The Dually Acylated NH$_2$-terminal Domain of G$_{i1\alpha}$ Is Sufficient to Target a Green Fluorescent Protein Reporter to Caveolin-enriched Plasma Membrane Domains

PALMITOYLATION OF CAVEOLIN-1 IS REQUIRED FOR THE RECOGNITION OF DUALLY ACYLATED G-PROTEIN α SUBUNITS *IN VIVO**

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Here we investigate the molecular mechanisms that govern the targeting of G-protein α subunits to the plasma membrane. For this purpose, we used G$_{i1\alpha}$ as a model dually acylated G-protein. We fused full-length G$_{i1\alpha}$ or its extreme NH$_2$-terminal domain (residues 1–32 or 1–122) to green fluorescent protein (GFP) and analyzed the subcellular localization of these fusion proteins. We show that the first 32 amino acids of G$_{i1\alpha}$ are sufficient to target GFP to caveolin-enriched domains of the plasma membrane *in vivo*, as demonstrated by co-fractionation and co-immunoprecipitation with caveolin-1. Interestingly, when dual acylation of this 32-amino acid domain was blocked by specific point mutations (G2A or C3S), the resulting GFP fusion proteins were localized to the cytoplasm and excluded from caveolin-rich regions. The myristoylated but nonpalmitoylated (C3S) chimera only partially partitioned into caveolin-containing fractions. However, both nonacylated GFP fusions (G2A and C3S) no longer co-immunoprecipitated with caveolin-1. Taken together, these results indicate that lipid modification of the NH$_2$-terminal of G$_{i1\alpha}$ is essential for targeting to its correct destination and interaction with caveolin-1. Also, a caveolin-1 mutant lacking all three palmitoylation sites (C133S, C143S, and C156S) was unable to co-immunoprecipitate these dually acylated GFP-G-protein fusions. Thus, dual acylation of the NH$_2$-terminal domain of G$_{i1\alpha}$ and palmitoylation of caveolin-1 are both required to stabilize and perhaps regulate this reciprocal interaction at the plasma membrane *in vivo*. Our results provide the first demonstration of a functional role for caveolin-1 palmitoylation in its interaction with signaling molecules.

The α subunits of heterotrimeric G$_i$, G$_o$, and G$_z$ proteins contain a common methionine-glycine-cysteine motif (MGC) at their extreme NH$_2$ terminus. This MGC motif is also shared by most members of the Src family of non-receptor tyrosine kinases (NRTKs),[1] with the exception of Src itself and Blk (1, 2). This motif represents a consensus sequence that specifies the covalent attachment of a 14-carbon lipid (myristate) and a 16-carbon lipid (palmitate) to glycine 2 and cysteine 3, respectively. Moreover, some NRTKs may possess an additional palmitoylated cysteine (*e.g.* cysteine 5 in Lck and cysteine 6 in Fyn). Point mutations that prevent myristoylation and/or palmitoylation of Gα subunits and NRTKs drastically reduce the membrane avidity of these polypeptides, indicating that these lipid modifications act cooperatively to anchor these proteins to the cytoplasmic leaflet of the plasma membrane (3, 4).

Because myristoylation is an irreversible modification, it has been proposed that it may play only a passive role as a hydrophobic handle or appendage for anchoring Gα subunits to membranes. In contrast, palmitoylation is a reversible modification and is therefore potentially regulatable, suggesting that it may play a more complex role in this process. In support of this hypothesis, palmitoylated G$_{z\alpha}$ and G$_{i1\alpha}$ are totally refractory to the GTPase-stimulating activities of the Gα-interacting protein and the regulator of G-protein signaling 4. Thus, palmitoylation may represent a mechanism for prolonging or potentiating G-protein signaling (5).

Moreover, palmitoylation is required for a productive interaction between G-protein-coupled receptors and Gα subunits. The myristoylated but palmitoylation-negative C3S mutant of G$_{i1\alpha}$ is unable to functionally couple with the α2A adrenergic receptor co-expressed in transfected COS-7 cells, even when the concentration of the mutant in the membrane is equivalent to that of wild type G$_{i1\alpha}$ (6). One possible explanation for these results is that the C3S mutant of G$_{i1\alpha}$ might be segregated into a distinct plasma membrane compartment that is separated from where the receptor is located.

Recent evidence suggests that G-proteins are not homogeneously distributed throughout the cell surface. Instead, they may be concentrated within vesicle-like microdomains of the plasma membrane termed caveolae that exist in most cell

[1] The abbreviations used are: NRTK, non-receptor tyrosine kinase; GFP, green fluorescent protein; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; Gα, G-protein α subunit; PBS, phosphate-buffered saline.

Targeting of Lipid-modified G-Protein α Subunits

types. It has been proposed that caveolae function as signaling depots where G-proteins and G-protein-coupled receptors, as well as other signaling molecules, are clustered (7–9).

Caveolae membranes fractionate as low buoyancy complexes on sucrose density gradients and are resistant to solubilization by nonionic detergents, such as Triton X-100, at low temperatures, due to their specialized lipid composition that is rich in cholesterol and glycosphingolipids (10, 11). The position of caveolae within these gradients can be tracked by immunoblotting with specific antibodies directed against the caveolin family of proteins, which are the principal structural protein components of caveolae membranes. Caveolins interact with each other to form homo- and hetero-oligomeric complexes, bind cholesterol, and interact with a variety of signaling molecules. For example, caveolin binding to Gα subunits is sufficient to maintain them in the inactive GDP-liganded conformation (12–14). According to the "caveolae signaling hypothesis," Gα subunits may become activated when G-protein-coupled receptors are recruited to caveolae upon agonist stimulation (15–17).

Recent studies have indicated that point mutations that abolish myristoylation and/or palmitoylation prevent the association of $G_{i1\alpha}$ and other dually acylated proteins (such as NRTKs and endothelial nitric oxide synthase) with caveolin-enriched membrane fractions (18–20). This raises the possibility that NH_2-terminal lipid modification can also specify membrane compartmentalization of dually acylated molecules within caveolae by acting as a targeting signal and/or through direct interactions with the lipid or protein components of caveolae (*e.g.* cholesterol, glycosphingolipids, or caveolins).

To evaluate the potential role of the NH_2-terminal region of Gα subunits and its lipid modification in targeting to the plasma membrane, we constructed a variety of lipid-modified and non-lipid-modified fusion proteins using GFP. We examined their subcellular distribution by immunofluorescence microscopy and their ability to co-fractionate and co-immunoprecipitate with caveolin-1 using transient expression in COS-7 or 293T cells. In addition, we studied the behavior of GFP fused to a short caveolin-binding domain (21) recently identified within Gα subunits (specifically, residues 186–200 in $G_{i1\alpha}$).

EXPERIMENTAL PROCEDURES

Materials—The GFP cDNA was a gift of Dr. J. Pines (Wellcome/CRC Institute, Cambridge, United Kingdom). It contains five mutations (F64L, S65T, V163A, I167T, and S175G) resulting in improved levels of fluorescence at 475 nm and is correctly folded at 37 °C. The cDNA encoding nonpalmitoylated caveolin-1 was as described previously (22). Antibodies and their sources were as follows: (i) anti-caveolin-1 IgG (mAb 2297 and mAb 2234; gifts of Dr. J. R. Glenney, Transduction Laboratories); (ii) anti-GFP IgG (rabbit polyclonal antibody; CLON-TECH); and (iii) rabbit anti-$G_{i1\alpha}$ polyclonal anti-serum (I1C; raised against a synthetic peptide corresponding to amino acids 160–169 of the $G_{i1\alpha}$ subunit). [9,10-^{3}H]Palmitic acid (40–60 Ci/mmol) and [9,10-^{3}H]myristic acid (40–60 Ci/mmol) were from Amersham. All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

Construction of $G_{i1\alpha}$-GFP Fusion Proteins—To generate GFP fusion proteins, the cDNA encoding GFP was placed at the 3′ end of the full-length rat $G_{i1\alpha}$ sequence (amino acids 1–355; FL-GFP) and of the following sequences of wild type rat $G_{i1\alpha}$: (i) amino acids 1–32 (32aaWT-GFP), (ii) amino acids 1–122 (122aa-GFP), and (iii) amino acids 186–200 (caveolin-binding domain; CBD-GFP). Two additional constructs were generated by fusing GFP to the 1–32 NH_2-terminal amino acid sequence of $G_{i1\alpha}$: (i) 32aaG2A-GFP (mutation at the myristoylation site by replacing glycine 2 with alanine), and (ii) 32aaC3S-GFP (mutation at the palmitoylation site by substituting cysteine 3 with serine). Finally, GFP fusions were constructed in which the first 6 of 10 NH_2-terminal amino acids of $G_{i1\alpha}$ were fused to GFP. All $G_{i1\alpha}$-GFP fusion proteins were generated by polymerase chain reaction amplifications using the appropriate primers and subsequently subcloned in pcDNA3 expression vector (Invitrogen). The correctness of intended base substitutions and the absence of unwanted mutations were verified by DNA sequencing.

All DNA manipulations, including ligations, bacterial transformation, and plasmid purification, were carried out using standard procedures.

Cell Culture and Transient Transfection—COS-7 and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells (30–50% confluent) were transfected using the calcium phosphate precipitation method and routinely analyzed 48 h after transfection.

Immunoblot Analysis—Cellular proteins were resolved by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose membranes. Blots were incubated for 2 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween 20) containing 2% powdered skim milk and 1% bovine serum albumin. After three washes with TBST, membranes were incubated for 2 h with the primary antibody (~1,000-fold dilution in TBST) and for 1 h with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (~5,000-fold dilution). Proteins were detected using the ECL detection kit (Amersham). For cell fractionation, cells were harvested and resuspended in 0.5 ml of hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM EDTA with 0.067 TIU/ml aprotinin (Sigma) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma) as protease inhibitors). Cell suspensions were incubated on ice for 30 min, freeze/thawed, and homogenized with a Teflon/glass homogenizer. After a low speed centrifugation to remove unbroken cells and the nuclear pellet, samples were centrifuged for 30 min at $200,000 \times g$ at 4 °C in a Beckman TL100 centrifuge. The pellets (particulate fraction) and the acetone-precipitated supernatants (soluble fraction) were separated by SDS-PAGE (12.5% acrylamide) and analyzed by Western blotting.

Metabolic Labeling and Immunoprecipitation—After transfection, COS-7 cells were labeled for 4 h with [9,10-^{3}H]palmitic acid (150 μCi/ml) or [9,10-^{3}H]myristic acid (50 μCi/ml) in Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal bovine serum, 5 mM sodium pyruvate, antibiotics, and L-glutamine. After washing with PBS, cells were lysed in 0.2 ml of 1% (w/v) SDS. After breakage of DNA by repeated pipetting and boiling for 4 min, 0.8 ml of the following mixture (Mix I) was added to each sample: 1.25% (w/v) Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.5, and protease inhibitors as indicated above. After centrifugation at $13,000 \times g$ for 10 min at 4 °C, 2 μl of GFP antibody were added to each supernatant, and the samples were incubated overnight at 4 °C. Twenty-five μl of a 1:1 suspension of protein A-Sepharose CL-4B beads (Pharmacia Biotech Inc.) in Mix II (4 parts of Mix I plus 1 part of 1% SDS) were added to each sample and incubated for ~5 h at 4 °C with continuous rotation. Immunoprecipitates were washed three times for 15 min at 4 °C with Mix II (1 ml) and then washed once with 50 mM Tris-HCl, pH 6.8, and heated in Laemmli sample buffer (23) containing 20 mM dithiothreitol. Samples were separated by SDS-PAGE, and gels were treated with 2,5-diphenyloxazole for fluorography (24). To maximize sensitivity, we used a gel miniaturization procedure that involved soaking the 2,5-diphenyloxazole-impregnated gels after water washing in 50% (w/v) polyethylene glycol 3000 (Sigma) at 70 °C for 15 min before drying (25). Dried gels were exposed on Kodak X-Omat AR-5 films at −70 °C.

Fluorescence Microscopy—Transfected 293T cells grown on glass coverslips were washed three times with PBS and fixed for 30 min at room temperature with 2% (w/v) paraformaldehyde in PBS. Fixed cells were rinsed with PBS and treated with 25 mM NH_4Cl in PBS for 10 min at room temperature to quench free aldehyde groups. Cells were washed three times with PBS, and slides were mounted with Slow-Fade antifade reagent (Molecular Probes, Eugene, OR) and observed under a Bio-Rad MR600 confocal microscope at an excitation wavelength of 488 nm.

Preparation of Caveolin-enriched Membrane Fractions—COS-7 cells were scraped into 2 ml of 2-(N-morpholino)ethanesulfonic acid-buffered saline (MBS; 25 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5, and 0.15 M NaCl) containing 1% (v/v) Triton X-100. Homogenization was carried out with 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homogenate and centrifuged at 39,000 rpm for 16–20 h in a SW41 rotor (Beckman Instruments). A light scattering band confined to the 15–20% sucrose region was observed that contained caveolin-1 but excluded most of the other cellular proteins. From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 12 fractions. An equal volume of each gradient fraction was separated by SDS-PAGE and subjected to immunoblot analysis.

Co-Immunoprecipitation—Cells were washed twice with PBS and lysed for 30 min at 4 °C in a buffer containing 10 mM Tris, pH 8.0, 0.15
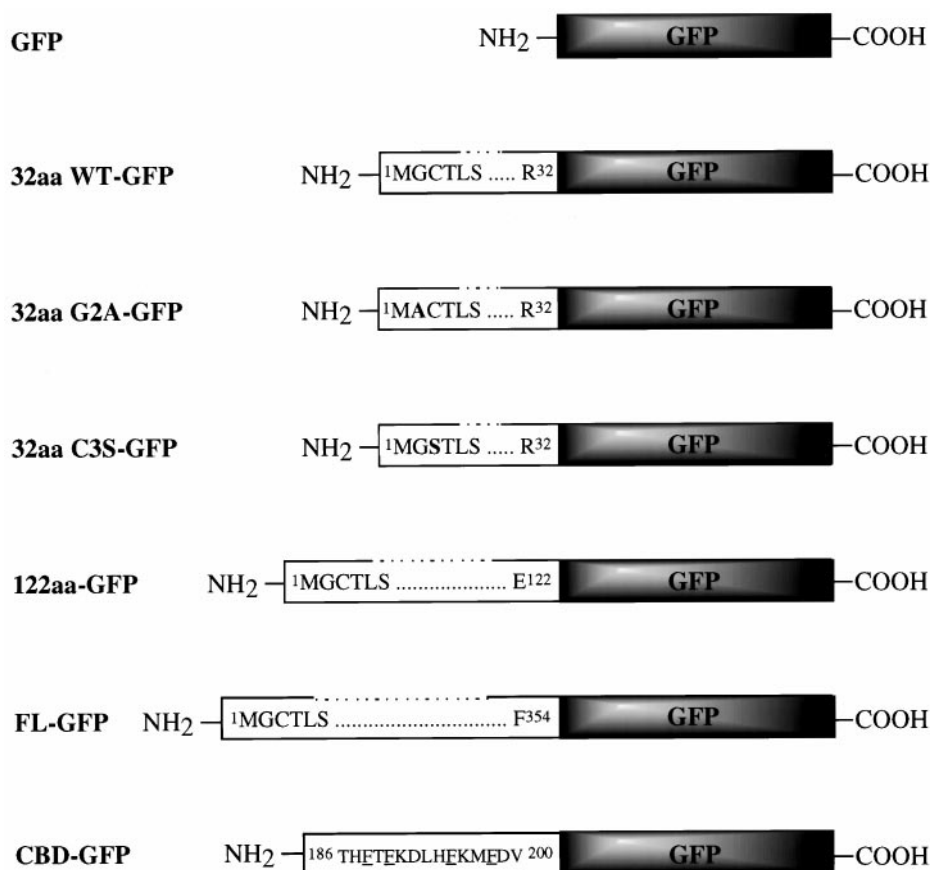
FIG. 1. **Schematic illustration of $G_{i1\alpha}$-GFP fusion proteins used in this study.** The full-length sequence (*FL*) or the first 32 (*32aaWT*) or 122 (*122aa*) NH$_2$-terminal amino acids of $G_{i1\alpha}$ were fused to the NH$_2$ terminus of GFP. In 32aaG2A-GFP and 32aaC3S-GFP, each of the 32 amino acid sequences of $G_{i1\alpha}$ fused to GFP was mutagenized to convert glycine 2 to alanine and cysteine 3 to serine, respectively (substitutions are shown as *bold letters*). In the CBD-GFP chimera, the caveolin-binding domain containing the phenylalanine repeat (*underlined*; Ref. 21) was NH$_2$ terminally fused to GFP.

M NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM octyl glucoside. Samples were pre-cleared for 1 h at 4 °C using protein A-Sepharose (20 μl; slurry, 1:1) and subjected to overnight immunoprecipitation at 4 °C using anti-caveolin-1 antibody (10 μl; mAb 2234) and protein A-Sepharose (30 μl; slurry, 1:1). After three washes with the immunoprecipitation buffer, samples were separated by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose. Blots were then probed with a polyclonal anti-GFP antibody.

RESULTS

The Dually Acylated NH$_2$-terminal Region of $G_{i1\alpha}$ Is Sufficient to Target Soluble GFP to the Plasma Membrane in Transfected COS-7 and 293T Cells—To date, all studies examining the role of lipid modification in the subcellular localization of $G_{i1\alpha}$ have relied on loss of function mutations in the context of the full-length $G_{i1\alpha}$ protein. To determine whether the acylated NH$_2$ terminus of $G_{i1\alpha}$ is sufficient to direct membrane localization, we constructed fusion proteins consisting of full-length wild type $G_{i1\alpha}$ or the first 6, 10, 32, or 122 amino acids of $G_{i1\alpha}$ attached to the NH$_2$ terminus of GFP, a normally cytosolic protein.

Moreover, to define the role of fatty acylation in the specific subcellular localization of the 32aa-GFP chimera, the known palmitoylation site was mutated by replacing cysteine 3 with serine (32aaC3S-GFP). Likewise, the known myristoylation site, glycine 2, was mutated to alanine to generate a nonmyristoylated fusion protein (32aaG2A-GFP). An additional fusion protein (CBD-GFP) was engineered by attaching amino acids 186–200 of $G_{i1\alpha}$ to the NH$_2$ terminus of GFP. Amino acids 186–200 of $G_{i1\alpha}$ contain the known caveolin-binding domain within G_{i1} (21).

The characteristics of all these engineered fusion proteins are detailed in Fig. 1. The cDNAs encoding wild type GFP and $G_{i1\alpha}$-GFP fusions were individually transiently transfected into COS-7 cells. Their expression was analyzed by Western blotting of whole cell lysates using an anti-GFP polyclonal antibody. Fig. 2 shows that 48 h after transfection, the cells produced comparable levels of all of the fusion proteins. In striking contrast, fusion proteins between GFP and the first 6 or 10 amino acids of $G_{i1\alpha}$ were expressed at very low levels (data not shown) and were not further characterized.

Subsequently, fatty acylation patterns of the $G_{i1\alpha}$-GFP fusions were determined by metabolic radiolabeling of transfected COS-7 cells with [^{3}H]myristate or [^{3}H]palmitate, followed by immunoprecipitation of cell lysates with the GFP antibody. As expected, GFP alone was neither myristoylated nor palmitoylated (data not shown), whereas FL-GFP, 122aa-GFP, and 32aaWT-GFP incorporated both fatty acids (Fig. 3). The 32aaC3S-GFP fusion incorporated [^{3}H]myristate but not [^{3}H]palmitate, whereas 32aaG2A-GFP lacked both fatty acids, thus behaving as the corresponding full-length $G_{i1\alpha}$ G2A mutant, in which myristoylated glycine was required for efficient palmitoylation (26).

The subcellular localization of GFP and each of the $G_{i1\alpha}$-GFP fusion proteins was next examined by fractionating postnuclear COS-7 cell homogenates into cytosolic (Fig. 4, *S*) and membrane (Fig. 4, *P*) fractions by ultracentrifugation at $200,000 \times g$. Each fraction was then analyzed by immunoblotting with an anti-GFP antibody.

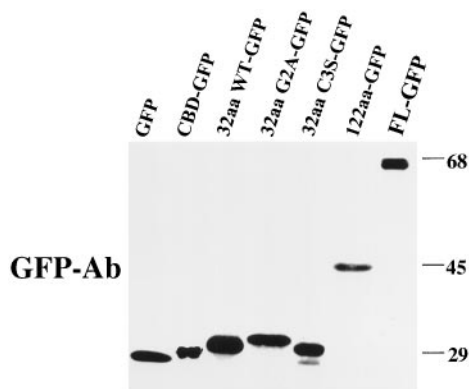As expected, GFP was entirely soluble, whereas FL-GFP,

FIG. 2. **Expression of GFP and $G_{i1\alpha}$-GFP fusion proteins in COS-7 cells.** COS-7 were transiently transfected with different constructs using the calcium phosphate precipitation method. Forty-eight h after transfection, cells were harvested and lysed in Laemmli sample buffer. Lysates were separated by 12.5% SDS-PAGE and immunoblotted with a polyclonal anti-GFP antibody.
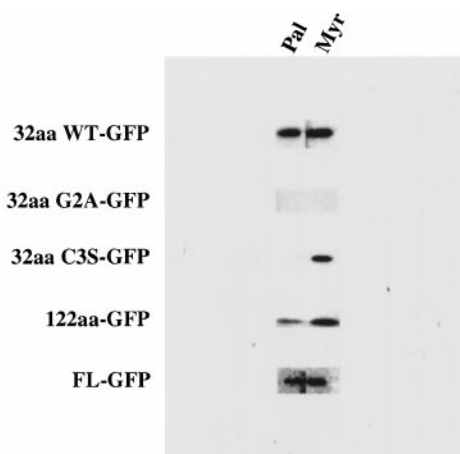

FIG. 3. **Fatty acylation of the $G_{i1\alpha}$-GFP fusion proteins.** Transfected COS-7 cells were metabolically labeled for 4 h with either [³H]palmitic acid (*Pal*) or [³H]myristic acid (*Myr*). Cell lysates were immunoprecipitated with an anti-GFP antibody and analyzed by SDS-PAGE and fluorography. To enhance signal detection, fluorographed gels were miniaturized as described under "Experimental Procedures."

122aa-GFP, and 32aaWT-GFP were primarily concentrated in the particulate fraction (80–90%; Fig. 4). In contrast, the amount of nonpalmitoylated 32aaC3S-GFP was greatly increased within the soluble fraction (>50%; Fig. 4). An even greater shift was observed with 32aaG2A-GFP, which is neither myristoylated nor palmitoylated, because ~90% of this protein was detected in the soluble fraction, as was CBD-GFP (Fig. 4). We conclude from these experiments that the NH_2-terminal $G_{i1\alpha}$ sequence is sufficient to target a cytosolic protein to cellular membranes and that myristic and palmitic acids cooperatively affect membrane binding. The results observed with CBD-GFP fusion suggest that binding to caveolin-1 and anchorage to cellular membranes are expressed by distinct domains of the $G_{i1\alpha}$ protein. It is worth noting that the particulate fraction in this assay contains total cell membranes, including plasma membrane and intracellular membranes. Thus, changes in the distribution among various cell membrane compartments will not be appreciated unless a morphological analysis of transfected cells is performed.

To investigate the intracellular localization of the various $G_{i1\alpha}$-GFP fusion proteins, we took advantage of the spontaneous fluorescence of GFP. 293T cells expressing wild type GFP and the $G_{i1\alpha}$-GFP fusions were fixed and examined by laser scanning confocal microscopy. The micrographs presented in
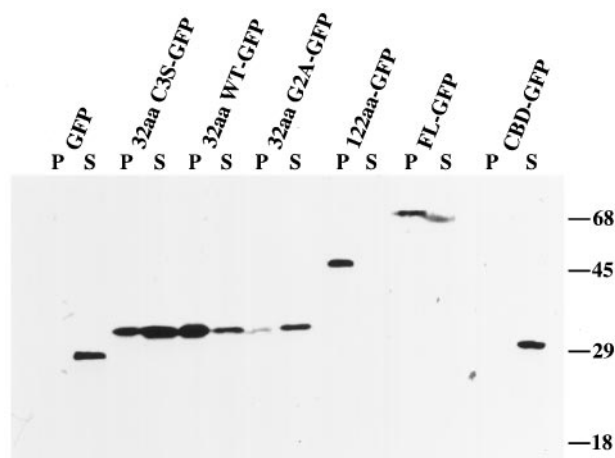

FIG. 4. **Subcellular distribution of GFP and $G_{i1\alpha}$-GFP fusion proteins in transfected COS-7 cells.** Postnuclear supernatants of transfected COS-7 cells were fractionated by ultracentrifugation at $200,000 \times g$ into soluble (*S*) and particulate (*P*) fractions. Partitioning of proteins between the fractions was determined by SDS-PAGE and immunoblotting with an anti-GFP antibody.

Fig. 5 illustrate that although GFP was cytosolic, it gained a plasma membrane localization after fusion with full-length $G_{i1\alpha}$ or its NH_2-terminal 122- and 32-amino acid sequences.

Interestingly, FL-GFP, 122aa-GFP, and 32aaWT-GFP exhibited the same pattern of cellular distribution as wild type $G_{i1\alpha}$ visualized by indirect immunofluorescence staining of 293T cells with a $G_{i1\alpha}$ anti-serum. In contrast, nonacylated 32aaG2A-GFP showed a diffuse cytosolic staining (Fig. 5), whereas nonpalmitoylated 32aaC3S-GFP exhibited a hybrid distribution, as illustrated by the staining of both cell edges and the cytoplasm. The fluorescence associated with the CBD-GFP fusion was diffuse, thus confirming its localization primarily within the cytosol, in agreement with our biochemical experiments (see Fig. 4). Mock-transfected 293T cells showed no detectable fluorescence (data not shown). These results clearly demonstrate that the first 32 amino acids of $G_{i1\alpha}$ contain information that is sufficient to specify a plasma membrane localization, provided that fatty acylation is successfully completed.

Lipid Modification of the NH_2-terminal Region of $G_{i1\alpha}$ Is Required to Deliver a GFP Fusion Protein to Caveolin-enriched Membrane Domains in COS-7 Cells—Recently, it has been shown that heterotrimeric G-protein subunits, as well as a number of other signaling molecules, are concentrated within a distinct subcompartment of the plasma membrane known as caveolae (7–9).

Due to their high content of cholesterol and glycosphingolipids, caveolar membranes are resistant to extraction at 4 °C by nonionic detergents such as Triton X-100 and float on bottom-loaded sucrose density gradients. Using a well-defined procedure that combines both of these physicochemical properties to separate low density, Triton-insoluble membranes containing caveolin-1 (7) from the bulk of cellular membranes and cytosolic proteins, we analyzed by Western blotting the content of GFP, $G_{i1\alpha}$, and $G_{i1\alpha}$-GFP fusions in sucrose density gradient fractions obtained from transfected COS-7 cells.

Caveolin-1, a structural protein of caveolar membranes, was used as a marker protein for the distribution of these detergent-resistant membrane domains. As shown in Fig. 6*A*, the bulk of cellular proteins, including soluble polypeptides, were concentrated in the bottom, high density fractions (40% sucrose layer of the gradient; *lanes 9–12*), whereas caveolin-1 immunoreactivity was exclusively detected in the caveolae fractions (~10–20% sucrose layer of the gradient; *lanes 4* and *5*).
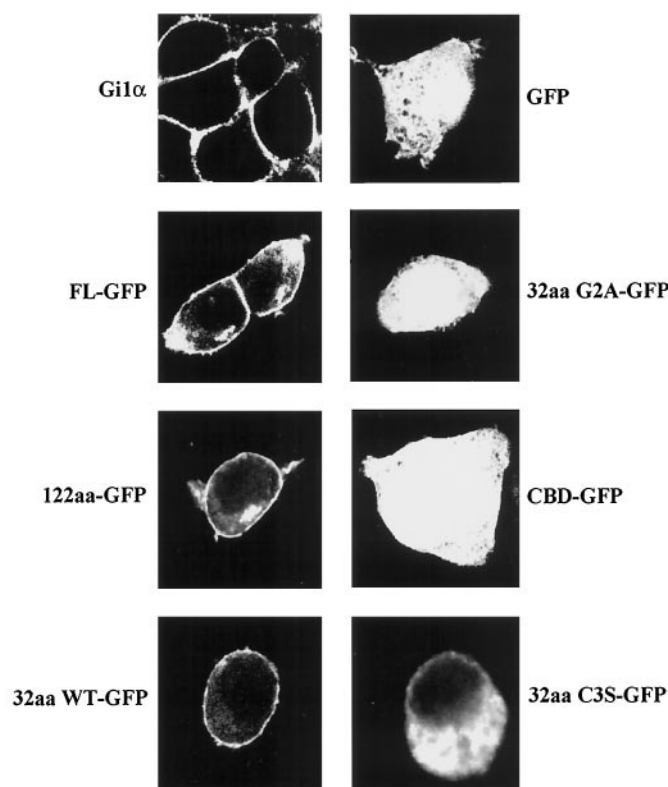
FIG. 5. **Subcellular targeting of $G_{i1\alpha}$-GFP fusion proteins in transfected 293T cells.** 293T cells expressing wild type GFP or $G_{i1\alpha}$-GFP fusion proteins were fixed with 2% (w/v) paraformaldehyde 48 h after transfection. Cells expressing endogenous $G_{i1\alpha}$ were stained with a rabbit antiserum (I1C) raised against a synthetic peptide corresponding to amino acids 160–169 of the $G_{i1\alpha}$ subunit. GFP fluorescence and $G_{i1\alpha}$ indirect immunofluorescence were visualized by confocal microscopy at an excitation wavelength of 488 nm.
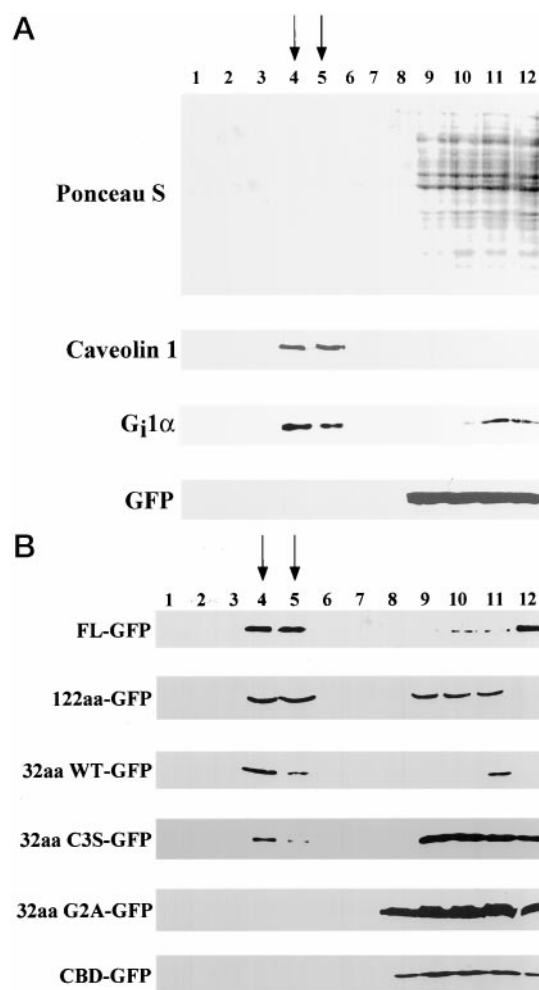

FIG. 6. **Subcellular fractionation of COS-7 cells expressing $G_{i1\alpha}$-GFP fusions.** COS-7 cells transfected with $G_{i1\alpha}$, GFP, and each of the $G_{i1\alpha}$-GFP fusion proteins were extracted in 1% (v/v) Triton X-100 at 4 °C and subjected to ultracentrifugation on sucrose density gradients. One-ml fractions collected from the top of the gradients were separated by SDS-PAGE and transferred to nitrocellulose. Fractions 1–4 (*lanes 1–4*) are the 5% sucrose layer, and fractions 5–8 (*lanes 5–8*) are the 30% sucrose layer. Fractions 9–12 (*lanes 9–12*), containing 40% sucrose, represent the "loading zone" of these bottom-loaded flotation gradients and contain the bulk of the cellular membrane and cytosolic proteins as described previously (14). *Upper panel*, Ponceau S staining of the total cellular proteins; *lower panels*, immunoblotting with anti-caveolin-1, anti-$G_{i1\alpha}$, or anti-GFP antibodies to visualize the distribution of endogenously expressed caveolin-1 and transfected $G_{i1\alpha}$ and $G_{i1\alpha}$-GFP fusion proteins.

Fig. 6*B* shows that FL-GFP, 122aa-GFP, and 32aaWT-GFP fusions exhibited a distribution pattern equivalent to that of wild type $G_{i1\alpha}$, distributing between both caveolin-containing fractions as well as fractions 9–12 (*lanes 9–12*). The palmitoylation-defective 32aaC3S-GFP fusion had a similar distribution; however, compared with the wild type, significantly less of the fusion protein was detected in the low density caveolae fractions and more was present in the soluble, higher density fractions, as might be anticipated by its increased solubility (see Fig. 4).

Accordingly, fully soluble 32aaG2A-GFP and CBD-GFP fusions were completely excluded from the caveolin-containing fractions and were concentrated at the bottom of the gradient, behaving essentially as soluble GFP alone. These observations suggest that amino acids 1–32 of $G_{i1\alpha}$, which contain the myristoyl and palmitoyl moieties, encode the complete address signal for subcellular targeting of the $G_{i1\alpha}$ polypeptide.

Lipid Modifications Are Required for the Binding of $G_{i1\alpha}$ to Caveolin—Caveolin interacts directly with Gα subunits, preferentially recognizing their inactive conformation (13). Thus, it has been suggested that caveolin functions as a scaffolding protein to spatially organize certain caveolin-interacting proteins within caveolae membranes. The scaffolding domain of caveolin consists of a 20-amino acid stretch (residues 82–101) within the NH$_2$-terminal cytoplasmic domain of caveolin-1 (27). A consensus sequence for caveolin binding has been deduced in many caveolae-associated polypeptides, including Gα subunits (21). Lipid modification of Gα subunits is not required for interaction with caveolin-1 *in vitro* (13); however, it remains

unknown whether lipid modification of Gα subunits is required for interaction with caveolin-1 *in vivo*.

In this study, we wanted to examine whether the NH$_2$ terminus of $G_{i1\alpha}$, although located a distance from the caveolin-binding motif (residues 186–200 of $G_{i1\alpha}$), might influence interactions with caveolin-1 *in vivo*. For this purpose, we immunoprecipitated lysates of COS-7 cells transfected with various $G_{i1\alpha}$-GFP fusions with a specific mAb probe that recognizes the extreme NH$_2$ terminus of caveolin-1 and analyzed the composition of these precipitates by Western blotting with a GFP-specific antibody.

Fig. 7*A* shows that FL-GFP, 122aa-GFP, and 32aaWT-GFP clearly co-immunoprecipitated with caveolin-1. In striking contrast, mutant fusions 32aaC3S-GFP, 32aaG2A-GFP, and GFP alone failed to co-immunoprecipitate with caveolin-1. Therefore, even in the absence of the known $G_{i1\alpha}$ caveolin-binding domain, as in 122aa-GFP and 32aaWT-GFP fusions, the NH$_2$-terminal domain of $G_{i1\alpha}$ played a key role in the association of
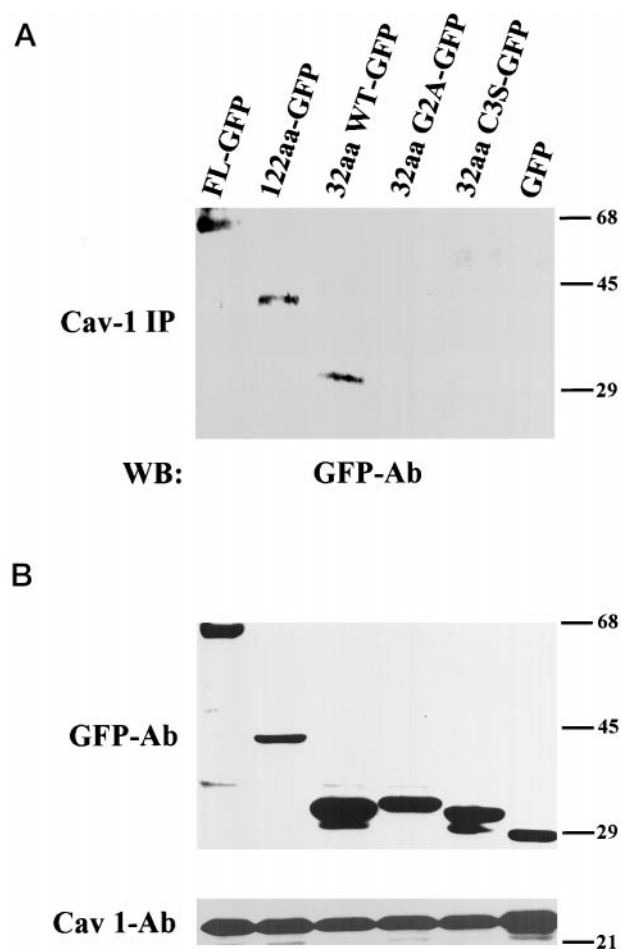
FIG. 7. **Interaction of G$_{i1\alpha}$-GFP fusion proteins with caveolin-1.** *A*, detergent extracts of transfected COS-7 cells were immunoprecipitated with anti-caveolin-1 mAb 2234 IgG and protein A-Sepharose beads. These immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting with anti-GFP antibody. *B*, COS-7 cell extracts contained equivalent amounts of each of the G$_{i1\alpha}$-GFP fusion proteins and of caveolin-1, as determined by Western blot analysis before immunoprecipitation.

FIG. 8. **Palmitoylation of caveolin-1 is required for interaction with G$_{i1\alpha}$.** 32aaWT-GFP was co-transfected in COS-7 cells with the wild type form of caveolin-1 (*cav-1 WT*) or with the palmitoylation-defective caveolin-1 mutant (*cav-1 Cys$^-$*). *A*, detergent extracts of transfected cells were immunoprecipitated with anti-caveolin-1 mAb 2234 IgG and protein A-Sepharose beads. Immunoprecipitates were subjected to SDS-PAGE and assayed by Western blotting with anti-GFP antibody. *B*, Western blot analysis of cell extracts before immunoprecipitation with anti-GFP and anti-caveolin-1 antibodies shows that equivalent levels of 32aaWT-GFP and of wild type and palmitoylation-defective caveolin-1 were expressed in COS-7 cells after transfection.

G$_{i1\alpha}$ with caveolin-1, provided that it is correctly dually acylated.

Palmitoylation of Caveolin-1 Is Required for Its Recognition of the Lipid-modified NH$_2$-terminal Domain of G$_{i1\alpha}$—Caveolin-1 is palmitoylated on three COOH-terminal cysteines, more specifically, residues 133, 143, and 156 (22). The loss of palmitoyl moieties does not affect the membrane insertion of the caveolin-1 polypeptide, its targeting to caveolae membranes, or its ability to form high molecular weight homo-oligomers (22, 28). Palmitoylation of caveolin-1 protects caveolin-1 homo-oligomers from dissociation by SDS denaturation during boiling. However, the physiological relevance of this finding remains unknown.

To determine whether 32aaWT-GFP binds equally well to palmitoylated or nonpalmitoylated forms of caveolin-1, we co-transfected COS-7 cells with this GFP fusion together with either wild type caveolin-1 or a mutant form of caveolin-1 that lacks all three palmitoylation sites (C133S, C143S, and C156S). Extracts of the transfected cells were then prepared and immunoprecipitated with a specific mAb directed against the unique NH$_2$ terminus of caveolin-1 (mAb 2234). These caveolin-1 immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot analysis with a specific GFP antibody. Interestingly, we observed that the 32aaWT-GFP fusion co-immunoprecipitated only from cells expressing wild type caveo-
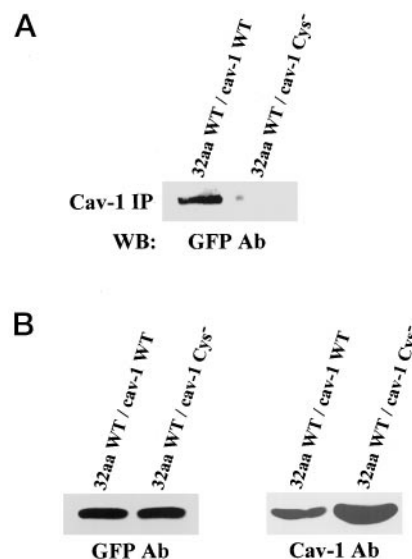
lin-1 (Fig. 8*A*), suggesting that the proper binding of G$_{i1\alpha}$ requires palmitoylation of caveolin-1.

DISCUSSION

There is a general consensus that the dually acylated NH$_2$ terminus of G$_i$/G$_o$/G$_z$ α subunits is required for their attachment to the cytoplasmic leaflet of the plasma membrane. This is based mainly on the observation that simultaneous mutations of glycine 2 and cysteine 3, respectively, the acceptor sites for the attachment of myristate and palmitate, considerably shift Gα subunits to the soluble fraction (26, 29, 30). Similarly, Src-like NRTKs (such as Lck and Fyn) share the same NH$_2$-terminal acylation motif with G$_i$/G$_o$/G$_z$ α subunits and an extra palmitoylated cysteine at position 5 or 6, respectively, and their membrane association is prevented by mutation of these key sites of fatty acylation (3).

However, in the case of Lck and Fyn, the NH$_2$-terminal domain plays a more complex role than merely serving as a membrane-anchoring device. Mutational analysis has demonstrated that the association of Lck and Fyn with the glycosylphosphatidylinositol-anchored membrane proteins mapped to the first 10 amino acids of the NRTKs, and the addition of myristate and palmitate is required for this reciprocal interaction (31). Subsequent studies have highlighted the key role of palmitoylated cysteines in targeting Lck and Fyn to Triton-insoluble, glycolipid-enriched, membrane subdomains (DIGs; Refs. 32–35) and in sorting these polypeptides to different subcellular membrane compartments (36). Thus, the NH$_2$-terminal domain of NRTKs accomplishes the dual function of targeting these polypeptides to a final membrane compartment and stabilizing their association with the cytoplasmic leaflet of the lipid bilayer.

By analogy with NRTKs, a similar role for the dually acylated NH$_2$-terminal domain of G$_i$/G$_o$/G$_z$ α subunits in targeting has been postulated. This view is supported by the finding that Gα subunits, as well as Src-like NRTKs, can be found in flattened (termed DIGs or rafts) or invaginated (termed caveolae)

specializations of the plasma membrane, which are rich in cholesterol and glycosphingolipids (7–9). In contrast, earlier reports have suggested that membrane anchoring and subcellular targeting of G_i α subunits are contained within distinct protein domains. The COOH terminus plays a role in the differential targeting of $G_{i2\alpha}$ and $G_{i3\alpha}$ subunits to the plasma and Golgi membranes, respectively, because intracellular localization of $G_{i2\alpha}/G_{i3\alpha}$ chimeras was dependent on which COOH-terminal half was used (37).

Here we consider the issue that the NH_2 terminus of dually acylated Gα subunits also contributes to their subcellular targeting. We analyzed the ability of various NH_2-terminal extensions of $G_{i1\alpha}$ to act as a membrane-anchoring and targeting signal for a normally soluble carrier protein, namely, GFP. The subcellular targeting of each $G_{i1\alpha}$-GFP fusion construct was evaluated after transient expression in transfected cells using confocal microscopy and biochemical techniques. The fluorescence associated with the full-length $G_{i1\alpha}$-GFP fusion protein localized at the edges of transfected cells behaving as wild type untagged $G_{i1\alpha}$, suggesting that the GFP reporter did not interfere with the proper localization of $G_{i1\alpha}$. Moreover, the full-length $G_{i1\alpha}$-GFP fusion protein co-localized with caveolin-1, a protein marker for caveolae membranes, in low buoyancy fractions purified by sucrose gradient centrifugation and was co-immunoprecipitated by caveolin-1 antibodies.

The same distribution pattern was exhibited by 122aa-GFP and 32aaWT-GFP fusions, indicating that the first 32 amino acids of $G_{i1\alpha}$ contain the signal to deliver polypeptides to low density, caveolin-enriched, plasma membrane domains and to interact with caveolin-1. We also attempted to define the targeting domain of $G_{i1\alpha}$ in greater detail by fusing only the first 6 or 10 residues to GFP. Unfortunately, these GFP fusions were expressed at much lower levels than 32aaWT-GFP in COS-7 cells (data not shown). This may reflect the structural instability of these shorter $G_{i1\alpha}$ fusions because 6 or 10 amino acids would not be long enough to allow the formation of the 30-amino acid residue NH_2-terminal α-helix present in wild type $G_{i1\alpha}$ (38).

As with NRTKs, the cellular targeting of 32aaWT-GFP was strictly dependent on fatty acylation. The 32aaG2A-GFP chimera, which lacked both fatty acids, was diffusely localized within the cytoplasm and behaved as wild type GFP. In contrast, the palmitoylation-defective 32aaC3S-GFP retained some membrane avidity due to the presence of myristate and displayed a reduced association with caveolin-enriched sucrose density fractions.

Interestingly, the binding of nonpalmitoylated 32aaC3S-GFP to caveolin-1 was completely abrogated, suggesting that: (i) co-fractionation with caveolin on sucrose gradients does not necessarily imply a direct physical interaction between the two polypeptides, and (ii) palmitoylation of cysteine 3 is an absolute prerequisite for the binding of $G_{i1\alpha}$ to caveolin *in vivo*. This may explain the results of Huang *et al.* (9), who failed to detect a substantial direct interaction between recombinant myristoylated but palmitoylation-defective $G_{o\alpha}$ and caveolin-1. However, it contrasts with the observed *in vitro* interaction between caveolin-1 and palmitoylated $G_{o\alpha}$ or $G_{i2\alpha}$ expressed by baculovirus-infected Sf9 cells (13).

Using the caveolin-1 scaffolding domain as a receptor to select caveolin-binding peptide ligands from peptide sequences displayed at the surface of the bacteriophage (phage display libraries), two related caveolin-binding motifs have been identified ($\phi X\phi XXXX\phi$ and $\phi XXXX\phi XX\phi$, with ϕ representing aromatic amino acids Trp, Phe, or Tyr) (21). These motifs exist within most caveolae-associated proteins, including $G_{i1\alpha}$ (21). Interestingly, when residues 186–200 of $G_{i1\alpha}$ containing the
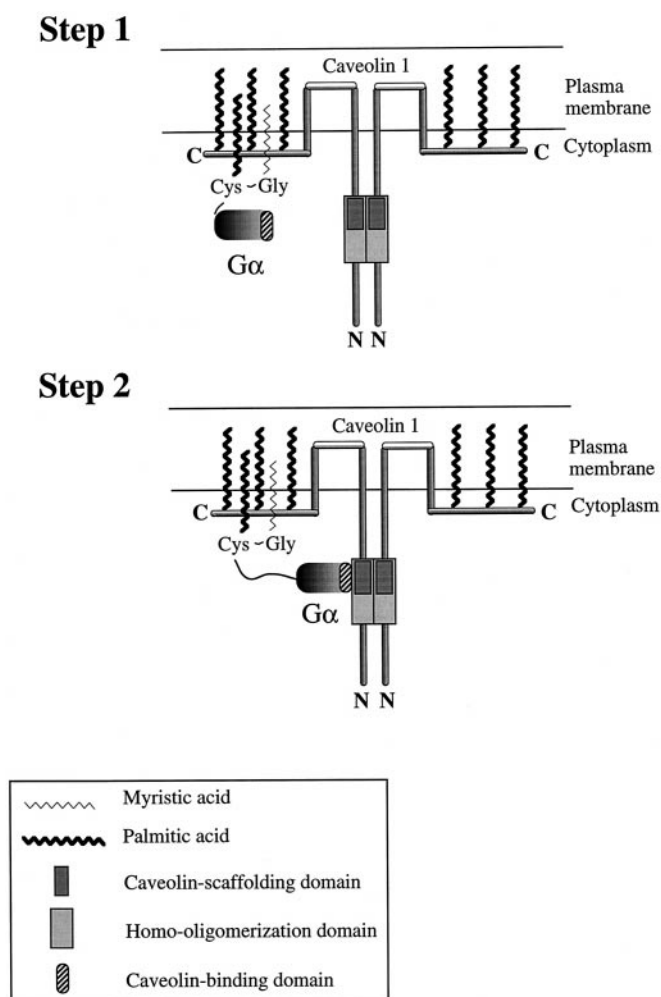

FIG. 9. **Schematic illustration of the caveolar targeting of $G_{i1\alpha}$.** In *Step 1*, palmitic acid of $G_{i1\alpha}$ is responsible for binding to the plasma membrane bilayer and to caveolin-1 oligomers. Palmitic acid residues in the COOH-terminal of caveolin-1 directly interact with palmitic acid residues in $G_{i1\alpha}$. In *Step 2*, the caveolin-binding domain of $G_{i1\alpha}$ interacts with the scaffolding domain of caveolin-1, stabilizing the mutual interaction.

defined motif for interaction with caveolin-1 (21) were fused to GFP, the resulting CBD-GFP chimera did not co-immunoprecipitate with caveolin-1. This may suggest that the fatty acylation of $G_{i1\alpha}$, by promoting an interaction with lipid and/or protein component(s) of the plasma membrane bilayer, must precede caveolin-1 recognition by the caveolin-binding domain *in vivo*. This implies a two-step mechanism for the caveolar targeting of $G_{i1\alpha}$. This proposed mechanism is summarized schematically in Fig. 9. In the first step, mutual interaction of palmitic acid residues in $G_{i1\alpha}$ and caveolin-1 are responsible for the binding of $G_{i1\alpha}$ to caveolin-1. In a second step, this interaction is stabilized by the binding of the caveolin-binding domain of $G_{i1\alpha}$ to the scaffolding domain of caveolin-1. A similar conclusion has been reached regarding the interaction of Ras with caveolin-1 (39). Lipid modification of Ras by prenylation is required for its correct targeting to caveolae *in vivo* but is not required for its *in vitro* interaction with caveolin-1 (39).

The unique role of palmitoylation in mediating the association of $G_{i1\alpha}$ with caveolin-1 is important because, unlike myristoylation, palmitoylation is a reversible modification. For example, in the case of $G_{s\alpha}$ (40–42) and $G_{q/11\alpha}$ (43), regulation of palmitoylation by direct or receptor-mediated activation has been demonstrated experimentally. Our results are also consistent with the previous observation that mutationally acti-

vated $G_{s\alpha}$ showing an accelerated rate of depalmitoylation (41) fails to interact with recombinant caveolin-1, as compared with its wild type counterpart, nonmutated $G_{s\alpha}$ (13).

Caveolin-1 can associate with itself to form Triton-insoluble high molecular mass homo-oligomers (300–400 kDa) *in vivo* as well as *in vitro* (28, 44). It has been proposed that caveolin-1 oligomers complexed with cholesterol and glycosphingolipids can form a matrix within membrane rafts, or caveolae, acting as a scaffold for lipid-modified signaling proteins (17). Although the oligomerization region of caveolin has been mapped to residues 61–101 of the NH_2-terminal cytoplasmic domain (44), recent observations (28) have shown that complete maturation of these high molecular mass complexes requires the palmitoylation of three cysteine residues located at positions 133, 143, and 156 within the COOH-terminal cytoplasmic tail. This COOH-terminal region of caveolin-1 has also been implicated in mediating interactions between individual caveolin-1 homo-oligomers, forming a caveolin-1 network or patch within the plasma membrane (17). Here, we show that the 32aaWT-GFP chimera, when co-expressed in COS-7 cells with a mutant form of caveolin-1 that lacks all three palmitoylation sites, can no longer be co-immunoprecipitated with caveolin-1 IgG, indicating a loss of interaction between the two polypeptides. This finding suggests that palmitoylation of caveolin-1 oligomers is necessary for complex formation with inactive $G\alpha$, and perhaps with other signaling molecules as well, within caveolae membranes *in vivo*.

In conclusion, our results demonstrate that both membrane-anchoring and targeting functions are encoded by the dually acylated NH_2-terminal domain of $G_{i1\alpha}$ and that palmitoylation of $G_{i1\alpha}$ cysteine 3 plays a key role by facilitating the interaction with caveolin-1. A recent study (45) has reached similar conclusions for $G_{z\alpha}$, suggesting specific roles for myristoylation and palmitoylation in membrane targeting. They observed that the attachment of myristate cooperates with $\beta\gamma$ dimer binding to promote the stable association of $G_{z\alpha}$ with membranes and that the addition of palmitate confers specific localization at the plasma membrane. However, these authors did not examine the interaction of $G_{z\alpha}$ with caveolae or caveolins. Thus, regulated palmitoylation of $G\alpha$, on one hand, and caveolin-1, on the other, adds further potential handles to control G-protein-mediated signal transduction.

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The Dually Acylated NH$_2$-terminal Domain of G$_{i1\alpha}$ Is Sufficient to Target a Green Fluorescent Protein Reporter to Caveolin-enriched Plasma Membrane Domains: PALMITOYLATION OF CAVEOLIN-1 IS REQUIRED FOR THE RECOGNITION OF DUALLY ACYLATED G-PROTEIN α SUBUNITS IN VIVO

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