THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 275, No. 3, Issue of January 21, pp. 2191–2198, 2000
Printed in U.S.A.

Lipid-dependent Targeting of G Proteins into Rafts*

(Received for publication, September 27, 1999, and in revised form, November 2, 1999)

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Domains rich in sphingolipids and cholesterol, or rafts, may organize signal transduction complexes at the plasma membrane. Raft lipids are believed to exist in a state similar to the liquid-ordered phase. It has been proposed that proteins with a high affinity for an ordered lipid environment will preferentially partition into rafts (Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G., and Brown, D. A. (1999) *J. Biol. Chem.* 274, 3910–3917). We investigated the possibility that lipid-lipid interactions between lipid-modified proteins and raft lipids mediate targeting of proteins to these domains. G protein monomers or trimers were reconstituted in liposomes, engineered to mimic raft domains. Assay for partitioning of G proteins into rafts was based on Triton X-100 insolubility. Myristoylation and palmitoylation of $G\alpha_i$ were necessary and sufficient for association with liposomes and partitioning into rafts. Strikingly, the amount of fatty-acylated $G\alpha_i$ in rafts was significantly reduced when myristoylated $G\alpha_i$ was thioacylated with *cis*-unsaturated fatty acids instead of saturated fatty acids such as palmitate. Prenylated $\beta\gamma$ subunits were excluded from rafts, whether reconstituted alone or with fatty-acylated α subunits. These results suggest that the structural difference between lipids that modify proteins is one basis for the selectivity of protein targeting to rafts.

Our view of the lateral organization of plasma membrane constituents has evolved in recent years from the conventional picture of membrane proteins diffusing freely in a sea of lipid (1). A large body of evidence from studies using cell biological and biophysical approaches suggests that there is selective confinement of lipids and proteins in discrete regions of the membrane (2–4). These domains, named lipid rafts, are rich in sphingolipids and cholesterol, and appear to be a ubiquitous feature of mammalian cells. Lipid rafts are likely to contribute to the structure and function of caveolae, plasma membrane invaginations that are implicated in a variety of cellular processes, including signal transduction, endocytosis, transcytosis, and cholesterol trafficking. It has been proposed that the spatial concentration of specific sets of proteins increases the efficiency and specificity of signal transduction by facilitating interactions between proteins and by preventing inappropriate cross-talk between pathways.

Raft lipids have been proposed to exist in a separate phase from the rest of the bilayer, in a state similar to the liquid-ordered (l_o)[1] phase described in model membrane (5, 6). Acyl chains of lipids in the l_o phase are tightly packed and highly ordered and extended, similar to those in the gel phase. Thus, lipid structural features (such as saturated acyl chains) that enhance formation of the gel phase can also enhance formation of the l_o phase when these lipids are mixed with cholesterol. The presence of unusually long saturated acyl chains on sphingolipids promotes phase separation and formation of the l_o phase in mixtures of phospholipids, sphingolipids, and cholesterol at concentrations similar to those in the plasma membrane at 37 °C (7), and is likely to do so in biological membranes as well.

Cholesterol- and sphingolipid-rich detergent-resistant membrane (DRMs) can be isolated from mammalian cells (8). Because there are good correlations between onset of formation of an ordered phase and acquisition of detergent insolubility in model membranes (7, 9), and because DRMs isolated from cells are present in the l_o phase (10), DRMs are thought to be derived from rafts in living cells. In contrast, cell membranes that are in the conventional disordered phase are fully solubilized by non-ionic detergents.

Based on the structural model of rafts described above, it has been postulated that proteins with a high affinity for an ordered lipid environment are selectively recruited to rafts (6). These might be expected to include proteins modified with saturated fatty acyl chains, which could partition favorably into l_o phase domains. Indeed, the best characterized DRM targeting signals on proteins are structures that include dual saturated acyl chains. These are glycosylphosphatidylinositol membrane anchors (11, 12) (which contain predominantly saturated fatty acids; Ref. 13), modification with tandem amide-linked myristate and thioester-linked palmitate (14, 15), and modification with tandem thioester-linked palmitate chains (16). Palmitoylation is also required for DRM association of the integral membrane proteins LAT (17) and influenza hemagglutinin (18). The contribution of the lipid modification to raft recruitment is not simply the addition of a hydrophobic moiety,

* This work was supported by a postdoctoral fellowship from the Missouri affiliate of the American Heart Association (to S. M.) and National Institutes of Health Grants GM51466 (to M. E. L.) and GM47897 (to D. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: l_o, liquid-ordered; PC:Chol, phosphatidylcholine (PC):cholesterol (Chol) liposomes (1:7, mol:mol); SCRL, phosphatidylcholine:phosphatidylethanolamine:sphingomyelin:cerebroside:cholesterol liposomes (1:1:1:1:2); ^{bromo}PC:Chol, (16:0–18:0(6–7Dibr) phosphatidylcholine PC:Chol; ^{bromo}SCRL, (16:0–18:0(6–7Dibr) phosphatidylcholine SCRL; G proteins, heterotrimeric guanine nucleotide-binding regulatory protein; $G\alpha_i$, unmodified α_{i1} subunit of a G protein, $^{M}G\alpha_i$, myristoylated $G\alpha_i$; $^{M/P}G\alpha_i$, myristoylated and palmitoylated $G\alpha_i$; $G\beta\gamma$, farnesylated $\beta_1\gamma_2$; GTPγS, guanosine 5′-3-*O*-(thio) triphosphate; NRTK, non-receptor tyrosine kinase; DRM, detergent-resistant membrane; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

as prenylated proteins are not enriched in DRMs (18).

Sphingolipid- and cholesterol-rich liposomes (SCRL), constructed to mimic the lipid composition of DRMs isolated from cells (8), contain l_o phase domains (7) and yield DRMs after detergent extraction (6). SCRL are useful for studying association of purified proteins with DRMs in a defined model system. Purified glycosylphosphatidylinositol-anchored proteins incorporated into SCRL are present in DRMs derived from the liposomes (6, 9), supporting the model that acyl-chain interactions are important in targeting.

The heterotrimeric GTP-binding proteins (G proteins) are fatty-acylated and prenylated, and thus are a useful model for examining the role of lipid modifications in targeting proteins to rafts. All G protein α subunits are fatty-acylated with amide-linked myristate, thioester-linked palmitate, or both (19). G protein γ subunits are modified by farnesyl or geranylgeranyl groups (19). In mammalian cells, an unambiguous role for *N*-myristoylation and prenylation in mediating membrane association of G protein subunits has been established. $G\alpha_i$ family members lacking *N*-myristate or $G\gamma$ subunits lacking prenylation are soluble (19). Palmitoylation of G protein α subunits does not appear to be a major determinant of membrane avidity, at least in the presence of $G\beta\gamma$ subunits (20–22), but may play a role in targeting $G\alpha$ specifically to the plasma membrane (20, 21, 23, 24), and potentially in targeting proteins to subdomains (25, 26). Palmitoylation-defective mutants of $G\alpha_z$, $G\alpha_o$, and Gpa1p, a G protein α subunit in *Saccharomyces cerevisiae*, are mislocalized to intracellular membranes (20, 22, 23). However, it is not possible to discern whether mislocalization is attributable to the lack of palmitate or mutation of the palmitoylated cysteine residue.

Biochemical and morphological evidence points to the organization of G protein pathways in subdomains of the plasma membrane. All of the components of the hormone-sensitive adenylyl cyclase system appear to be enriched in preparations of low density plasma membrane fragments (27). $G\alpha_i$ and $G\beta$ subunits have a punctate appearance in plasma membrane fragments when detected by immunofluorescence. This punctate distribution of $G\alpha_i$ is corroborated by its clustered appearance when decorated with gold particles in electron micrographs of plasma membrane (22). $G\alpha$ subunits are enriched in DRM isolated from cells, but there is disagreement as to the extent of enrichment of $G\beta\gamma$ subunits in these preparations (18, 28, 29). Two nonexclusive mechanisms have been proposed for the targeting of $G\alpha$ subunits to lipid rafts; fatty acylation (25, 30), or interactions with caveolin, a coat protein of caveolae that may function as a scaffolding molecule (31, 32). Most $G\alpha_i$ family members contain the DRM-targeting signal, Met-Gly-Cys, that directs *N*-myristoylation at Gly-2 and palmitoylation at Cys-3 (19). As with similarly modified nonreceptor tyrosine kinases, mutation of either site in $G\alpha_{i1}$ reduces association with DRM isolated from transfected cells (25). It is assumed that the failure of the mutant proteins to cofractionate with DRM is due to the loss of the lipid modification. However, this has not been directly demonstrated. A direct protein-protein interaction between $G\alpha_i$ subunits and caveolin has also been proposed as a mechanism for targeting proteins to lipid rafts (33). Caveolin is reported to bind to an amino acid motif in $G\alpha_{i2}$, fXfXXXXf (where f represents aromatic amino acids Trp, Phe, or Tyr) (32), and also to interact either directly or indirectly with the amino terminus of $G\alpha_{i1}$ (30). The direct binding interaction between purified $G\alpha_i$ and caveolin was not reproduced with purified components (27), raising questions about the nature of the interaction of caveolin with the fXfXXXXf motif. Co-immunoprecipitation of $G\alpha_{i2}$ with caveolin from transfected cells requires only the NH_2-terminal domain of $G\alpha_{i2}$, is independent of the fXfXXXXf motif, and is dependent upon an intact palmitoylation site (30). The studies published to date do not discriminate whether targeting of proteins to rafts by the Met-Gly-Cys motif is mediated by interactions of the protein's fatty acyl chains with membrane lipids, with other proteins present in domains, or through a mechanism that is dependent upon the primary amino acid sequence of the protein rather than the lipid modification.

In this paper, we address the question of whether interactions between the lipid modifications present on G proteins and raft lipids account for their distribution in rafts in cells. The affinity of differentially lipid-modified G proteins for rafts was examined by reconstituting purified subunits into liposomes engineered to mimic rafts. Membrane partitioning was evaluated using susceptibility of the protein to Triton X-100 extraction. We present evidence that the selectivity of protein targeting to rafts relies on the lipid structure that modifies the protein.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylcholine (liver), phosphatidylethanolamine (liver), cerebrosides (brain), sphingomyelin (brain), cholesterol (wool grease), and (16:0–18:0 (6–7Dibr)-phosphatidylcholine were obtained from Avanti Polar Lipids Inc. [2-*palmitoyl*-9,10-^{3}H]Phosphatidylcholine and [^{3}H]palmitate were purchased from NEN Life Science Products. The myristoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA, and linoleoyl-CoA lipid derivatives were purchased from Sigma. The detergent Triton X-100 was purchased as an aqueous solution (10%) from Roche Molecular Biochemicals, CHAPS from Calbiochem, and polyoxyethylene 10-lauryl ether from Sigma. The nucleotides guanosine 5′-diphosphate and guanosine 5′-triphosphate were obtained from Sigma. GTPγS was from Roche Molecular Biochemicals.

G_i Protein Expression and Purification—Recombinant nonlipidated $G\alpha_{i1}$ ($G\alpha_i$) was expressed and purified from *Escherichia coli* (34). To obtain myristoylated $G\alpha_{i1}$ ($^MG\alpha_i$), the *S. cerevisiae N*-myristoyltransferase was expressed together with $G\alpha_i$ in bacteria and $^MG\alpha_i$ purified as described (35). Activity of the different purified $G\alpha_i$ preparations was evaluated by quantification of bound GTPγS (34). Recombinant $G\beta_1$ and hexahistidine-tagged $G\gamma_2$ subunits ($G\beta\gamma$) were produced in Sf9 cells and purified by sequential chromatography with nickel-nitrilotriacetic acid (Qiagen) and Mono Q (Amersham Pharmacia Biotech) as described (36). To evaluate the purity of the preparation, the proteins were resolved by SDS-PAGE and stained with silver nitrate.

Fatty Acylation of $^MG\alpha_i$ in Vitro—The procedure was adapted from the protocol of Duncan and Gilman (37). Purified recombinant $^MG\alpha_i$ (4 μM) was incubated in Buffer A (20 mM NaHepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA) containing 7.5 mM CHAPS and 80 μM myristoyl-CoA (C14:0), palmitoyl-CoA (C16:0), palmitoleoyl-CoA (C16:1), stearoyl-CoA (C18:0) or linoleoyl-CoA (C18:2). For $G\alpha_i$ monomer, $^MG\alpha_i$ was preincubated for 30 min at 30 °C in the presence of GDP (10 μM) and NaF (10 mM), or GTPγS (10 μM). For experiments with trimeric G proteins, heterotrimer was formed by incubating $^MG\alpha_i$ (4 μM) with $G\beta\gamma$ (4 μM) in the presence of GDP (10 μM) for 30 min at 30 °C. The acylation reaction was started with the additions of a mix of the other reagents. The solution was then further incubated for 90 min. All concentrations shown are the final concentrations in the autoacylation reaction.

To monitor the efficiency of palmitoylation, GTPγS-bound $^MG\alpha_i$ (1 μM) was incubated with [^{3}H]palmitoyl-CoA (20 μM, 3300 dpm/pmol) in Buffer A containing 7.5 mM CHAPS. [^{3}H]Palmitoyl-CoA was prepared as detailed by Dunphy *et al.* (24). Aliquots of the reaction (10 μl) were removed at different time points and mixed with 30 μl of a solution containing 1% SDS and 2 mg/ml aldolase. Samples were then precipitated at room temperature by the addition of 500 μl of 15% trichloroacetic acid, 2% SDS. Following a 45-min incubation, the samples were filtered on BA85 nitrocellulose filters (Schleicher & Schuell) using a 10-place filter manifold (Hoefer). Each tube was rinsed twice with 4 ml of 6% trichloroacetic acid, 2% SDS. The filters were washed twice with 2 × 2 ml of 6% trichloroacetic acid, 2% SDS, followed by 2 × 2 ml of 6% trichloroacetic acid. The filters were dried, placed in 4 ml of scintillation fluid, and counted by liquid scintillation spectrometry. To monitor the acylation of $^MG\alpha_i$ when using acyl-CoAs other than palmitoyl-CoA, an indirect method of labeling was used. $^MG\alpha_i$ (1 μM) was acylated with 20 μM acyl-CoA (C14:0, C16:0, C16:1, C18:0, C18:2) as described above. The solution was then spiked with [^{3}H]palmitoyl-CoA (20 μM, 6000 dpm/pmol) and the incubation continued for 90 min. The reaction was

stopped and quantitated as above. Sequential acylation reactions allowed us to estimate the level of acylation in the first reaction by measuring the inhibition of incorporation of radioactive palmitate.

Liposome Preparation—Lipids (6 mg) for making phosphatidylcholine and cholesterol (PC:Chol, 7:1 mol:mol) liposomes or phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, cerebrosides, and cholesterol (SCRL, 1:1:1:1:2) (6) were mixed in a glass screw cap vial, dried under nitrogen gas, lyophilized for 1 h, and stored under argon gas at −20 °C. For the sucrose gradient sedimentation assay, the liposomes were brominated (^{bromo}PC:Chol, ^{bromo}SCRL) in order to create a heavier liposome, by replacing half of the phosphatidylcholine with brominated phosphatidylcholine (16:0–18:0(6–7Dibr)-PC). To make [^{3}H]phosphatidylcholine labeled PC:Chol or SCRL, 16 nCi/mg lipids of [2-*palmitoyl*-9,10-^{3}H]phosphatidylcholine was incorporated in the lipid mixture prior to lyophilization.

To prepare liposomes, a vial of dried lipids was first brought to room temperature and lyophilized for 30 min. The lipids were suspended in buffer B (100 mM NaCl, 50 mM NaHepes (pH 8.0), 5 mM $MgCl_2$, 2 mM EDTA) at 2 mg/ml, transferred to a Corning glass screw cap tube, and the tube filled with argon gas. All the buffers used for liposome experiments were bubbled with nitrogen gas for a few minutes to reduce solubilized oxygen. The lipid suspension was sonicated in a bath ultrasonicator (Laboratory Supplies, Inc.) until the solution became partially clear (between 5 and 20 min). The suspension was then frozen and thawed three times by alternative immersion of the tube in baths of methanol/dry ice and water at 30 °C. The liposomes were recovered in the pellet by ultracentrifugation for 30 min at 200,000 × *g* and suspended in buffer B at 20 mg/ml. Liposomes were discarded after 36 h.

G Protein Reconstitution into Liposomes—Freshly acylated G proteins (40 pmol of $^{M/P}G\alpha_i$ or $^{M/P}G\alpha_i\beta\gamma$), or mock-acylated $G\alpha_i$, $^{M}G\alpha_i$, $G\beta\gamma$, and $^{M}G\alpha_i\beta\gamma$ were mixed with CHAPS (375 nmol) in a total volume of 12 μl. The protein solution was then mixed with 500 μg of PC:Chol or SCRL, and incubated for 45 min at room temperature. Under those conditions, the molar ratio of CHAPS:phospholipids is 0.72 and only partial solubilization of the liposomes occurs (38). The mixture was then diluted stepwise at room temperature with buffer B containing either 10 μM GDP in the presence or the absence of 10 mM NaF or 10 μM GTPγS, until the CHAPS concentration reached 2 mM. Equal volumes of buffer were added to the partially solubilized liposomes every 2 min and the solution gently vortexed. To recover the liposomes, the sample was fractionated by centrifugation at 200,000 × *g* for 30 min at 4 °C and the pellet suspended in nucleotide-supplemented buffer B at 2.2 mg of lipids/ml. A sample of the dilution prior to the fractionation, input (I), and the resulting supernatant (S) and suspended pellet (P) were kept to evaluate the efficiency of G protein incorporation into the liposomes. Quantification of G proteins in the fractions was determined either by GTPγS binding for $G\alpha_i$-containing samples and/or by image analysis (NIH Image) of the gels resulting from protein resolution by SDS-PAGE and stained with Coomassie Blue. Recoveries of the protein in the supernatant (S) and the suspended pellet (P) following the fractionation averaged more than 90% of the starting material (I), (S+P)/I. The reconstitution efficiencies of G protein was expressed as the percentage of protein associated with the liposome pellet over the total amount of protein recovered, P/(S+P).

Sedimentation Assay of Liposomes and Total Lipid Analysis—Sucrose gradients were prepared by freezing (−80 °C) a 15% sucrose solution in buffer B supplemented with 10 μM amounts of the appropriate nucleotide in ultracentrifugation tubes (Beckman), and thereafter allowing the solution to slowly thaw at room temperature. This method gives rise to a linear gradient from ~5% to 20% sucrose, as determined by the sucrose refractive index measured in the fractions recovered. A sample of reconstituted protein in ^{bromo}PC:Chol and ^{bromo}-SCRL or protein alone in buffer B containing 7.5 mM CHAPS was applied to the top of the gradient and centrifuged at 200,000 × *g* for 8 h at 4 °C. Fractions were collected and analyzed for total lipid and protein content. Lipids were isolated by chloroform extraction (1:1). Following vigorous mixing, the solution was centrifuged in a tabletop centrifuge and the organic phase was recovered and evaporated under nitrogen gas to ~10 μl. The concentrated lipid extracts were spotted side by side on a HPTLC plate (Whatman) and allowed to dry. Lipids were revealed by charring (39). Proteins were detected by immunoblot and visualized by chemiluminescence (Pierce).

Membrane Partitioning of G Protein—Reconstituted G proteins were chilled on ice for 10 min. Cold Triton X-100 was added to the suspension at a final concentration of 1% and the preparation incubated on ice for 25 min. The solution was then fractionated by centrifugation at 200,000 × *g* for 30 min. An equivalent sample from the resulting supernatant and the suspended detergent-resistant fraction were resolved by SDS-PAGE, and proteins were detected by Coomassie Blue staining of the gel. The gel was scanned and the intensity of the bands evaluated by densitometry using NIH Image. The amount of protein in the detergent-resistant fraction was expressed as a percentage of the protein recovered following the fractionation, P/(P+S).

Effect of Nucleotide on Partitioning of $G\alpha_i$ Subunits—$^{M}G\alpha_i$ was palmitoylated *in vitro* in the presence of GDP and NaF and reconstituted in SCRL as described above. To compare the effect of the activation state of $G\alpha_i$ on its membrane partitioning, NaF and $MgCl_2$ were removed from half of the preparation to return the protein to a GDP-bound inactive state. Accordingly, the solution of reconstituted liposomes was divided, and fractionated by ultracentrifugation. The resulting pellets were washed once in a large volume of buffer B containing 5 μM GDP and 10 mM NaF or buffer B (lacking $MgCl_2$) and 5 μM GDP. The washed SCRL pellets were then assayed for membrane partitioning as described above. In parallel, the samples were subjected to a trypsin protection assay (36), to monitor the activation state of $G\alpha_i$ following the wash step. $G\alpha_i$ is substantially protected from tryptic proteolysis when activated with either GTPγS or GDP/NaF (40, 41). Briefly, aliquots (20 μl) of the reconstituted liposomes were mixed with 50 mM NaHepes (pH 8.0), 1 mM EDTA, 3 mM dithiothreitol, and 0.05% polyoxyethylene 10-lauryl ether, together with 150 ng of tosylphenylalanyl chloromethyl ketone-treated trypsin, in a final volume of 40 μl. The mixture was incubated at 30 °C and terminated 20 min later by the addition of 40 μg of soybean trypsin inhibitor and then transferred to ice. The samples were subjected to SDS-PAGE on 14% acrylamide gel. The gel was stained with Coomassie Blue to visualize the proteins.

RESULTS

Lipid Modifications Are Required for Reconstitution of G Proteins in Liposomes—An *in vitro* approach was chosen to determine the minimal molecular requirement for targeting membrane-anchored G proteins to lipid rafts. G proteins were reconstituted into PC:Chol and SCRL. The former are present in a disordered state and are fully solubilized by Triton X-100 (6). G protein subunits were purified using recombinant expression systems to permit manipulation of the lipid modifications. The experiments were performed with $G\alpha_{i1}\beta_1\gamma_2$ (42).

It has previously been reported that $G\alpha_o$ and $G\alpha_t$ subunits bind only weakly to liposomes unless co-reconstituted with prenylated $G\beta\gamma$ subunits, suggesting that $G\alpha$ requires $G\beta\gamma$ as a membrane anchor (43, 44). However, the ability of $G\alpha$ modified with both myristate and palmitate to bind to liposomes has not been tested. As predicted, nonacylated $G\alpha_i$ did not associate with either vesicle preparation (Fig. 1). The addition of myristate to $G\alpha_i$ increased its association with vesicles. However, consistent with the previous reports, the reconstitution efficiency was not as high as that of prenylated $G\beta\gamma$ subunits. Approximately 20% of $^{M}G\alpha_i$ was found in the pellet of PC:Chol or SCRL, compared with more than 50% of prenylated $G\beta\gamma$ subunits. The addition of the second lipid modification to $^{M}G\alpha_i$ increased the reconstitution efficiency to levels comparable with $G\beta\gamma$ subunits. The efficiency of reconstitution of the dually acylated proteins may be an underestimate, as the stoichiometry of chemical acylation was approximately 75% (data not shown). The reconstitution efficiency of the heterotrimer was similar whether $G\alpha_i$ was modified with one or two acyl chains. To ensure that the protein remained in its native form during the time-consuming palmitoylation and reconstitution processes, we monitored the activity of $G\alpha_i$ by GTPγS binding at different steps during the procedure. No significant binding activity was lost (data not shown). These results show that functional G proteins can be reconstituted efficiently into liposomes in a lipid-dependent manner. $G\alpha_i$ can associate directly with liposomes if it is sufficiently acylated. The results also argue that the liquid-ordered domains present in SCRL do not interfere with the ability of the protein to become incorporated in the membrane, as similar efficiency of protein reconstitution was obtained with the single phase liquid-crystalline PC:Chol.

To confirm that the pelleted proteins were associated with the liposomes, $^{M/P}G\alpha_i$ in ^{bromo}PC:Chol was subjected to sedi-

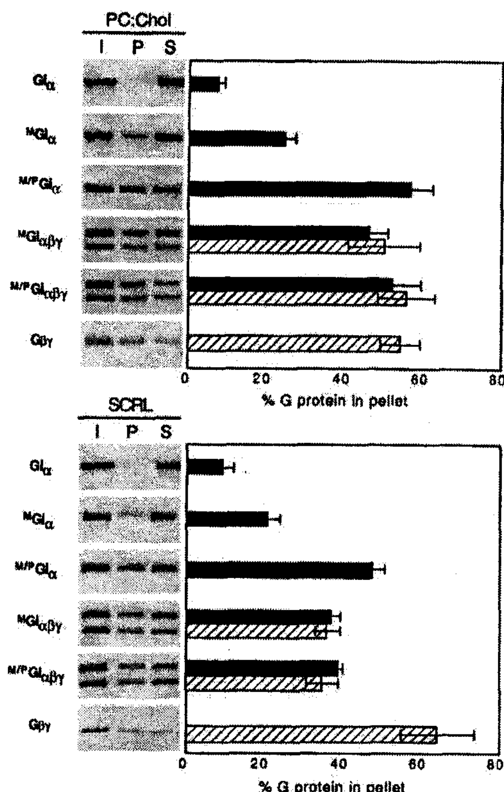

FIG. 1. **Reconstitution of G proteins into PC:Chol and SCRL.** Monomeric $G\alpha_{i1}$ protein (unmodified ($G\alpha_i$), myristoylated ($^{M}G\alpha_i$), myristoylated and palmitoylated ($^{M/P}G\alpha_i$), prenylated $G\beta\gamma$ ($G\beta\gamma$)), or heterotrimeric G proteins ($^{M}G\alpha_i\beta\gamma$, $^{M/P}G\alpha_i\beta\gamma$) were reconstituted in PC: Chol (*upper panel*) or in SCRL (*lower panel*) as described under "Experimental Procedures." To evaluate the proportion of protein reconstituted, a sample of the suspension, before (*I*, input) and after isolation of the reconstituted vesicles by centrifugation (*S*, supernatant; *P*, suspended pellet), was subjected to SDS-PAGE and the proteins detected by Coomassie Blue staining. A representative gel of each type of reconstitution is shown on the *left* of the *panels*. For the trimeric G protein reconstitutions, the upper band on the gels represents $G\alpha_i$ and the lower band, $G\beta\gamma$. To quantitate the reconstitution efficiency, the gels were scanned and the intensity of the bands quantitated by densitometry. The amount of protein in the pellet was expressed as a percentage of the total material recovered from the starting material, P/(S+P) (*right panels*; *filled column*, $G\alpha_i$; *hatched column*, $G\beta\gamma$). The recovery of protein from the starting material, (S+P)/I, averaged 95.5% ±3.3 (PC:Chol) and 101.7% ±5.7 (SCRL). The results shown represent the mean ± S.E. of 4–15 independent experiments.

mentation through sucrose gradients (Fig. 2). Most of the reconstituted $^{M/P}G\alpha_i$ (Fig. 2*A*, *top panel*) sedimented in the same fractions as the lipids (Fig. 2*A*, *bottom panel*), in the middle of the gradient. In contrast, when nonacylated $G\alpha_i$ was included in the reconstitution mixture (Fig. 2*B*), the protein was found at the top of the gradient (Fig. 2*B*, *top panel*), whereas the lipids were in fractions 4–6 (Fig. 2*B*, *lower panel*). As a control, mock-reconstituted $^{M/P}G\alpha_i$ was also found in the fractions at the top of the gradient (Fig. 2*C*). Similar results were obtained for proteins reconstituted into SCRL (data not shown). The fact that $G\alpha_i$ and the liposomes co-sedimented in the sucrose gradient only when $G\alpha_i$ was acylated strongly suggests a lipid-dependent anchoring of the protein to liposomes.

Only Fatty-acylated G Proteins Partition into Rafts—We next determined the Triton X-100 insolubility of $^{M/P}G\alpha_i$, $G\beta\gamma$, $^{M}G\alpha_i\beta\gamma$, and $^{M/P}G\alpha_i\beta\gamma$ reconstituted in PC:Chol or SCRL (Fig. 3) as a way of measuring the membrane compartmentalization of these proteins. Only the amount of insoluble $G\alpha_i$ recovered from liposomes containing $^{M/P}G\alpha_i$, $^{M}G\alpha_i\beta\gamma$, and $^{M/P}G\alpha_i\beta\gamma$ is shown in the figure, while the amount of insoluble $G\beta\gamma$ in liposomes containing $G\beta\gamma$ alone is shown. As predicted, all G proteins reconstituted in PC:Chol were completely solubilized by the Triton X-100 treatment. No lipids (data not shown) and only a marginal amount (less than 10%) of $^{M/P}G\alpha_i$, $G\beta\gamma$, $^{M}G\alpha\beta\gamma$, or $^{M/P}G\alpha\beta\gamma_i$ could be detected in the Triton X-100-insoluble pellet.

Different results were obtained when the G proteins were incorporated into SCRL. When SCRL containing $^{M/P}G\alpha_i$ alone were extracted, more than 50% of the protein was found associated with the Triton-insoluble pellet. In contrast, more than 90% of the farnesylated $G\beta\gamma$ subunit was solubilized when it was incorporated into SCRL in the absence of a $G\alpha_i$ subunit. This striking difference in the distribution between fatty-acylated $G\alpha_i$ and prenylated $G\beta\gamma$ points to selective lipid-lipid interactions as a mechanism for targeting proteins to rafts.

Because $^{M/P}G\alpha_i$ and $G\beta\gamma$ had different solubility behavior when incorporated into SCRL independently, we then looked at the compartmentalization of G protein heterotrimers (Fig. 3). $G\beta\gamma$ was almost completely solubilized from SCRL (data not shown), whereas approximately 25% of $^{M}G\alpha_i$ or $^{M/P}G\alpha_i$ was found in the detergent-insoluble pellet (Fig. 3), a substantial reduction from what was observed for $^{M/P}G\alpha_i$ reconstituted alone. These results suggest a dominant effect of the $G\gamma$ prenyl group over the fatty acid(s) of $G\alpha_i$ on partitioning of the heterotrimer. Interestingly, both monoacylated and diacylated $G\alpha_i$ had the same partitioning. The presence of a fraction of $G\alpha_i$ in the detergent-resistant fraction is a consequence of partial dissociation of the heterotrimer, as shown by the fact that the ratio of $G\alpha_i$:$G\beta\gamma$ reconstituted in SCRL (1.0 for $^{M}G\alpha_i/G\beta\gamma$ and 0.9 for $^{M/P}G\alpha_i/G\beta\gamma$) changed to 1.5 ($^{M}G\alpha_i/G\beta\gamma$) and 1.4 ($^{M/P}G\alpha_i/G\beta\gamma$) in the detergent-resistant pellet. We were not able to find conditions where heterotrimer dissociation was minimized and the liposomes were efficiently solubilized. The fact that only ~25% "trimeric" $G\alpha_i$ compared with more than 50% $^{M/P}G\alpha_i$ alone remained with the detergent-resistant pellet argues that $G\beta\gamma$ plays a significant role in restraining fatty-acylated $G\alpha_i$ from associating with rafts. The results also suggest that $^{M}G\alpha_i$ and $^{M/P}G\alpha_i$, when released from $G\beta\gamma$, can move into rafts. However, this observation remains to be addressed clearly.

Detergent Treatment of SCRL Does Not Induce Diffusion of Lipids or Proteins Containing Saturated Fatty Acids into DRM—Triton treatment of $^{M/P}G\alpha_i$ reconstituted in SCRL could have artifactually driven the protein into the detergent-resistant fraction, even if it was not present in l_o phase domains before extraction. To address this possibility, we determined whether either $^{M/P}G\alpha_i$ or a saturated chain phospholipid could "hop" from Triton-soluble liposomes into DRMs derived from separate liposomes, if the two liposomes were mixed during lysis. This strategy was previously used to show that both sphingomyelin and a glycosylphosphatidylinositol-anchored protein must be in membranes containing l_o-phase domains before detergent extraction to be recovered in DRMs (9). Trace amounts of [2-*palmitoyl*-9,10-^{3}H]phosphatidylcholine were incorporated into PC:Chol. These vesicles were treated with 1% Triton X-100 in the presence of unlabeled PC:Chol or SCRL (Fig. 4, *left panel*). If Triton X-100 induces an artifactual redistribution of lipids containing saturated fatty acids into rafts, then the [2-*palmitoyl*-9,10-^{3}H]phosphatidylcholine should be found in the detergent-resistant fraction of the PC:Chol/SCRL solution. The results of such an experiment show that nearly 100% of the [2-*palmitoyl*-9,10-^{3}H]phosphatidylcholine was solubilized by Triton upon mixing the labeled PC:Chol with PC: Chol or SCRL, arguing that the saturated lipid did not diffuse in rafts upon solubilization of the liposomes. However, when the [2-*palmitoyl*-9,10-^{3}H]phosphatidylcholine was first incor-

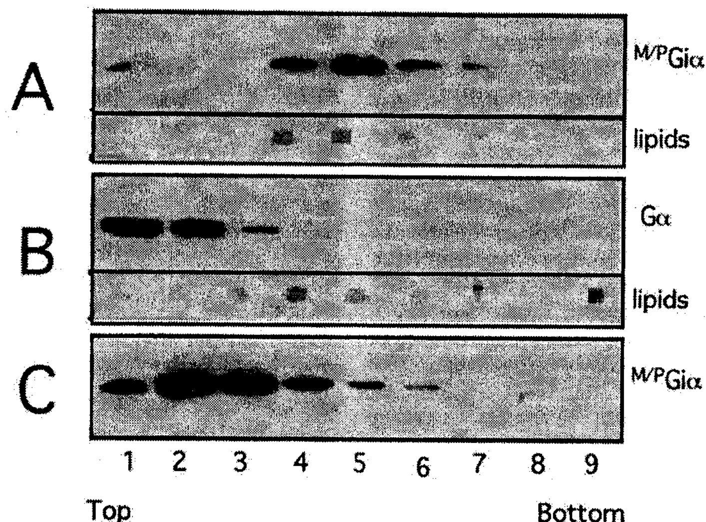

FIG. 2. **Lipid-dependent association of $G\alpha_i$ with PC:Chol.** Myristoylated and palmitoylated $G\alpha_i$ ($^{M/P}G\alpha_i$) or unmodified $G\alpha_i$ ($G\alpha_i$) were reconstituted in ^{bromo}PC:Chol (*A* and *B*) as described under "Experimental Procedures." An aliquot of liposomes with $^{M/P}G\alpha_i$ (*A*) or $G\alpha_i$ (*B*) and of mock-reconstituted $^{M/P}G\alpha_i$ (*C*) was applied to the top of a 5–20% sucrose gradient and centrifuged at 200,000 × *g* for 8 h. Fractions of equal volumes were collected and subjected to SDS-PAGE, followed by protein immunoblotting with $G\alpha_i$ antiserum (*top panel, A* and *B*, and *C*). The *lower panels* in *A* and *B* show the lipid content of the fractions. An aliquot of each fraction was spotted on a HPTLC plate, and lipids were detected by charring as described under "Experimental Procedures." The results shown are representative of two independent experiments.

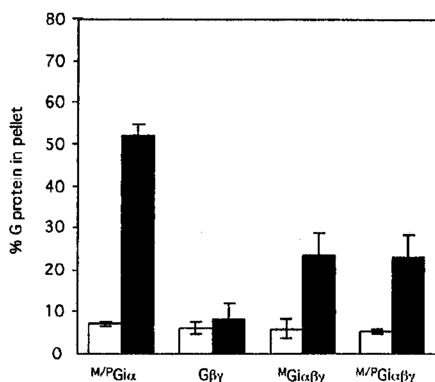

FIG. 3. **Membrane partitioning of lipidated G proteins in SCRL.** G protein subunits ($^{M/P}G\alpha_i$, $G\beta\gamma$) or heterotrimeric G proteins ($^{M}G\alpha_i\beta\gamma$, $^{M/P}G\alpha_i\beta\gamma$) were reconstituted in PC:Chol (*hollowed bars*) or in SCRL (*solid bars*). The reconstituted liposomes were collected by centrifugation and treated with 1% ice-cold Triton X-100 for 25 min. The mixture was fractionated by ultracentrifugation, and the proteins in the detergent-resistant pellet and the supernatant were subjected to SDS-PAGE and detected by Coomassie Blue staining. The gels were scanned and the intensity of the bands analyzed by densitometry. The percentage of $G\alpha_i$ in the detergent-insoluble fraction when liposomes containing $^{M/P}G\alpha_i$, $^{M}G\alpha_i\beta\gamma$, or $^{M/P}G\alpha_i\beta\gamma$ were extracted is reported. Quantitation of $G\alpha_i$ in the detergent-insoluble fraction by GTPγS binding gave similar results to those by densitometry (data not shown). The percentage of $G\beta\gamma$ in the detergent-insoluble fraction when liposomes containing only $G\beta\gamma$ were extracted is reported. Less than 10% of $G\beta\gamma$ was detergent-insoluble when liposomes containing heterotrimers were extracted (data not shown). The results shown represent the mean ± S.E. of three to eight independent experiments.

porated into SCRL, and then mixed with unlabeled PC:Chol or SCRL, most of the labeled phospholipid was resistant to solubilization, suggesting that the labeled phosphatidylcholine was present in l_o-domains. Therefore, there appears to be little redistribution of lipids induced by detergent.

Qualitatively similar results were obtained when using $^{M/P}G\alpha_i$ (Fig. 4, *right panel*). In these experiments, 77% of $^{M/P}G\alpha_i$ was associated with DRM. In contrast, the addition of a Triton solution containing $^{M/P}G\alpha_i$ to SCRL resulted in only 16% incorporation of protein into the detergent-resistant pellet. This fraction was increased to 36% when $^{M/P}G\alpha_i$ reconstituted in PC:Chol was treated with Triton in the presence of empty SCRL. These results suggest that treatment with detergent induces some transfer of $^{M/P}G\alpha_i$ into DRM in the presence of empty SCRL. However, the amounts of $^{M/P}G\alpha_i$ found in the the detergent-resistant pellets under these conditions is significantly less than the amounts associated with DRM when $^{M/P}G\alpha_i$ is reconstituted directly into SCRLs. The presence of $^{M/P}G\alpha_i$ in the detergent-insoluble pellet is not likely to be due to nonspecific aggregation because the protein retains GTPγS binding activity. These results argue that proteins exist in l_o domains prior to treatment with Triton X-100.

The Activation State of $^{M/P}G\alpha_i$ Does Not Influence Its Partitioning into Detergent-resistant Domains—We next tested the possibility that the activation state of $^{M/P}G\alpha_i$ might influence its distribution in SCRL. In the previous experiments, $^{M/P}G\alpha_i$ reconstituted in SCRL was in the GTPγS-bound form. The protein was activated with GTPγS to maintain its stability during the autoacylation and reconstitution protocols. To determine if GDP-bound $^{M/P}G\alpha_i$ also partitioned into DRM, we took advantage of the fact that $G\alpha_i$ can be activated by NaF and $MgCl_2$ in a reversible manner, in the GDP-bound state (45). GDP-bound $^{M}G\alpha_i$ was palmitoylated and reconstituted into SCRL in the presence of NaF and $MgCl_2$. Activation was reversed by washing the liposomes in buffer lacking NaF and $MgCl_2$. Recovery of $G\alpha_i$ in the inactive GDP-bound state was confirmed by its susceptibility to proteolysis with trypsin (data not shown). The activated form of $G\alpha_i$ is partially protected from tryptic digestion, whereas the GDP-bound form is digested completely. The distribution of GDP-bound and NaF-activated $^{M/P}G\alpha_i$ in detergent-resistant membranes isolated from SCRL is shown in Fig. 5. Approximately 50% of the protein in either state was resistant to detergent extraction. Therefore, the activation state of the $^{M/P}G\alpha_i$ monomer does not influence its association with rafts.

Fatty Acid Saturation Influences Protein Targeting to Detergent-resistant Membranes—As shown in the previous experiments, one of the determinants for association of lipidated proteins with DRM is the structural compatibility of the lipid that modifies the protein with membrane lipids. Only fatty-

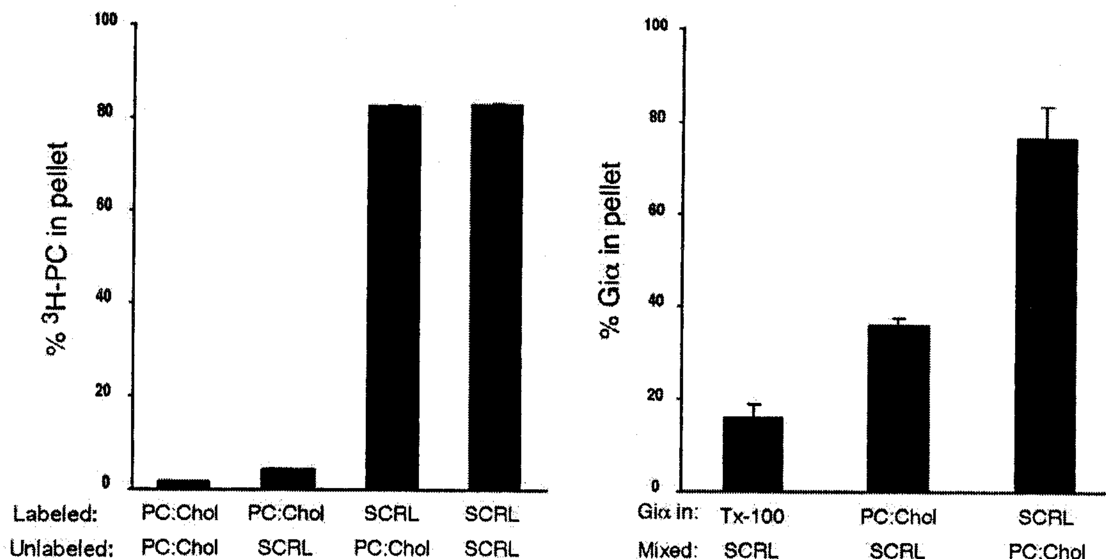

FIG. 4. **Characterization of the Triton X-100 treatment of SCRL.** *Left panel*, trace amounts of [2-*palmitoyl*-9,10-^{3}H]phosphatidylcholine were incorporated into PC:Chol or SCRL (labeled) as described under "Experimental Procedures." The radiolabeled liposomes were mixed with equal amounts of the indicated unlabeled liposomes, and treated with 1% ice-cold Triton X-100. The mixture was fractionated by ultracentrifugation, and radioactivity present in the supernatant and pellet was quantitated by liquid scintillation spectrometry. The *graph* shows the percentage of radioactivity in the detergent-resistant pellet. The figure shows the average ± S.D. of two experiments. *Right panel*, $^{M/P}G\alpha_i$ was reconstituted in PC:Chol or SCRL and the reconstituted liposomes isolated, as described under "Experimental Procedures." The reconstituted liposomes or soluble $^{M/P}G\alpha_i$ in Triton X-100 (2%) were then mixed with equal volume of PC:Chol or SCRL, as indicated, and treated with ice-cold Triton X-100 for 25 min. The final Triton X-100 concentration in all samples was 1%. The mixture was fractionated by ultracentrifugation and the amount of $^{M/P}G\alpha_i$ quantitated in the supernatant and the detergent-resistant fraction by GTPγS binding. The *graph* shows the percentage of $^{M/P}G\alpha_i$ that is Triton-resistant. The results shown represent the mean ± S.E. of 5–14 independent experiments.

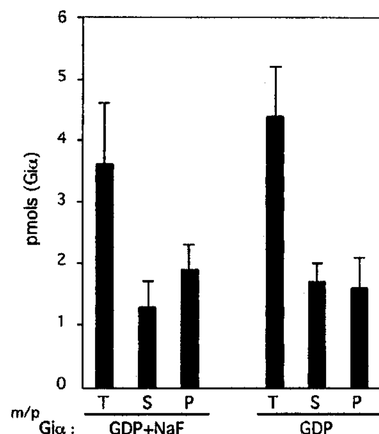

FIG. 5. **Effect of $^{M/P}G\alpha_i$ activation state on its membrane distribution in SCRL.** $^{M}G\alpha_i$ was activated with NaF and $MgCl_2$ in the presence of GDP to stabilize the protein during the autopalmitoylation reaction and reconstituted into SCRL. The resulting liposome suspension was divided, and the liposomes were washed twice in buffer containing GDP, NaF, and $MgCl_2$ or in buffer containing GDP but lacking $MgCl_2$ to inactivate $G\alpha_i$. The membrane distribution of $^{M/P}G\alpha_i$ under both conditions was evaluated by treatment of the preparations with 1% ice-cold Triton X-100 for 25 min. The solution was fractionated by ultracentrifugation, and $^{M/P}G\alpha_i$ in the soluble (*S*), the detergent-resistant pellet (*P*), and the total starting material (*T*) was quantitated by GTPγS binding. The results shown represent the mean ± S.E. of four independent experiments.

acylated proteins were targeted to DRM in SCRL. To explore further the lipid-lipid interactions between the modified protein and the rafts, membrane partitioning of $G\alpha_i$ modified with different isomers of fatty acids was tested. We hypothesized that a protein modified with unsaturated fatty acids would behave similarly to a prenylated protein and be excluded from DRM. A *cis*-double bond results in an increase in the cross-sectional area of the hydrocarbon chain (46), making it more difficult to pack in the ordered lipid environment present in rafts. $^{M}G\alpha_i$ was acylated with fatty acids of different chain length and degrees of *cis*-unsaturation (C14:0, C16:0, C16:1, C18:0, C18:2) and reconstituted in SCRL. The *in vitro* acylation method used allowed modification of $^{M}G\alpha_i$ with an efficiency similar to that of palmitate (C14:0 = 80.2% ±3.9, C16:1 = 79.7% ±12.1, C18:0 = 74.8% ±8.2, C18:2 = 87.0% ±8.3). Unsaturation in the fatty acyl chain did not affect the reconstitution of $G\alpha_i$ in SCRL, as similar efficiencies of reconstitution were obtained to that of $^{M/P}G\alpha_i$ (Table I). DRM partitioning of the different dually acylated $G\alpha_i$ in SCRL are shown in Fig. 6. Similarly to C16:0-modified $^{M}G\alpha_i$, 45–55% of the proteins acylated with C14:0 and C18:0 were resistant to detergent extraction, suggesting that acyl chain length does not significantly affect the distribution of $G\alpha_i$. However, proteins modified with the unsaturated lipids C16:1 and C18:2 were relatively less resistant to the Triton extraction. Indeed, less than 30% of the protein was found in the insoluble fraction. These results highlight the fact that structural differences between lipids are at the origin of the selectivity for raft association.

DISCUSSION

In addition to their well characterized role as hydrophobic anchors for otherwise soluble proteins, covalent lipid modifications appear to be important determinants of the lateral distribution of proteins in subdomains of the plasma membrane (5, 47). In this study, we have addressed the role of fatty acylation and prenylation of G protein subunits in membrane binding and in membrane partitioning using a reconstituted system with purified components. We confirmed previous studies demonstrating that $G\beta\gamma$ and the G protein heterotrimer associate with membranes, presumably through prenylation of $G\gamma$ and that myristoylated $G\alpha_i$ binds relatively weakly to liposomes in the absence of $G\beta\gamma$ (43, 44). Here we demonstrate that $G\alpha_i$ tandemly modified with myristate and palmitate binds

TABLE I
Reconstitution efficiency of dually acylated $G\alpha_i$ in SCRL

${}^{M}G\alpha_i$ was autoacylated with the acyl-CoAs shown below and reconstituted in SCRL. In each experiments, the recovery of material after the centrifugation was within 104.7 ± 2% of the starting material. Results shown represent the mean ± S.E. of four independent experiments.

Lipid modifying cysteine 3 of $G\alpha_i$	$G\alpha_i$ in SCRL
	%
C14:0	45.6 ± 4.6
C16:0	45.9 ± 3.1
C16:1	46.4 ± 7.3
C18:0	44.4 ± 3.9
C18:2	46.1 ± 3.6

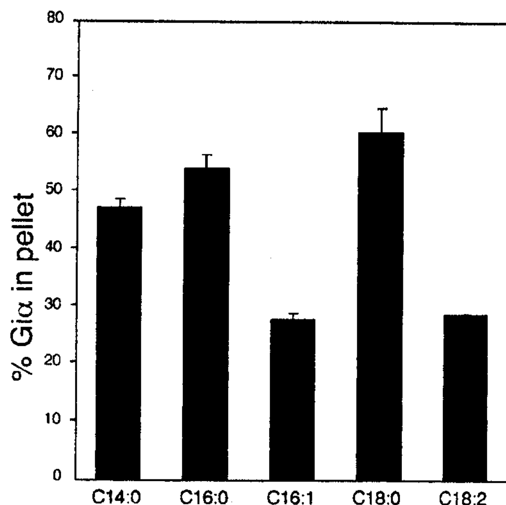

FIG. 6. **Effect of saturation of thioester-linked fatty acids on membrane partitioning of $G\alpha_i$.** ${}^{M}G\alpha_i$ was fatty-acylated *in vitro* (C14:0, C16:0, C16:1, C18:0, C18:2) and reconstituted in SCRL, as described under "Experimental Procedures." The reconstituted liposomes were isolated, treated with 1% ice-cold Triton X-100, and processed for quantitation as described in the legend for Fig. 3. The results shown represent the percentage of protein that remains associated with the detergent-resistant fraction. The data shown are the mean ± S.E. of three to four experiments.

efficiently to the lipid bilayer in the absence of any other proteins. These results are consistent with measurements of the affinities of lipidated peptides for artificial membranes where the presence of tandem acyl groups results in a very long-lived membrane association in contrast to that mediated by a myristoyl group alone (48–50).

The effect of palmitoylation on membrane association of $G\alpha_i$ family members in cells has been studied using palmitoylation-defective mutants. In stable cell lines expressing modest amounts of palmitoylation-defective $G\alpha_z$ (C3A), the total membrane distribution of the mutant protein is not significantly different from wild type, although a fraction of the mutant protein is mislocalized to intracellular membranes (20, 21). $G\beta\gamma$ subunits stabilize membrane association of the mutant protein in the inactive state. Activation of the G protein results in subunit dissociation, but this apparently does not result in release of the protein from membranes. Native $G\alpha_i$ in membranes that has been depalmitoylated enzymatically remains associated with membranes (22). This suggests that myristate is sufficient to hold $G\alpha_i$ at the membrane or that other protein interactions stabilize its membrane association in the absence of $G\beta\gamma$. Thus, in intact cells, the contribution of palmitate to the membrane avidity of $G\alpha_i$ is probably secondary to other functions for this modification (21, 22). A role for palmitoylation in regulating $G\alpha$ interactions with $G\beta\gamma$ subunits and regulators of G protein signaling is indicated by *in vitro* studies (51, 52). In addition, studies of $G\alpha_i$ (C3A) also point to a role for palmitate in targeting proteins to rafts (25).

The reconstituted system was used to evaluate a model for how fatty acylation facilitates protein association with sphingolipid cholesterol-rich domains. Lipids found in rafts are thought to exist in a more ordered state than those in the surrounding fluid phase (5–7). The physical parameters of raft lipids that govern the formation of l_o domains may also apply to lipidated proteins that are present in rafts. Proteins modified with fatty acids could associate with ordered domains by spontaneous insertion of the fatty acyl chains into the l_o phase (6). In this study, we present evidence that directly supports this hypothesis. We report that $G\alpha_i$ modified with amide-linked myristate and thioester-linked palmitate acquires detergent resistance when reconstituted in liposomes that contain lipids in the l_o phase. These results establish that lipid-lipid interactions are sufficient to mediate raft localization of proteins containing the DRM targeting signal, Met-Gly-Cys. Thus, interactions with caveolin or unidentified resident proteins of caveolae or rafts are not a prerequisite for targeting dually acylated $G\alpha_i$ to rafts, although this does not exclude the possibility that these protein interactions exist in rafts.

In the model membranes, fatty-acylated $G\alpha_i$ exhibited detergent resistance, whereas prenylated $G\beta\gamma$ was solubilized. This result recapitulates the distribution of G protein subunits in DRM isolated from Madin-Darby canine kidney cells (18). The branched and bulky prenyl group that modifies $G\gamma$ is not predicted to fit easily in the compact lipid organization of rafts (18), even though the hydrophobicity of farnesyl and geranylgeranylated groups is similar to that of myristate and palmitate (49). The finding that $G\alpha_i$ modified with unsaturated fatty acid was more easily solubilized than protein modified with its saturated counterpart supports the idea that the ability of the lipids to pack into an ordered environment is a critical factor for sorting proteins into liquid-ordered domains.

The solubility of $G\beta\gamma$ suggests that the protein resides outside l_o domains. However, we cannot rule out the possibility that $G\beta\gamma$ binds to rafts with lower affinity, and this interaction is susceptible to disruption by Triton X-100. Interestingly, the partitioning behavior of prenylated $G\beta\gamma$ was dominant when the heterotrimer was analyzed. The fraction of ${}^{M/P}G\alpha_i$ in DRM was significantly reduced from that of ${}^{M/P}G\alpha_i$ reconstituted alone. That ${}^{M/P}G\alpha_i$ remaining in DRM could be accounted for by subunit dissociation, since little $G\beta\gamma$ was seen in DRM. A possible explanation for the dominance of the prenyl group in membrane partitioning is the preferential interaction of the fatty acyl chains on ${}^{M/P}G\alpha_i$ with the prenyl group on $G\gamma$. In transducin, the $T\alpha$ acyl chain binds cooperatively with the $T\gamma$ farnesyl chain in membrane lipids (44). This cooperativity could represent lipid-lipid interactions between the farnesyl and myristoyl moieties. The three-dimensional x-ray crystal structure of transducin and of $G\alpha_{i1}\beta_1\gamma_2$ has been solved using proteins that lack their respective lipid modifications (42, 53). The positions of the observed NH_2 terminus of $G\alpha_i$ and the COOH terminus of $G\gamma$ within the structures are consistent with the hydrophobic modifications inserting simultaneously at a single locus in the lipid bilayer. In SCRL, the cooperativity between lipids may favor partitioning of the heterotrimer into regions outside of l_o domains. An alternative model for the domain organization of G proteins that is also consistent with our data is the disposition of the heterotrimer at the edge of rafts. This would permit insertion of the lipids into their respective domains. Whether the heterotrimer is located at the edge or outside of DRM, the different distribution of monomeric

and trimeric $G\alpha_i$ in DRM raises the possibility that partitioning of $G\alpha_i$ into rafts is dynamic and regulated by its activation state. This may represent another regulatory input into G protein signaling.

The behavior of $G\alpha_i$ in this model system is likely to be representative of a large number of proteins that undergo sequential modification by amide-linked myristate and thioester-linked palmitate and are enriched in DRM. These include other G protein α subunits, many nonreceptor tyrosine kinases (NRTK), and endothelial nitric-oxide synthase (reviewed in (47)). Myristoylation is necessary, but not sufficient for inclusion of these proteins in DRM. Abrogation of palmitoylation by mutation decreases the enrichment of these proteins in DRM. Furthermore, the introduction of a palmitoylation site (Cys-3) into the nonpalmitoylated kinases, $p60^{src}$ or $p61^{hck}$, results in palmitate incorporation and inclusion in DRM (15, 54). A function for Cys-3 independent of palmitoylation could not be excluded as an alternative explanation for the targeting of proteins to DRM, particularly for $p56^{lck}$, where there is disagreement as to whether Cys-3 is the primary site of palmitoylation of the protein (12, 55). In the *in vitro* system, we have been able to manipulate the status of fatty acylation without changing the primary amino acid sequence of the protein. This allowed us to demonstrate directly that fatty acylation at Cys-3 is the critical parameter for targeting $G\alpha_i$ to rafts. This finding is likely to apply to other proteins that are similarly modified.

Evidence for the functional importance of compartmentalizing signaling proteins into lipid rafts is accumulating. T cell activation leads to the compartmentation of the T cell receptor with downstream NRTKs into rafts (56). Disruption of raft structure by cholesterol-removing reagents or by internalization interferes with the earliest steps in T cell activation (56). T cells treated with polyunsaturated fatty acids also exhibit diminished calcium responses to activation of CD3 or CD59 (57). Loss of signaling is strictly correlated with the displacement of $p56^{lck}$ and $p59^{fyn}$ from detergent-resistant domains. Whether polyunsaturated fatty acid disrupts raft structure through incorporation of unsaturated acyl chains into NRTKs, raft lipids, or both is not known. Our finding that introduction of an unsaturated acyl chain into $G\alpha_i$ significantly reduces its partitioning into DRM demonstrates that changes in the acylation status of the protein are sufficient to shift the distribution of the protein. The effect of acyl chain saturation on partitioning of $G\alpha_i$ into DRM may have other important biological correlates. $G\alpha_i$ incorporates radiolabeled arachidonic acid in platelets, suggesting that the protein is posttranslationally modified with unsaturated as well as saturated fatty acids (58). Our data suggest that the lateral distribution of $G\alpha_i$ in platelet membranes will be affected accordingly.

In this study, we have provided evidence using well defined, purified components that selective interaction between saturated fatty acids on lipid-modified proteins with lipids in DRM is a targeting mechanism for localization in membrane subdomains. Our data are consistent with the hypothesis that packing order conferred by the structure of the lipid modification rather than hydrophobicity is the primary determinant of whether a lipidated protein is targeted to DRM. The heterotrimeric G protein is both prenylated and fatty-acylated, and thus harbors conflicting signals. In the model system, this manifests as the exclusion of heterotrimer from DRM and suggests that dynamic association of fatty-acylated $G\alpha_i$ with prenylated $G\beta\gamma$ may regulate $G\alpha_i$ targeting to rafts.

Acknowledgment—We thank Wendy K. Greentree for providing purified $G\alpha_{i1}$ used in this study.

REFERENCES

1. Singer, S. J., and Nicolson, G. L. (1972) *Science* **175,** 720–731
2. Brown, R. E. (1998) *J. Cell Sci.* **111,** 1–9
3. Anderson, R. G. W. (1998) *Annu. Rev. Biochem.* **67,** 199–225
4. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
5. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell. Dev. Biol.* **14,** 111–136
6. Schroeder, R., London, E., and Brown, D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12130–12134
7. Ahmed, S. N., Brown, D. A., and London, E. (1997) *Biochemistry* **36,** 10944–10953
8. Brown, D. A., and Rose, J. K. (1992) *Cell* **68,** 533–544
9. Schroeder, R. J., Ahmed, S. N., Zhu, Y., London, E., and Brown, D. A. (1998) *J. Biol. Chem.* **273,** 1150–1157
10. Ge, M., Field, K. A., Aneja, R., Holowka, D., Baird, B., and Freed, J. (1999) *Biophys. J.* **77,** 925–933
11. Arreaza, G., and Brown, D. A. (1995) *J. Biol. Chem.* **270,** 23641–23647
12. Rodgers, W., Crise, B., and Rose, J. K. (1994) *Mol. Cell. Biol.* **14,** 5384–5391
13. McConville, M. J., and Ferguson, M. A. J. (1993) *Biochem. J.* **294,** 305–324
14. Milligan, G., Parenti, M., and Magee, A. (1995) *Trends Biochem. Sci.* **20,** 181–187
15. Schenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994) *J. Cell Biol.* **126,** 353–364
16. Arni, S., Keilbaugh, S. A., Ostermeyer, A. G., and Brown, D. A. (1998) *J. Biol. Chem.* **273,** 28478–28485
17. Zhang, W., Trible, R. P., and Samelson, L. E. (1998) *Immunity* **9,** 239–246
18. Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G., and Brown, D. A. (1999) *J. Biol. Chem.* **274,** 3910–3917
19. Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995) *J. Biol. Chem.* **270,** 503–506
20. Morales, J., Fishburn, S., Wilson, P. T., and Bourne, H. R. (1998) *Mol. Biol. Cell* **9,** 1–14
21. Fishburn, C. S., Herzmark, P., Morales, J., and Bourne, H. R. (1999) *J. Biol. Chem.* **274,** 18793–18800
22. Huang, C., Duncan, J. A., Gilman, A. G., and Mumby, S. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 412–417
23. Song, J., and Dohlman, H. G. (1996) *Biochemistry* **35,** 14806–14817
24. Dunphy, J. D., Greentree, W. K., Manahan, C. L., and Linder, M. E. (1996) *J. Biol. Chem.* **271,** 7154–7159
25. Song, K. S., Sargiacomo, M., Galbiati, F., Parenti, M., and Lisanti, M. P. (1997) *Cell. Mol. Biol.* **43,** 293–303
26. Mumby, S. M. (1997) *Curr. Opin. Cell Biol.* **9,** 148–154
27. Huang, C., Hepler, J. R., Chen, L. T., Gilman, A. G., Andersone, R. G. W., and Mumby, S. M. (1997) *Mol. Biol. Cell* **8,** 2365–2378
28. Chang, W., Ying, Y., Rothberg, K., Hooper, N., Turner, A., Gambliel, H., DeGunzburg, J., Mumby, S., Gilman, A., and Anderson, R. (1994) *J. Cell Biol.* **126,** 127–138
29. Rehm, A., and Pleogh, H. L. (1997) *J. Cell Biol.* **137,** 305–317
30. Galbiati, F., Volonte, D., Meani, D., Milligan, G., Lublin, D. M., Lisanti, M. P., and Parenti, M. (1999) *J. Biol. Chem.* **274,** 5843–5850
31. Li, S., Couet, J., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 29182–29190
32. Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272,** 6525–6533
33. Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J., Hansen, S., Nishimoto, I., and Lisanti, M. (1995) *J. Biol. Chem.* **270,** 15693–15701
34. Linder, M. E., Ewald, D. A., Miller, R. J., and Gilman, A. G. (1990) *J. Biol. Chem.* **265,** 8243–8251
35. Mumby, S. M., and Linder, M. E. (1993) *Methods Enzymol.* **237,** 254–268
36. Kozasa, T., and Gilman, A. (1995) *J. Biol. Chem.* **270,** 1734–1741
37. Duncan, J. A., and Gilman, A. G. (1996) *J. Biol. Chem.* **271,** 23594–23600
38. Cladera, J., Rigaud, J.-L., Villaverde, J., and Dunach, M. (1997) *Eur. J. Biochem.* **243,** 798–804
39. Macala, L. J., Yu, R. K., and Ando, S. (1983) *J. Lipid Res.* **24,** 1243–1250
40. Fung, B. K., and Nash, C. R. (1983) *J. Biol. Chem.* **258,** 10503–10510
41. Sternweis, P. C., and Robishaw, J. D. (1984) *J. Biol. Chem.* **259,** 13806–13813
42. Wall, M., Coleman, D., Lee, E., Iniguez-Lluhi, J., Posner, B., Gilman, A., and Sprang, S. (1995) *Cell* **83,** 1047–1058
43. Sternweis, P. C. (1986) *J. Biol. Chem.* **254,** 3333–3340
44. Bigay, J., Faurobert, E., Franco, M., and Chabre, M. (1994) *Biochemistry* **33,** 14081–14090
45. Antonny, B., Sukumar, M., Bigay, J., Chabre, M., and Higashijima, T. (1993) *J. Biol. Chem.* **268,** 2393–2402
46. Gennis, R. B. (1989) in *Biomembranes, Molecular Structures and Function* (Cantor, C. R., ed) p. 49, Springer Verlag, New York
47. Dunphy, J. T., and Linder, M. E. (1998) *Biochim. Biophys. Acta* **1436,** 245–261
48. Peitzsch, R. M., and McLaughlin, S. (1993) *Biochemistry* **32,** 10436–10443
49. Silvius, J. R., and l'Heureux, F. (1994) *Biochemistry* **33,** 3014–3022
50. Shahinian, S., and Silvius, J. R. (1995) *Biochemistry* **34,** 3813–3822
51. Iiri, T., Backlund, Jr., P. S., Jones, T. L. Z., Wedegaertner, P. B., and Bourne, H. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14592–14597
52. Tu, Y., Wang, J., and Ross, E. M. (1997) *Science* **278,** 1132–1135
53. Lambright, D., Sondek, J., Bohm, A., Skiba, N., Hamm, H., and Sigler, P. (1996) *Nature* **379,** 311–320
54. Robbins, S. M., Quintrell, N. A., and Bishop, J. M. (1995) *Mol. Cell. Biol.* **15,** 3507–3515
55. Kwong, J., and Lublin, D. M. (1995) *Biochem. Biophys. Res. Commun.* **207,** 868–876
56. Xavier, R., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998) *Immunity* **8,** 723–732
57. Stulnig, T. M., Berger, M., Sigmind, T., Raederstorff, D., Stockinger, H., and Waldhausl, W. (1998) *J. Cell Biol.* **143,** 637–644
58. Hallak, H., Muszbek, L., Laposata, M., Belmonte, E., Brass, L. F., and Manning, D. R. (1994) *J. Biol. Chem.* **269,** 4713–4716