\), 0.5 mM EGTA, 1 mM glutaraldehyde, 1 \(\mu\)g/ml aprotinin, 0.1% Triton X-100, 3.7% paraformaldehyde) for 15 min at room temperature. Coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories). An LSM510 confocal laser scanning microscope (Zeiss) was used for imaging, and pictures were taken using a 100\(\times\) oil immersion objective. Images were processed using the Zeiss LSM image browser (version 2.801123) and Adobe Photoshop 7.0 (Adobe Systems).

Measurement of Inositol Phosphate Accumulation—Inositol phosphate formation was measured as described elsewhere (30). In brief, 4.75 \(\times\) 10\(^5\) HEK 293 cells per well in 12-well plates were transfected as described with EE-tagged Ga-Q/L ± C/S or pCDNA3.1, G\(_{16}\beta\) and G\(_{12}\gamma\) cDNA in a ratio of 6:3:1 Ga\(_{14}\beta\), G\(_{12}\gamma\). The following amounts of Ga cDNA were used per well: 2 \(\mu\)g of G\(_{14}\alpha\)-Q/L WT, -C4S, -C6S, and G\(_{16}\alpha\)-Q/L WT; 0.6 \(\mu\)g of G\(_{14}\alpha\)-Q/L-C5S,C6S and -C4S,C5S,C6S; 0.3 \(\mu\)g/well of all other Ga-Q/L cDNAs were used for transfection. cDNA amounts of G\(_{16}\beta\) and G\(_{12}\gamma\) were adjusted to maintain the 6:3:1 ratio and pCDNA3.1 was transfected to bring total amount of cDNA/well up to 0.5 \(\mu\)g (G\(_{14}\alpha\)-Q/L-C5S,C6S and -C4S,C5S,C6S transfections had an approximate total of 1 \(\mu\)g/well). cDNA amounts were determined according to similar protein expression levels determined by Western blot and densitometry analysis. Cells were transfected for 5 h and then metabolically labeled overnight in inositol-free complete medium containing 4 \(\mu\)Ci/ml [\(^{3}H\)inositol. The following day, cells were incubated for 50 min at 37 °C in DMEM buffered with 25 mM HEPES, pH 8.0, and containing 10 mM LiCl\(_{2}\). After solubilization of cells with 20 mM formic acid and neutralization with 0.7 \(\times\) NH\(_{4}\)OH, [\(^{3}H\)]inositol phosphates were separated by anion exchange chromatography (AG 1-X8 Dowex, Bio-Rad) using increasing amounts of ammonium formate. Total [\(^{3}H\)]inositol phosphate content was assessed by liquid scintillation spectrometry. Cell lysates were prepared for immunoblotting by extracting protein directly in the plate wells with 1\(\times\) Laemmli sample buffer. Collected lysates were sonicated for 5 s, and proteins were denatured at 95 °C for 5 min, and 20% of each lysate was loaded on an 11% polyacrylamide gel for electrophoretic separation, followed by Western transfer and immunoblotting. Protein levels were quantitated by densitometry analysis, and inositol phosphate accumulation was plotted as a ratio of total inositol phosphates to adjusted Ga protein expression level.

RESULTS

The N Termini of G\(_{14}\alpha\) and G\(_{16}\alpha\) Are Membrane Targeting Domains—The primary objective of this study was to determine the potential for the G\(_{14}\alpha\) and G\(_{16}\alpha\) N termini to direct plasma membrane localization, with the specific goals of identifying hydrophobic and ionic signals within this region important for localization and Ga signaling function. Therefore, we utilized two complementary approaches to compare biochem-
ical properties that regulate the isolated Gα N-terminal peptides and the corresponding full-length G14α and G16α proteins in cells. In this way, we were able to dissect membrane targeting signals that are native to the specific region of the Gα N terminus and independent of Gβγ interactions, and then evaluate the importance of these signals for normal biological activity of the full-length proteins.

To begin, we isolated the N-terminal domains of G14α and G16α by PCR (Fig. 1) and expressed them as fusion proteins with enhanced green fluorescent protein (EGFP). The fusion proteins express robustly in HEK 293 and HeLa cells and with enhanced green fluorescent protein (EGFP). The fusion signals that are native to the specific region of the Gα family members is highly diverse. A, amino acid sequence alignment of the N termini of the Gα family members. Conserved residues are indicated below the alignment with an asterisk. Palmitoylated cysteine residues (Gα/C1α) and putative sites for palmitoylation (Gα/C1α) are identified in boldface type. Basic positively charged residues are identified above the alignment with +, B, schematic illustrating the structure of the NTGα-EGFP-FLAG fusion constructs. C, NTGα-EGFP fusion protein expression in lysates of transfected HEK 293 cells. Cells were lysed in 1× Laemmli Sample Buffer 24 h post-transfection. 20% of each lysate was loaded onto a 15% polyacrylamide gel for PAGE separation, followed by Western blot analysis using an anti-FLAG antibody, and chemiluminescence was used for visualization.

To test if addition of the Gα N termini promotes membrane localization of EGFP, we examined the subcellular distribution properties of EGFP-FLAG and the NTGα-EGFP-FLAG fusion proteins expressed in HEK 293 cells (Fig. 2). Immunoreactivity in samples of cytosolic and particulate fractions indicates that the N termini of G14α and G16α drive membrane association of the otherwise soluble EGFP (Fig. 2A). We believe that the lower protein band in the cytosol fraction is N-terminally degraded fusion protein because this protein band is found exclusively in cytosol, similar to EGFP alone (Fig. 2A). Confocal fluorescence imaging of the EGFP fusion proteins in HeLa cells supports the fractionation data, indicating that the fusion proteins, but not EGFP, are targeted to the plasma membrane (Fig. 2B). Similar results were obtained with HEK 293 cells (data not shown).

Hydrophobic Interactions Mediate Association between the Plasma Membrane and the N Terminus of G14α and G16α—To identify the biochemical nature of the membrane association of the NTGα-EGFP-FLAG fusion proteins, we subjected membranes from transfected cells to serial extractions in low ionic, high ionic, and hydrophobic buffers. Membranes were washed first three times with physiological salt (100 mM NaCl) and then three times with high salt (1 M NaCl) to disrupt nonspecific and ionic protein interactions, respectively, with membrane lipids. These washes were followed by three extractions with high salt (1M NaCl), or 1% Triton X-100 (TX-100) (three washes in each buffer). Equivalent sample volumes of total membranes (T), first supernatant (S1), second supernatant (S2), and post-extraction pellet (P) were resolved by SDS-PAGE, and Western blot analysis was performed using an anti-FLAG antibody. Proteins were visualized with chemiluminescence. Results are representative of at least three separate experiments. A, NTGα-EGFP-FLAG protein localization in HeLa cells. Membranes were fixed on coverslips for immunofluorescent staining, and adjacent P and S1 bands were duplicated using confocal microscopy as described under “Experimental Procedures.” Bar indicates 10 μm. Images are representative of many cells observed in at least three separate transfections.
Importance of Gα N-terminal Cysteines and Polybasic Region

G14α and G16α Are Palmitoylated at Their N Termini—Although the N-terminal domains of G14α and G16α exhibit hydrophobic properties in membrane preparations, the primary sequences appear to encode soluble proteins. The most likely source of hydrophobicity for soluble proteins is incorporation of long chain fatty acids. Many Gα subunits are lipid-modified at the N terminus with myristate and/or palmitate (1), although it is unknown whether or not G14α and G16α incorporate lipid. G14α and G16α are unique among Gαs because they each have three cysteine residues in their respective N termini that are putative acceptor sites for palmitate (27, 32) (Fig. 1A). Because G14α and G16α, like Gαq and G11α, lack the +2 glycine sequence requirement for myristoylation (27), we screened the Gα N-terminal fusion proteins for incorporation of [3H]palmitate. HEK 293 cells transiently expressing EGFP-FLAG, NTG14α-EGFP-FLAG, or NTG16α-EGFP-FLAG were metabolically labeled with [3H]palmitic acid. Membrane and cytosolic fractions were prepared from labeled cells, and EGFP-FLAG or NTGα-EGFP-FLAG fusion constructs were immunoprecipitated from each fraction. Although EGFP-FLAG was entirely cytosolic, NTG14α- and NTG16α-EGFP-FLAG were found in both cytosol and membrane fractions by immunoblotting (as shown previously (28)). Only membrane-bound NTG14α and NTG16α-EGFP-FLAG, however, yielded a [3H] radiolabel signal by autoradiography (Fig. 4A). Again, we believe the lower protein band in cytosol immunoprecipitates is fusion protein-degraded at the N terminus because it is purely cytosolic protein and does not incorporate palmitate.

Thioester bonds that attach palmitate to target proteins are sensitive to NH2OH cleavage (28). Therefore, to confirm the source of the autoradiograph band as a [3H]palmitate signal, we subjected gels from a similar labeling experiment to washes with either 0.5 M Tris, pH 7, or 0.5 M NH2OH, pH 7. After washing immunoprecipitates with NH2OH, the [3H] radiolabel signals for NTG14α- and NTG16α-EGFP-FLAG were lost, whereas Tris Buffer did not affect either signal (Fig. 4B), thereby confirming the attachment of [3H]palmitate as the source of the signals. Cells were labeled in parallel with [35S]methionine to control for loss of protein during gel washes.

Palmitoylation Requires Different Sequence-specific Sites in G14α and G16α N Termini—With three cysteine residues each in the N termini of G14α and G16α, there are seven combinations of cysteines that potentially could be important for palmitoylation. Two cysteines of the three in G14α and G16α (Cys-5, Cys-6 and Cys-9, Cys-10, respectively) are conserved across Gα family members (Fig. 1A). Each of these cysteines in G14α and G11α are palmitoylated because mutation of both residues together is necessary to eliminate palmitoylation (7, 16). For this reason, we began identification of sites important for palmitoylation by using site-directed mutagenesis to change the pair of conserved cysteines in NTG14α- and NTG16α-EGFP-FLAG to serines. Similar to G14α and G11α, the double cysteine-to-serine (C/S) point mutants of both fusion constructs failed to incorporate palmitate (Fig. 5). Accordingly, mutation of all three cysteine residues in both NTG14α- and NTG16α-EGFP-FLAG also eliminated palmitoylation. These results established the requirement of one or both of the conserved cysteines for palmitoylation of the N termini of G14α and G16α. The necessity of the third, nonconserved cysteine residue, however, was unresolved. Unexpectedly, we found that this third cysteine is differentially required for palmitoylation of the G14α and G16α N termini. Mutating each cysteine individually in NTG14α-EGFP-FLAG revealed that all three amino acids are required for palmitoylation.
acids are required for palmitoylation of the Gα \textit{N}-terminal domain. Similar analysis of the cysteines in the N terminus of Gα\textsubscript{16} indicated that whereas both Cys-9 and Cys-10 are necessary for palmitoylation, the third cysteine (Cys-13) is dispensable under these conditions (Fig. 5). These findings are summarized in Table 1. Of note, NTG\textsubscript{14α} and NTG\textsubscript{16α}-EGFP-FLAG fusion proteins containing multisite C/S mutations remain in the membrane fraction to a degree (Fig. 5). We believe this is nonspecific membrane association because these mutants do not incorporate palmitate. Furthermore, much of this protein partitions to the cytosol with additional washes in physiological salt (see Fig. 6B).

**The Palmitoylation State of the Gα \textit{N}-Terminus Correlates with Plasma Membrane Localization Patterns**—Consistent with a role for palmitoylation in promoting membrane attachment, immunoprecipitation studies revealed a corresponding decrease in fusion protein in cell membranes for all cysteine point mutants displaying a loss of palmitoylation (Fig. 5). To verify these findings, we used confocal fluorescence imaging to examine subcellular localization of the C/S mutants in intact HeLa cells. Consistent with our earlier results, each of the C/S single or multisite mutants of NTG\textsubscript{14α} and NTG\textsubscript{16α}-EGFP-FLAG that failed to incorporate palmitate also did not localize to the plasma membrane but instead displayed diffuse cytosolic and nuclear staining (supplemental Fig. 1 and Table 1). Of note, the single cysteine point mutation of NTG\textsubscript{16α}-EGFP-FLAG that did not interfere with protein palmitoylation, C13S, retained the plasma membrane targeting pattern seen with the wild-type Gα\textsubscript{16} fusion construct.

**Critical Cysteine Residues, but Not the Polybasic Region, Are Necessary for Plasma Membrane Binding of Gα \textit{N} Termini**—The addition of palmitate to the EGFP fusion proteins provides a mechanism for membrane binding and likely is responsible for the hydrophobic nature of the membrane interaction shown in Fig. 3. However, it also has been postulated that basic residues in the N termini of Gα\textsubscript{14} and Gα\textsubscript{16} form a positively charged structural patch on one surface of the N-terminal α-helix and help mediate membrane binding through ionic interactions (22), although this has never been tested experimentally. To test this hypothesis, we introduced combinations of multiple point mutations in the polybasic regions of NTG\textsubscript{14α}-EGFP-FLAG and NTG\textsubscript{16α}-EGFP-FLAG that eliminated increasing amounts of this charge. Ultimately, we selectively changed nine and five basic amino acids in NTG\textsubscript{14α}-EGFP-FLAG and NTG\textsubscript{16α}-EGFP-FLAG, respectively, to glutamine (NTG\textsubscript{14α}-9Q and NTG\textsubscript{16α}-5Q; Fig. 6A). Cell membranes expressing wild-type fusion protein, nonpalmitoylated C/S point mutants, or polybasic region point mutants were subjected to serial protein extraction with washes of increasing concentrations of salt or detergent. The nonpalmitoylated C/S fusion proteins were washed from the membranes with a single rinse in physiological salt buffer (150 mM NaCl; Fig. 6B). Residual NTG\textsubscript{14α}-C4S,C5S,C6S-EGFP-FLAG was removed in a single high salt wash, and whereas a relatively small amount of NTG\textsubscript{14α}-C9S,C10S,C13S-EGFP-FLAG was retained in membranes until detergent treatment, we suggest that this residual protein is aggregated and diluted to undetectable levels following multiple washes. The large majority of both proteins, however, were removed in the initial wash, suggesting that associa-

### TABLE 1

| Plasma membrane | Cytosol | Palmitate incorporation |
|-----------------|--------|-------------------------|
| **NTG14α-EGFP-FLAG** |       |                         |
| WT              | ++     | +                       | Yes                     |
| C5S,C6S        | -      | +                       | No                      |
| C5S,C6S,C6S    | -      | +                       | No                      |
| C4S            | -      | +                       | No                      |
| C5S            | -      | +                       | No                      |
| C6S            | -      | +                       | No                      |
| 3Q             | ++     | +                       | ND                      |
| 7Q             | ++     | +                       | ND                      |
| 9Q             | ++     | +                       | Yes                     |
| **NTG16α-EGFP-FLAG** |       |                         |
| WT              | ++     | +                       | Yes                     |
| C9S,C10S      | -      | +                       | No                      |
| C9S,C10S,C13S | -      | +                       | No                      |
| C9S            | -      | +                       | No                      |
| C10S           | -      | +                       | No                      |
| C13S           | +      | +                       | Yes                     |
| 2Q             | ++     | +                       | ND                      |
| 3Q             | ++     | +                       | ND                      |
| 5Q             | ++     | +                       | Yes                     |

* Cytosol localization includes apparent nuclear localization.

**FIGURE 6. Specific cysteine residues, but not basic residues, are important for membrane association. A.**, Gα \textit{N}-terminal amino acid sequences of mutated NTG\textsubscript{14α} and NTG\textsubscript{16α}-EGFP-FLAG constructs. Mutated amino acids are indicated in **boldface** type. B, serial extraction of wild-type (WT), C/S, and polybasic mutants of NTG\textsubscript{14α} and NTG\textsubscript{16α}-EGFP-FLAG from HEK 293 membranes. Twenty-four hours after transfection, membranes expressing the EGFP fusion proteins were prepared by ultracentrifugation at 100,000 × g and subjected to serial washes in buffers supplemented with 150 mM NaCl, 1 mM NaCl, or 1% Triton X-100 (TX-100) (three washes in each buffer). Equivalent sample volumes of total membranes (T), first supernatant (S1), second supernatant (S2), and post-extraction pellet (P) were resolved by SDS-PAGE (15% polyacrylamide). Western blot analysis was performed using an anti-FLAG antibody. To assist the reader in interpretation of the data, adjacent P and T bands were duplicated using Adobe Photoshop 7.0 (Adobe Systems) as described under "Experimental Procedures."
Importance of Gα N-terminal Cysteines and Polybasic Region

![Image](324x26 to 388x38)

**FIGURE 7.** N-terminal basic residues are not important for plasma membrane localization or palmitoylation of NTG14α-EGFP-FLAG. Wild-type (WT) and polybasic mutant NTG14α-EGFP-FLAG (A) and NTG14α-EGFP-FLAG (B) localization and palmitoylation in whole cells. Cells were transfected with indicated DNAs and fixed 24 h post-transfection. Images were taken with an LSM510 confocal laser scanning microscope and processed as described under “Experimental Procedures.” Images are representative of many cells observed in at least three separate transfections. 24 h after transfection, parallel cells were labeled with [3H]palmitic acid for 2 h. Cell lysates were separated into membrane and cytosol fractions by ultracentrifugation at 100,000 x g and FLAG-tagged proteins were extracted from membranes with 2% DDM (3 h, 4 °C) and immunoprecipitated from membrane extracts overnight at 4 °C with anti-FLAG affinity gel. Immunoprecipitates were resolved by SDS-PAGE (15% polyacrylamide). Top boxes, Western blot analysis of membrane-immunoprecipitated proteins with an anti-GFP antibody. Bottom boxes, autoradiography of labeled proteins. Proteins were fixed as described under “Experimental Procedures” and were exposed to film for 5 days at ~80 °C. Results represent at least two independent experiments with each DNA construct.

As mentioned previously, various constructs with increasing numbers of polybasic point mutations were generated for both NTG14α- and NTG16α-EGFP-FLAG. These constructs and their subcellular distributions and palmitoylation state are listed in Table 1. The specific mutations are denoted under “Experimental Procedures.” Of note, plasma membrane localization patterns did not change for any of the polybasic mutants of NTG14α- or NTG16α-EGFP-FLAG; each of the polybasic region mutants showed nearly exclusive localization at the plasma membrane (Table 1).

Based on the hydrophobic properties the mutant proteins displayed in membrane extraction experiments (Fig. 6B), we then tested the polybasic region mutants for incorporation of palmitate. Cells transiently expressing NTG14α-9Q or NTG16α-5Q were metabolically labeled with [3H]palmitic acid, and the fusion constructs were isolated from prepared membranes by immunoprecipitation. Both NTG14α-9Q and NTG16α-5Q incorporated palmitate like the wild-type G14α or G16α N-terminal domains (Fig. 7). We did note that consistently less NTG14α-9Q was immunoprecipitated from membranes compared with the amount of wild-type NTG14α-EGFP-FLAG immunoprecipitated under the same conditions, and this corresponded to a less robust autoradiography band when cells were labeled with [3H]palmitic acid. We believe the decreased recovery of NTG14α-EGFP-FLAG was because of altered interaction with the anti-FLAG affinity gel used for immunoprecipitation rather than decreased expression or altered lipid modification of NTG14α-9Q. This is supported by normal expression levels and membrane extraction properties of NTG14α-9Q demonstrated in imaging and extraction experiments (Fig. 6B and Fig. 7).

**FIGURE 8.** Full-length G14α and G16α are palmitoylated at N-terminal cysteine residues. In vivo palmitoylation of full-length Gα proteins in HEK 293 cells and the effects of multistatic C/S mutations. Cells were co-transfected with the Gβ1 and Gγ2 constructs and the indicated wild-type (WT) or C/S mutants of G14α and G16α. Twenty four hours after transfection, cells were labeled with [3H]palmitic acid for 2 h. Cell lysates were separated into membrane and cytosol fractions by ultracentrifugation at 100,000 x g, and EE-tagged Gα proteins were extracted from membranes with 2% DDM (3 h, 4 °C) and immunoprecipitated from each fraction with an anti-EE antibody overnight at 4 °C. Immunoprecipitates were resolved by SDS-PAGE (11% polyacrylamide). Top boxes, Western blot analysis of cytosol (C) and membrane (M)-immunoprecipitated proteins with Z811, an anti-Gα13/14/16/25 antibody. Bottom boxes, autoradiography of labeled proteins. Proteins were fixed as described under “Experimental Procedures” and were exposed to film for an average of 35 days at ~80 °C. Results are representative of at least two independent experiments with each DNA construct.

Full-length G14α and G16α Are Palmitoylated at N-terminal Cysteine Residues—To compare our findings with the GαEE-EGFP-FLAG fusion proteins, we examined [3H]palmitate labeling of full-length G14α and G16α in intact cells (Fig. 8). Gα family members are unusual among Gα proteins in that they are intrinsically unstable and exhibit a strong propensity to misfold and aggregate when expressed alone as recombinant protein. Full-length G14α and G16α aggregate and are inactive when
expressed in either bacteria or eukaryotic cells and only fold properly as active protein when co-expressed with Gβγ or when expressed as a chimera with Gα (34, 35). For these reasons, we co-expressed Gβγ and Gγ with EE-tagged versions of G1α and G16α in HEK 293 cells for biochemical analyses of the full-length proteins. We transfected the Gαβγ subunit cDNAs in a ratio of 7:2:1 as has been done similarly for other functional analyses of Gα in cell expression systems (19, 21, 36). The EE-tagged Gα constructs were expressed in HEK 293 cells and immunoprecipitated with an anti-EE antibody from cytosol and membrane fractions 24 h later. Both G1α and G16α from membranes incorporated palmitate, whereas multisite C/S mutations eliminated palmitoylation in both Gα (Fig. 8).

Similar to its N-terminal fusion protein counterpart, nonpalmitoylated G16α-C9S,C10S showed enhanced cysteonic accumulation and reduced membrane binding compared with wild-type G1α. The distribution of G1α-C4S,C5S,C6S between membranes and cytosol, however, appeared identical to that of wild-type G1α. G1α in particular has been recalcitrant to metabolic labeling with [3H]palmitate in these studies for unknown reasons, and so the amount of G1α-expressing cells was increased by 3-fold for these experiments, with a proportional increase in [3H]palmitate label. Therefore, the large amount of cytosolic and membrane-associated G1α likely reflects the 3-fold concentration of protein in each fraction. We hypothesize that the membrane association of G1α-C4S,C5S,C6S is nonspecific and includes nonfunctional, aggregated protein because this mutant does not incorporate palmitate and is relatively inactive in functional assays of second messenger signaling (see Fig. 10, A and B). Furthermore, confocal microscopic imaging of both G1α-C4S,C5S,C6S and G16α-C9S,C10S,C13S indicates that these proteins are not present at the plasma membrane but rather are concentrated in discrete intracellular locations, likely as aggregated protein or as targets for proteolytic degradation (data not shown). In support of this idea, one study reports that Gα and G1α are targeted for degradation through the proteasome, and a nonpalmitoylated C/S mutant of Gα, Gα-CCSS, is more rapidly degraded by proteasomal enzymes than is wild-type Gα (37). Whether or not the subcellular locations where we observe G1α-C4S,C5S,C6S and G16α-C9S,C10S are proteasomes, however, remains to be tested.

**Individual Cysteine Residues and the G1α N-terminal Polybasic Sequence Differentially Affect Ga Membrane Binding and Palmitoylation**—To determine the importance of individual cysteine residues for Gα palmitoylation and membrane association, each of the single C/S mutations was introduced into full-length G1α and G16α, and these mutants were co-expressed with Gβγ cells. Membrane and cytosol distribution patterns of the various C/S mutants were different depending on the particular Gα and were surprisingly different from the results acquired with the NGTα-GEGF proteins (Fig. 9). Individual C/S mutations in full-length G1α had a greater apparent effect on the amount of Gα in the membrane fraction than did single-site C/S mutations in G16α. Although a large amount of G16α ± C/S consistently was cystolic, each C/S mutation, including C13S, resulted in a decrease in membrane-bound G16α (Fig. 9B). By contrast, the membrane pool of G1α was only slightly decreased by single-site mutations in the N terminus, with the C5S mutation having the greatest apparent effect (Fig. 9A). These observations are supported by densitometry analysis of relative Gα protein expression levels in membrane and cytosol fractions (data not shown). Contrary to our findings with the N-terminal fusion constructs harboring the same mutations (see Fig. 5), palmitoylation of G1α and G16α was preserved in each of the individual C/S Gα mutants (Fig. 9).

Although the signal from [3H]palmitate was visibly reduced for each protein, the signal strength for most of the C/S mutants appeared consistent with the reduction in protein associated with the membrane fraction as represented in the corresponding immunoblots. These findings are summarized in Table 2. We also noted that G1α-C4S ± C/S and G16α-C6S ± C/S immunoprecipitated from cytosol fractions incorporated [3H]palmitate. Ultracentrifugation should have separated all cellular membranes from the cytosol, so it is possible that this represents a small amount of Gα ± C/S that is palmitoylated but is not associated with intracellular vesicles or other lipid bilayers. We hypothesize that this cystolic pool of G1α and G16α represents protein being trafficked through the cytosol by an unidentified mechanism, although it should be noted that the [3H]palmitate signal is relatively weak compared with the much larger amount of protein recovered from the cytosol fraction as represented in the corresponding immunoblots, and this signal may in fact be an experimental artifact. Of note, the smeared signals in the cytosol lanes for G1α and G16α-C4S represent extensive nonspecific immunoprecipitation of cystolic proteins in this particular experiment, as this smear was evenly distributed throughout the length of each lane (Fig. 9A, Ist box, and other data not shown).
Importance of Gα N-terminal Cysteines and Polybasic Region

Table 2

| Membrane association, palmitoylation, and signaling properties of G_{1α} and G_{16κ} site mutants |
|---------------------------------------------------------------|
| HEK 293 cells were co-transfected with Gβ and Gγ, and the indicated Gα DNAs, followed by biochemical analysis at 24 h post-transfection (see “Experimental Procedures”). Evaluations of membrane association and palmitoylation are based on subcellular fractionation results and autoradiography results, respectively, as presented in Figs. 8 and 9. For membrane association, protein levels are indicated by + or ++ and are based on intensity of immunoreactivity in membrane fractions. Additional or fewer + indicate more or less immunoreactivity, respectively, relative to each wild-type (WT) Gα protein. − indicates minimal immunoreactivity. PLC-β activation levels are based on data shown in Fig. 10, B and D. +++ + + + + + represents maximal activity of WT Gα-Q/L; +++ represents >60% maximal activity; + + represents >30 but <60% maximal activity; + represents >0 but <30% maximal activity; − represents <0% maximal activity. ND, no data; constructs were not tested. |
| Membrane association | Palmitate incorporation | PLC-β activation |
|----------------------|------------------------|------------------|
| G_{1α} WT            | +++                    | Yes              | +++ + + + + |
| C5S,C6S              | ND                     | ND               | +              |
| C4S,C5S,C6S          | + + +                  | Yes              | + + +          |
| C4S                  | + +                    | Yes              | + +            |
| CS5                  | +                      | Yes              | +              |
| C6S                  | + + +                  | Yes              | + + +          |
| G_{16κ} WT           | +++                    | Yes              | +++ + + + + +  |
| C9S,C10S             | −                      | No               | +              |
| C9S                  | +                      | Yes              | + +            |
| C10S                 | +                      | Yes              | + +            |
| C13S                 | +                      | Yes              | + +            |
| 5Q                   | +                      | Yes              | + + + + + +    |

* Gα ≥ 2 C/S constructs contain a constitutively activating point mutation (Q/L) in the GTPase domain.
* Intracellular protein verified by confocal immunofluorescence imaging; data not shown.

Additionally, we tested the biochemical properties of a polybasic region mutant of full-length G_{1α} by mutating the same five basic amino acids that are mutated in the N{T}{G}_{1α}-5Q construct (G16α-Q5Q, see Fig. 6A). Like each of the C/S mutants of G_{1α}, the G_{16α}-5Q construct exhibited diminished membrane binding (Fig. 9B), although by densitometry analysis, the relative activity of G_{16α}-5Q protein in the membrane fraction was only slightly less than relative levels of membrane-bound wild type G_{1α} (data not shown). Importantly, G_{16α}-5Q was still a target for palmitoylation (Fig. 9B).

**Palmitoylated Polycysteine Sequences and N-terminal Positively Charged Amino Acids Are Important for Gα-mediated Activation of Inositol Lipid Signaling in Intact Cells**—Both G_{1α} and G_{16α} directly activate PLC-β to initiate phosphatidylinositol (4,5)-biphosphate hydrolysis and inositol lipid signaling (5, 6). To see if the same sequences important for palmitoylation and plasma membrane association of G_{1α} and G_{16α} are also important for the signaling function of the full-length proteins, we introduced second-site C/S mutations into backgrounds of mutationally activated G_{1α} and G_{16α}. These constructs contain a point mutation (Q/L) in the guanine nucleotide binding domain that abrogates the GTPase activity of the Gα protein (38, 39) and allows for constitutive effector stimulation (G_{16α}-Q/L and G_{1α}-Q/L). As a read-out of recombinant G_{1α}-Q/L- and G_{16α}-Q/L-induced PLC-β activation, we measured accumulation of radiolabeled inositol phosphates in transfected HEK 293 cells following an overnight labeling period with myo-[3H]inositol (Fig. 10). Although expression of G_{1α}-Q/L-stimulated inositol phosphate production more than 7-fold over unstimulated levels, mutation of two or three cysteines inhibited inositol phosphate production equal to or slightly above basal levels, respectively (Fig. 10, A and B; Table 2). Of note, we observed that G_{1α}-Q/L-C4S,C5S,C6S was consistently expressed at much lower levels than the other G_{1α}-Q/L proteins, and transfection of increasing amounts of this cDNA indicated an upper limit to expressed protein levels that was markedly reduced relative to the other Gα proteins. We speculate that this likely is because of increased degradation of the triple C/S mutant of G_{1α} Q/L, because similar findings have been reported for the double C/S mutant of G_{1α} (37). We believe this accounts for the apparent increase in activity of G_{1α}-Q/L-C4S,C5S,C6S above basal when adjusted for protein expression levels and does not reflect a real difference in activity between the double and triple C/S mutants of G_{1α} Q/L (Fig. 10, A and B).

Like the double mutant of G_{1α}-Q/L, G_{16α}-Q/L-C9S,C10S also exhibited minimal stimulation of PLC-β activity, whereas G_{1α}-Q/L increased accumulation of inositol phosphates by 5-fold (Fig. 10, C and D; Table 2). These data are supported by confocal microscopic immunofluorescence images that show localization of wild-type G_{1α}-Q/L and G_{16α}-Q/L at the plasma membrane and in discrete intracellular locations but G_{1α}-Q/L- C4S,C5S,C6S and G_{16α}-Q/L-C9S,C10S,C13S in intracellular spaces only and not at the plasma membrane (data not shown).

Consistent with the changes individual C/S mutations cause in Gα membrane association properties (see Fig. 9), single C/S mutations in G_{1α}-Q/L had a greater impact on PLC-β activation than did single C/S mutations in G_{1α}-Q/L. Interestingly, whereas the C13S mutation was without effect on membrane distribution and palmitoylation of NT G_{1α}-EGFP-FLAG, the same mutation reduced function of G_{1α}-Q/L by ∼50% (Fig. 10, C and D; Table 2). Mutation of the polybasic sequence in G_{16α}-Q/L resulted in a retained capacity of G_{1α}-Q/L-5Q to induce a slight measurable increase in inositol phosphate accumulation over basal levels, although despite similar Gα expression levels, this protein was less active than all the G_{1α}-Q/L single-site C/S mutants (Fig. 10, C and D; Table 2).

**DISCUSSION**

Proper positioning of Gα at the plasma membrane is the critical first level of regulation for G protein-mediated signal transduction. Despite this importance, molecular mechanisms underlying plasma membrane targeting of most Gα are only partially understood, and for G_{1α} and G_{16α}, nothing at all is known about how these Gα are functionally regulated. We had two primary goals with this study. The first was to determine whether the isolated N termini of G_{1α} and G_{16α} alone possess the capacity to drive plasma membrane localization of soluble protein, and to identify possible lipid signals within this region that mediate this process. The second goal was to evaluate roles for the adjacent N-terminal polybasic regions in determining Gα membrane localization, lipid modification, and functional properties. Here we show that the polycysteine and polybasic motifs in the N termini of G_{1α} and G_{16α} serve essential yet differential roles in Gα palmitoylation, membrane localization, and signaling function.

The isolated N termini of G_{1α} and G_{16α} Utilize Distinct Polycysteine Sequences but Not the Polybasic Region for Plasma...
Importance of Go N-terminal Cysteines and Polybasic Region

Membrane Localization and Palmitoylation in Cells—With complementary biochemical and confocal microscopic imaging experiments, we have determined that the isolated N termini of G_{14}α and G_{16}α are palmitoylated at polycysteine sequences, and palmitoylation of these sequences alone provides membrane targeting and binding properties sufficient to direct a cytosolic protein, in this case EGFP, to the plasma membrane. Surprisingly, the sequences required for palmitate incorporation are not conserved across the two Go proteins; NTG_{14}α-EGFP-FLAG requires three contiguous cysteine residues (Cys-4, Cys-5, and Cys-6), whereas NTG_{16}α-EGFP-FLAG requires only Cys-9 and Cys-10 but not the downstream Cys-13. Palmitoylation of the G_{14}α and G_{16}α N termini is the necessary and sufficient signal for plasma membrane localization of the EGFP fusion proteins because interruption of the sequences required for palmitoylation completely eliminates the targeting function of each Go N terminus. By contrast, disruption of the polybasic region, in part or entirely, had no effect on plasma membrane targeting or binding of either NTG_{14}α- or NTG_{16}α-EGFP-FLAG. Furthermore, the polybasic region is not a signal for palmitoylation of the EGFP fusion proteins, indicating that this region per se is not required for productive interaction with an acyltransferase.

Unexpectedly, we found that the isolated N termini of G_{14}α and G_{16}α and their full-length Go counterparts exhibit differential dependence on the polycysteine sequences and the polybasic region for localization and palmitoylation, suggesting that there exist distinct molecular requirements for palmitoylation and membrane localization of a targeted peptide sequence versus a large, multimeric Go protein heterotrimer complex. Specifically, individual C/S mutations had a dominant negative effect on palmitoylation and plasma membrane localization of the EGFP fusion proteins, whereas loss of the polybasic region was without effect. By contrast, the same C/S mutations only partially altered the properties of full-length Go in biochemical and functional assays, whereas the polybasic region of G_{16}α was required for normal levels of membrane binding and G_{16}α-mediated PLC-β activation. The primary molecular property underlying the biochemical behavior of full-length Go, which likely is absent for the EGFP fusion proteins, is the interaction in cells with the Gβγ dimer. N-terminal point mutations in G_{14}α and G_{16}α prevent binding to Gβγ also prevent Go palmitoylation and plasma membrane targeting of the G protein (10, 19), suggesting that heterotrimer formation is a prerequisite to Go palmitoylation and membrane trafficking. The results presented here suggest that co-expressed Gβγ, which also is lipid-modified, can promote palmitoylation and can drive plasma membrane localization of Goα-C/S to an extent. These findings are consistent with reports showing that the other Go family members, G_{q}α and G_{11}α, are targeted for palmitoylation and exhibit normal signaling capacity providing at least one of the two conserved N-terminal cysteine residues (Cys-9 or Cys-10) is intact (7, 8, 16).

Palmitoylation of Distinct N-terminal Polycysteine Sequences Is Critical for G_{14}α and G_{16}α Membrane Localization and Functional Activity—Unequivocally, the different polycysteine sequences in the N termini of G_{14}α and G_{16}α are required for Go palmitoylation and plasma membrane localization necessary for efficient induction of PLC-β signaling pathways in cells. Alteration of these sequences at any one of the three cysteines in each results in reduced interaction with cellular mem-
branes and impaired functional responses, and loss of at least two of the three cysteine residues in either G14ɛ or G16ɛ eliminates signaling function entirely. In general, the functional activity of G14ɛ-Q/L is more sensitive to a single C/S point mutation than is the activity of G14ɛ-Q/L. A small subset of each C/S single mutant of G14ɛ binds membranes and is palmitoylated, although in functional assays, the two preserved cysteine residues are not sufficient to maintain normal levels of signaling by G14ɛ-Q/L; for G16ɛ in particular, all three N-terminal cysteine residues are necessary for full signaling function. A single C/S mutation in G14ɛ, on the other hand, has a lesser impact on membrane binding and effector activation, although at least two of the three cysteines are required for any detectable functional activity. Consistently, the palmitoylation state of G14ɛ ± C/S or G16ɛ ± C/S and the capacity for constitutive PLC-β activation closely correlate with the apparent distribution of each G protein in the membrane fraction. Surprisingly, although the third cysteine in the G16ɛ polycysteine sequence, Cys-13, is unimportant for palmitoylation and plasma membrane targeting of the isolated G16ɛ N terminus, mutating this residue in full-length G16ɛ reduces membrane binding and signaling capacity to approximately the same extent as the C10S mutation in G16ɛ, suggesting that whereas Cys-13 is not palmitoylated in the context of the G16ɛ N-terminal peptide, this residue is of functional importance for the G protein as a whole. Whether or not there are differences in the molecular requirements for acyltransferase activity involving the variant polycysteine sequences in G14ɛ and G16ɛ, which could account for the observed effects of different C/S mutations, should be examined in future studies.

The correlative decrease in membrane binding of G14ɛ or G16ɛ with a single C/S mutation and functional activity of G14ɛ-Q/L and G16ɛ-Q/L with a single C/S mutation could reflect diminished palmitate incorporation. C/S mutations inherently reduce the available putative palmitate acceptor sites on Gα and therefore may alter the stoichiometry of palmitate-bound Gα in the membrane. Fewer palmitate molecules could reduce membrane affinity or increase the rate of depalmitoylation of the Gα protein. Whether or not palmitate binds each cysteine residue in question is unknown. The metabolic labeling techniques used in this study indicate the palmitoylation state of isolated proteins but cannot provide quantitative information regarding stoichiometry or identify specific sites of lipid incorporation in the protein target. Although the single C/S mutants of both G14ɛ and G16ɛ display much weaker signals of [3H]palmitate incorporation relative to wild-type Gα, we can conclude that these proteins are substrates for palmitoylation, but not that fewer lipid molecules are bound. The functional effects of each single C/S mutation in G14ɛ and G16ɛ suggest that all three residues in each Gα may be separate binding sites for palmitate. Our findings with the isolated N terminus of G14ɛ support this idea, although experiments with NTG16ɛ-EGFP-FLAG suggest that Cys-13 in particular is not important for lipid modification of the upstream dual cysteine sequence in G16ɛ and may not be a site of palmitate incorporation, at least in the context of the isolated N terminus. If in fact all three cysteine residues in G14ɛ are palmitoylated as our findings suggest, then G14ɛ has a unique profile of lipid incorporation among all heterotrimeric G proteins, as no other Gα subunits have been shown to have three palmitate-binding sites.

**The Polybasic Region Is Important for G16ɛ Signaling Activity at the Plasma Membrane**—Our studies with full-length Gα provide the first evidence that the polybasic region in the N terminus of G16ɛ is an important determinant of Gα signaling function but not palmitoylation. These findings support a proposed multisignal model for plasma membrane targeting of palmitoylated Gα proteins like those of the Gε family in which surface electrostatic charges and lipid modifications together directly bind the protein to membrane lipids (22). Such a mechanism is utilized by proteins like RGS4 and RGS16, which require both palmitoylation and positively charged protein surfaces in their N-terminal α-helix for functional interaction with the plasma membrane (40–42). Other signaling molecules, including the nonreceptor tyrosine kinase c-Src, the small G protein K-Ras4B, and neuromodulin/GAP-43, also rely on hydrophobic lipid molecules and polybasic motifs for binding to the plasma membrane and cannot actively participate in signaling reactions without the cooperativity of these dual membrane targeting mechanisms (43–48). Similarly, full-length G16ɛ lacking most of this positive charge in the N terminus is markedly reduced in the membrane fraction and exhibits minimal functional activity. The polybasic region in G14ɛ includes several additional positively charged amino acids (nine total) compared with the polybasic region of G16ɛ (six total). Although we were able to compare the effects of substitution mutations in the polybasic region on localization and palmitoylation of the isolated N termini of G14ɛ and G16ɛ, functional experiments with full-length G14ɛ were not included due to the limiting technical barriers of introducing all nine point mutations into the full-length G14ɛ sequence. As Gα and G11ɛ each contain 10 positively charged amino acids in their N termini (Fig. 1A), it will be important to identify the functional importance of these larger regions of basic charge for the other members of the Gε family.

**Implications for G Protein Membrane Localization and Signaling Function**—Previous work indicates that Gα membrane localization and signaling function is dictated by lipid-modifying groups on the Gα N terminus and by interaction with the Gβγ dimer. Here we provide evidence that the polybasic region on G16ɛ also may contribute to membrane binding and signaling activity of palmitoylated Gα. Precisely how these residues are utilized by the protein, however, is unclear. Palmitoylation of the isolated N termini of G14ɛ and G16ɛ and full-length G16ɛ do not require these residues, but full-length G16ɛ has altered subcellular localization and diminished function without them. Therefore, it is likely that this sequence of amino acids is not an intrinsic requirement for palmitoylation of targeted Gα cysteines, but instead it may indirectly affect palmitoylation by regulating localization of the G protein at the plasma membrane.

The polybasic region potentially may be important for maintaining G16ɛ localization at the membrane during palmitate turnover, because palmitoylation of G proteins has been shown to increase during active signaling cycles (49–51). A growing body of evidence demonstrates that Gαε, G11ɛ, and other palmitoylated Gα like Gε dynamically associate with the
plasma membrane during signaling cycles. Agonist activation induces redistribution of G\(_1\alpha\) laterally within the membrane, followed by internalization and, depending on treatment time, either membrane recycling or degradation of G\(_\alpha\) (52, 53). Real time imaging of GFP-tagged G\(_\alpha\) shows only a minor translocation to cytosol following receptor stimulation or direct G protein activation with ALF\(_4\) (13), and other studies show that direct activation or enzymatic depalmitoylation of G\(_\alpha\) and G\(_{16}\alpha\) does not release these G\(_\alpha\) from membranes (54–57). Together these reports suggest that conditions sufficient for palmitate turnover on targeted G\(_\alpha\), in some cases, can induce spatially limited changes in membrane localization of G\(_\alpha\) without causing an extensive loss of G protein from the plasma membrane. It is possible that electrostatic interactions between membrane lipids and the positively charged surface of the G\(_\alpha\) N terminus may be one of the molecular properties preserving G\(_\alpha\) localization at the plasma membrane in these studies.

It is also likely that persistent interaction with G\(_{16}\) during G\(_\alpha\)-mediated effector activation contributes to membrane localization of the G\(_\alpha\) that is repeatedly undergoing depalmitoylation and palmitate reloading. A recent study shows that site mutants of G\(_\alpha\) that cannot bind G\(_{16}\beta\gamma\) also cannot activate PLC-\(\beta\) and inositol lipid signaling, even when targeted to the plasma membrane by a myristoylated peptide sequence (36), and adds to a growing list of reports demonstrating that some heterotrimeric G protein subunits may not fully dissociate in vivo during the GTPase cycle as previously thought (58–60). Although neither the N-terminal cysteines nor the polybasic region amino acids are among the contact sites in the G\(_\alpha\) N terminus for G\(_{16}\) binding based on available heterotrimeric crystal structures (31, 61), it is important to consider that the G\(_{14}\alpha\) and G\(_{16}\alpha\) point mutations utilized in this study could introduce structural changes in the G\(_\alpha\) N terminus that alter the affinity of the G\(_\alpha\)-G\(_{16}\) interaction, and this deficiency could contribute to the observed reduction in G\(_\alpha\) function and membrane localization. Reduced interaction with G\(_{16}\beta\gamma\) in fact may explain the localization and functional differences of the single-site C/S mutants of G\(_{14}\alpha\) ± Q/L and G\(_{16}\alpha\) ± Q/L, because G\(_{16}\beta\gamma\) binding is required for G\(_\alpha\) palmitoylation (10, 19); a less stable heterotrimer could result in reduced G\(_\alpha\) palmitoylation, and both mechanisms could account for reduced G\(_\alpha\) functional activity at the plasma membrane. However, our data suggest that the functional differences measured for the polybasic region mutant of G\(_{16}\alpha\) are independent of G\(_{16}\beta\gamma\) based on the capacity for G\(_{16}\alpha\)-5Q to incorporate palmitate like wild-type G\(_{16}\alpha\). Of note, mutation of Arg-21 in G\(_\alpha\) was shown to inhibit heterotrimer formation by an unknown mechanism (62), but the corresponding amino acid in G\(_{14}\alpha\), Arg-27, was dispensable for G\(_{14}\alpha\) palmitoylation and therefore does not seem to be involved in G\(_{16}\beta\gamma\) binding (10). Interestingly, G\(_{14}\alpha\)-R27A exhibited a measurable decrease in receptor-stimulated PLC-\(\beta\) activation (10). This study, together with the results presented here, supports the idea that N-terminal basic residues in G\(_\alpha\) family members have a discrete role in mediating G\(_\alpha\) functional activity at the plasma membrane.

Conclusions—In this study we have established that distinct polycysteine sequences in the N-terminal domains are critical for palmitoylation of G\(_{14}\alpha\) and G\(_{16}\alpha\) and are sufficient for plasma membrane binding when expressed alone. The full-length G proteins utilize a combination of biochemical mechanisms, including, but not restricted to, palmitoylation of the G\(_\alpha\) N terminus for membrane localization and signaling function. N-terminal basic residues, which include an hypothesized positively charged basic patch on the G\(_\alpha\) surface, appear to be important for fully functional G\(_{14}\alpha\)-mediated inositol lipid signaling at the cell membrane but not for palmitoylation of the adjacent polycysteine sequences. Mutation of these basic amino acids in G\(_{14}\alpha\) or of N-terminal cysteine residues in G\(_{14}\alpha\) and G\(_{16}\alpha\) reduces the cellular pool of active and palmitoylated membrane-bound G\(_\alpha\) to varying degrees. Although the precise roles of G\(_\alpha\) palmitoylation, heterotrimer formation, and the charged G\(_\alpha\) polybasic domain during the G protein signaling cycle remain to be resolved, this study presents the first evidence that G\(_{14}\alpha\) and G\(_{16}\alpha\) function may be regulated by all three of these mechanisms.

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REFERENCES

1. Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995) J. Biol. Chem. 270, 503–506
2. Zhang, Z., Melia, T. J., He, F., Yuan, C., McGough, A., Schmid, M. F., and Wenesel, T. G. (2004) J. Biol. Chem. 279, 33937–33945
3. Gilman, A. G. (1987) Ann. Rev. Biochem. 56, 615–649
4. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Science 252, 802–808
5. Rhee, S. G. (2001) Ann. Rev. Biochem. 70, 281–312
6. Hubbard, K. B., and Hepler, J. R. (2006) Cell. Signal. 18, 135–150
7. Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Leis, M. J., and Bourne, H. R. (1993) J. Biol. Chem. 268, 25001–25008
8. Edgerton, M. D., Chabert, C., Chollet, A., and Arkinstall, S. (1994) FEBS Lett. 354, 195–199
9. Wise, A., Parenti, M., and Milligan, G. (1997) FEBS Lett. 407, 257–260
10. Evanko, D. S., Thiyagarajan, M. M., and Wedegaertner, P. B. (2000) J. Biol. Chem. 275, 1327–1336
11. Kosloff, M., Elia, N., Joel-Almagor, T., Timberg, R., Zars, T. D., Hyde, D. R., Minke, B., and Selinger, Z. (2003) EMBO J. 22, 459–468
12. Takida, S., and Wedegaertner, P. B. (2004) FEBS Lett. 567, 209–213
13. Hughes, T. E., Zhang, H., Logothetis, D. E., and Berlot, C. H. (2001) J. Biol. Chem. 276, 4227–4235
14. Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, A. G., and Mumby, S. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3675–3679
15. Parenti, M., Vigano, M. A., Newman, C. M., Milligan, G., and Magee, A. I. (1993) Biochem. J. 291, 349–353
16. Hepler, J. R., Bidlcome, G. H., Kleuss, C., Camp, L. A., Hofmann, S. L., Ross, E. M., and Milligan, A. G. (1996) J. Biol. Chem. 271, 496–504
17. Smotrys, J. E., and Linder, M. E. (2004) Ann. Rev. Biochem. 73, 559–587
18. Resh, M. D. (1999) Biochim. Biophys. Acta 1451, 1–16
19. Evanko, D. S., Thiyagarajan, M. M., Siderovski, D. P., and Wedegaertner, P. B. (2001) J. Biol. Chem. 276, 23945–23953
20. Michaelson, D., Ahearn, I., Bergo, M., Young, S., and Philips, M. (2002) Mol. Biol. Cell 13, 3294–3302
21. Takida, S., and Wedegaertner, P. B. (2003) J. Biol. Chem. 278, 17284–17290
