Enrichment of G-protein Palmitoyltransferase Activity in Low Density Membranes

IN VITRO RECONSTITUTION OF Gαi TO THESE DOMAINS REQUIRES PALMITOYLTRANSFERASE ACTIVITY*

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Many signaling proteins are targeted to low density, sphingomyelin- and cholesterol-enriched membranes, also called lipid rafts. These domains organize receptor-mediated signaling events at the plasma membrane. Fatty acylation is one mechanism for targeting proteins to rafts. It was therefore of interest to determine if protein palmitoyltransferase activity is also present in these domains. In this study, protein palmitoyltransferase activity, assayed using G-protein alpha subunits as a substrate, was found to be highly enriched in low density membranes derived from cells that express caveolin as well as those that do not. Depletion of cellular cholesterol with the drug methyl-β-cyclodextrin resulted in inhibition of palmitoyltransferase activity and a redistribution of the remaining activity to membranes of higher density. This effect was reversed by adding cholesterol to cyclodextrin-treated cells. When reconstituted into cell membranes, the population of purified recombinant Gαi that was palmitoylated was highly enriched in the low density membrane fractions, whereas the bulk unmodified Gαi-protein was largely excluded. This effect required palmitoyltransferase activity and was abolished if the palmitoylated cysteine was mutated. Thus, palmitoyltransferase facilitates the enrichment of fatty acylated signaling molecules in plasma membrane subdomains.

Efficient transmission of extracellular signals relies on the localization of signaling molecules at the plasma membrane (PM). Signal transducers such as nonreceptor tyrosine kinases (NRTK) and heterotrimeric G proteins are positioned at the inner leaflet of the PM to respond to receptor-generated signals and relay them to intracellular effectors. Increasing evidence indicates that signaling molecules are clustered in specialized domains of the PM (reviewed in Refs. 1–3). These domains have been characterized biochemically as regions that are enriched in cholesterol and glycosphingolipids (4, 5), are insoluble in Triton X-100, and exhibit unique buoyancy with respect to other cellular membranes (5–7). Low density membrane domains are likely to be present in all cell types and have been termed lipid rafts, detergent-insoluble glycolipid-enriched membranes, and detergent-resistant membranes. Caveolae, small nonclathrin-coated membrane invaginations, are likely to represent a specialized lipid raft with a characteristic protein coat of oligomerized caveolin (8).

Numerous signaling molecules are enriched in lipid raft preparations. Characterization of hormone-stimulated adenyl cyclase in S49 lymphoma cells demonstrates that all components of the signaling pathway are enriched in low density PM fragments (9). Morphological evidence is consistent with G-protein localization in subdomains of the plasma membrane. G-protein αi and β subunits exhibit a punctate distribution on the PM when visualized by immunofluorescence (9). This non-uniform distribution of Gαi in the PM is corroborated by its clustered appearance when detected by immunogold in electron micrographs (10). In T cells, the nonreceptor tyrosine kinases Fyn and Lck and the palmitoylated, transmembrane protein LAT are localized in lipid rafts. Activation of the T-cell receptor results in the recruitment of other components of this signaling pathway into rafts, including the zeta chain of the T-cell receptor, the tyrosine kinase Zap-70, Vav, and phospholipase Cγ1 (reviewed in Ref. 11).

The functional importance of organizing signaling pathways in PM subdomains has been demonstrated in several systems, most notably in hematopoietic cells. Transmembrane signaling through the high affinity IgE receptor FcεRI in mast cells requires a cholesterol-dependent association of the receptor with lipid rafts where the receptor is tyrosine-phosphorylated by the NRTK Lyn (12). In T cells, perturbation of lipid rafts disrupts early steps in T cell activation (11). Examples can be found in other cell types as well (13–15), suggesting that concentration of proteins in cholesterol-rich domains may be a common feature required for the function of many signaling pathways.

The best-characterized signals that target proteins to lipid rafts are those that involve fatty acylation. These are glycosphatidylinositol (16, 17), tandem amide-linked myristate and thioester-linked palmitate (18, 19), and tandem-linked palmitate chains (20). Palmitoylation of the cytoplasmic domains of the transmembrane proteins LAT (21) and influenza hemagglutinin (22) is also an important determinant of their raft association. Blocking palmitoylation of NRTKs by mutation (19) or with inhibitors such as 2-bromopalmitate results in the delocalization of NRTKs from lipid rafts (23).

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1 The abbreviations used are: PM, plasma membrane; NRTK, nonreceptor tyrosine kinase; PAT, protein acyltransferase; DTT, dithiothreitol; FBS, fetal bovine serum; SFM, serum-free medium; MES, 4-morpholineethanesulfonic acid; MBS, MBS-buffered saline; DEPC, diethylpyrocarbonate; CD, methyl-β-cyclodextrin; MEM, minimal essential medium; KBC cells, KB cells stably expressing caveolin; RGS, regulator of G-protein signaling.
Palmitoyltransferase Enrichment in Low Density Membranes

Proteins are modified with palmitate by membrane-bound palmitoyltransferases (reviewed in Refs. 11, 24). Using a variety of proteins as substrates, palmitoyltransferase activities have been characterized biochemically, but their molecular entities remain elusive. There is considerable heterogeneity in the protein motifs that direct palmitoylation. Furthermore, this modification occurs both on intracellular membranes and at the PM, suggesting that multiple enzymes are present in cells. We have studied a protein acyltransferase activity (PAT) using G-protein α subunits as a substrate (25, 38). We have previously shown that PAT activity for myristoylated Gα subunits is highly enriched in the PM (25) where it facilitates the stable association of Gα with the PM (26). In this study, we sought to determine how PAT is distributed in the PM to address whether it has a role in the localization of fatty acylated proteins in lipid rafts. We found that PAT is enriched in lipid raft preparations. Furthermore, we have reconstituted recruitment of Gα to these domains through a mechanism that requires PAT activity.

EXPERIMENTAL PROCEDURES

Materials—Solute—350 was obtained from Packard Instruments. Diethylpyrocarbonate (DEPC) was purchased from Sigma Chemical Co. as a 6.9 M stock in isopropanol. DEPC was diluted in assay buffer and used immediately in experiments. [3H]Palmitoyl-CoA was prepared and purified as described previously (25, 27). Myristoylated recombinant G-protein α subunits were purified after co-expression in Escherichia coli with Saccharomyces cerevisiae N-myristoyltransferase (28). Recombinant βγC68S was purified from insect cells infected with recombinant b1 and γC68S viruses (29).

Assay for PAT Activity—A source of PAT (20 µl) was mixed with 10 µl of G-protein α (2 µg) and βγC68S (2 µg) subunits that had been diluted in 50 mM sodium Hepes (pH 8.0), 1 mM EDTA, and 1 mM DTT before addition. The reaction was started by the addition of 20 µl of a mixture containing [3H]palmitoyl-CoA, MES (pH 6.4), Triton X-100, and DTT such that the final concentrations in the assay were 0.4–1.0 µM, 100 mM, 0.04%, and 1 mM, respectively. Assays were incubated for 10 min at 30 °C, terminated by the addition of 1 ml of 15% trichloroacetic acid and 2% SDS, incubated at room temperature for at least 15 min, and collected on glass fiber filters. The filters were washed twice with 2-ml aliquots of 6% trichloroacetic acid containing 2% SDS, followed by four washes of 1.5 ml 6% trichloroacetic acid. The filters were dried, and bound radioactivity was quantitated using liquid scintillation counting.

Cell Culture—KBC cells (KB cells stably expressing caveolin) were cultured in MEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 150 units/ml penicillin, 50 µg/ml streptomycin, and MEM amino acids (Life Technologies, Inc.). FBS was heat-inactivated at 56 °C for 30 min. KBC cells were the gift of Dr. Linda Pike (Washington University).

Cyclodextrin Treatment—Confluent cells in D150 dishes were rinsed once with serum-free medium (SFM). Cells were then treated with 10 mM methyl-β-cyclodextrin (CD) (Aldrich) made fresh in SFM for 30 min at 37 °C. The cells were then rinsed once with cold SFM and harvested. Cholesterol reconstitution was carried out by incubating cells in CD–cholesterol complexes (0.2 mM cholesterol) in SFM for 30 min at 37 °C. Complexes were prepared by dissolving 200 µg of CD in 2.2 ml of water, heating to 80 °C, and slowly adding 6 mg of cholesterol dissolved in 2:1 isopropanol:CHCl3. This yielded a solution of 6.8 mM cholesterol.

Detergent-free Preparation of Low Density PM Domains—Low density plasma membrane domains were prepared as described previously (7, 13). Cells were seeded in D150 dishes and allowed to grow until confluent. Following treatment when appropriate, cells were rinsed once with cold SFM and scraped into 1-ml ice-cold high pH buffer (150 mM Na2CO3, pH 11, 1 mM EDTA). The cells were subject to 30 s sonication pulses by a Virsonic sonicator set at the maximum setting for a microprobe, or by a Branson sonicator at 25% power, or 30 s sonication pulses by a Virsonic sonicator set at the maximum setting for a microprobe, or by a Branson sonicator at 25% power. The samples were then sonicated while suspended in ice water, and each pulse was followed by a 1-min rest period, during which the probe was immersed in ice water. The lysate (1 ml) was diluted with 1 ml of 80% sucrose in MES-buffered saline (MBS, 25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA) in the bottom of an SW41 centrifuge tube. This solution was overlaid with 30% sucrose in MBS (6 ml) followed by 5% sucrose in MBS (4 ml). The three-step gradient was subject to centrifugation at 175,000 × g for 3 h at 4 °C in an SW41 rotor. Fractions of 1.2 ml were harvested from the top of the gradient. Prior to assaying PAT activity, the total cell lysate and fractions 9 and 10 were neutralized with 1 N HCl.

G-protein Reconstitution into Low Density PM Domains—Cells were washed and scraped into high pH buffer as described above. The lysate was subject to Dounce homogenization and shearing followed by centrifugation for 30 min at 200,000 × g. The membrane pellet was resuspended in MBSD (MBS + 1 mM DTT) and subjected to homogenization, shearing, and sonication as for the standard preparation of low density membranes. Palmitoylation reactions were performed in volumes of 100 µl containing 5 µM membranes, 5 µM [3H]palmitoyl-CoA, and ~8 µg of G-protein heterotrimer. The reaction was allowed to proceed for 15 min at 30 °C, after which it was placed at the bottom of a thick-walled TWS55 centrifuge tube. The reaction was mixed with 100 µl of 80% sucrose in MBS and then overlaid with 600 µl of 30% sucrose in MBS and 400 µl of 5% sucrose in MBS. The samples were subject to centrifugation at 45,000 rpm in a TSW55 rotor for 3 h. Fractions of 120 µl were collected from the top of the gradient, and portions were resolved by SDS-polyacrylamide gel electrophoresis. The Gα band identified by Coomassie Blue staining was cut out of the gel and solubilized with Soluene-350 for 3 h at 50 °C, followed by liquid scintillation counting. For DEPC treatment, 10 µl of membranes was mixed with 2 µl of DTT-diluted free in MBSD (final 5 µM in 12 µl) and incubated at 30 °C for 10 min. The sample was then diluted with MBSD prior to addition of PAT substrates.

Immunoblots and Miscellaneous Procedures—Equal volumes of fractions from the sucrose gradients were transferred to nitrocellulose and analyzed by immunoblotting. Caveolin-1 antibody was purchased from Transduction Laboratories and used at a 1:10,000 dilution. Antibody to the transferrin receptor was purchased from Zymed Laboratories Inc. Laboratories and used at a 1:1000 dilution. Immune complexes were detected using goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000) followed by enhanced chemiluminescence. Protein concentrations were determined by staining with Amido Black (30).

RESULTS

PAT Activity Is Highly Enriched in Low Density Membranes—To determine the distribution of PAT activity in membrane fractions, cell lysates were prepared from KBC cells using a detergent-free method and separated on a discontinuous sucrose gradient (7, 13). Fractions were collected from the top of the gradient and analyzed for PAT activity, total protein, and markers for membrane compartments (Fig. 1). PAT activity was highest at the interface of 5 and 35% layers (fraction 4). This enrichment of PAT activity was significant; a 30- to 50-fold increase in PAT-specific activity was measured when compared with the starting cell lysate. Fraction 4 represents the accumulation of low density membranes as indicated by the presence of caveolin (Fig. 1A) and the absence of the transferrin receptor (Fig. 1B), a marker for plasma membrane outside lipid rafts (15). Similarly, the ER resident protein, grp94 (31), as well as the Golgi-localized p58 (32), were found exclusively in fractions 9 and 10 (not shown). Although both PAT activity and caveolin always co-enriched in the low density fraction, there was variability in the amounts of caveolin and PAT activity in fractions 8–10 (compare Figs. 1A and 2A). This was likely due to experimental variation in the efficiency of isolation of low density membranes. It is notable that the amounts of caveolin and PAT activity in fractions containing higher density membranes did not always correlate well (compare Figs. 1A and 2A). This may reflect an overlapping distribution of PAT activity and caveolin in subdomains of the plasma membrane (see "Discussion").

The presence of PAT activity in low density membranes was independent of caveolin-1 expression. KBC is an epithelial cell line that has been stably transfected with caveolin-1 and exhibits large numbers of caveolae on the inner surface of the PM. However, PAT was also enriched in low density domains isolated from cell lines that do not have morphological caveolae,
including the parental KB cell line, and neuroblastoma cell lines NG108 and N2A (data not shown).

**PAT Activity in Low Density Membrane Domains Is Dependent on Cholesterol**

Having established that PAT was enriched in low density membranes, we assessed the dependence of this localization on the presence of cholesterol. Previous work has shown that the removal of cholesterol by the drug methyl-β-cyclodextrin (CD) results in disruption of the integrity of these domains and the delocalization of raft proteins and lipids (13). Depletion of cholesterol by CD significantly reduced PAT activity present in fraction 4 (Fig. 2A). The distribution of caveolin-1 was similarly affected. Compared with the control, more PAT activity was consistently found in the bottom of the gradient after cyclodextrin treatment, suggesting at least a partial redistribution of enzyme activity to membranes of higher density. However, we also consistently observed inhibition of PAT activity by CD treatment. These effects were reversed by reconstituting the cells with exogenously added cholesterol following CD treatment (Fig. 2B and data not shown). These data demonstrate that cholesterol is an important determinant of PAT activity and are consistent with the distribution of the enzyme in cholesterol-enriched subdomains of the PM.

**PAT Activity and the Enrichment of G_{ia} in Low Density Membranes**—Palmitoylation of newly synthesized G_{ia} is coincident with arrival at the PM (33). The enrichment of PAT in low density membranes suggested that the enzyme might directly recruit its substrates into rafts through the attachment of palmitate. Therefore, we tested whether exogenously added myristoylated G_{ia} could be stably reconstituted into lipid rafts through the action of PAT. A sonicated preparation of total cell membranes was incubated with purified G-protein heterotrimer and [3H]palmitoyl-CoA to allow palmitoylation to occur. Following the reaction, low density membranes were isolated on a sucrose gradient, and the G protein present in each fraction was analyzed for abundance and [3H]palmitate incorporation. As shown in Fig. 3A, a significant portion (typically 30–50%) of palmitoylated G_{ia} was present in the low density fractions. However, nearly all of the G-protein mass remained at the bottom of the gradient. Thus, palmitoylated G protein was significantly enriched in the low density membranes. To establish that this enrichment was dependent on the palmitoylation site, Cys-3, the same experiment was performed using recombinant G_{ia}C3A. Little [3H]palmitate was incorporated,
and no protein was detected in the low density membrane fractions (Fig. 3B). The small amount of radiolabeled protein detected is most likely due to spurious palmitate incorporation into one or more other cysteine residues. These data clearly demonstrate that palmitoylated G$_{ai}$ is preferentially and selectively recruited to low density membranes when compared with protein that is not palmitoylated.

To establish that the enrichment of palmitoylated G$_{ai}$ required PAT activity, it was necessary to demonstrate that the [3H]palmitate incorporation was due to an enzymatic process. Prior to the palmitoylation reaction, PAT activity was inactivated with diethylpyrocarbonate (DEPC). [3H]Palmitate incorporation was almost entirely abolished (Fig. 4), indicating that the incorporation observed in the absence of DEPC was due to active PAT. Furthermore, the protein that did incorporate [3H]palmitate was almost entirely confined to fractions 9 and 10, which contained the nonraft membranes. Thus, nonenzymatic acylation of G$_{ai}$ results in association with bulk membranes, whereas enzymatic acylation appears to be responsible for the enrichment of palmitoylated proteins in the low density domains. The selective recruitment of palmitoylated G$_{ai}$ to rafts in vitro suggests that PAT activity is a critical part of the mechanism by which its substrates are targeted to lipid rafts.

**DISCUSSION**

In this report, we examined the role of PAT in the distribution of G$_{ai}$ in lipid rafts in the PM. We found that PAT activity for G$_{ai}$ is highly enriched in low density membranes, suggesting that it has an important functional role in targeting proteins to these domains. Indeed, we were able to selectively recruit palmitoylated G$_{ai}$ to rafts in vitro, suggesting that PAT activity is a critical part of the mechanism by which its substrates are enriched in lipid rafts. We have proposed previously that palmitoylation serves as a targeting signal for PM localization of G$_{ai}$ subunits that are myristoylated and palmitoylated (24). This hypothesis is based on the kinetic membrane-trapping model of Silvius and co-workers (35). In this model, a protein modified with a myristoyl group will cycle on and off membranes until it encounters a membrane where the protein is palmitoylated. Although a myristoyl group does not provide sufficient hydrophobicity for stable membrane association, two lipid modifications yield a protein that is “trapped” at the membrane. The model has been extended and refined by studies of the targeting of G$_{ai}$, a myristoylated, palmitoylated G$_{ai}$ subunit. In this “dock and lock” modification, binding to G$_{ai}$ subunits targets G$_{ai}$ to the PM where palmitoylation then stabilizes G$_{ai}$ association with the PM (26). Our current study extends this model further, suggesting that palmitoylation occurs in lipid rafts, trapping G$_{ai}$ in these subdomains.

The temporal relationship between palmitoylation of G-protein $\alpha$ subunits and their localization in lipid rafts has not been studied in cells, but our data suggest that they would coincide. Interestingly, the NRK Fyn is palmitoylated at the PM but does not acquire detergent resistance, a hallmark of raft localization, until 10–20 min later (36). Resolution of this apparent discrepancy will require kinetic analysis of G-protein entry into detergent-resistant membranes and a better understanding of the substrate range of the PAT activity we have characterized. PAT may have an overlapping distribution with caveolin in subdomains of the plasma membrane, similar to that seen for G$_{ai}$. Caveolin and G$_{ai}$ biochemically co-fractionate in low density membranes derived from a variety of cell types and tissues (2). Immunogold electron microscopy reveals that some G$_{ai}$ is associated with caveolae, however, most are clustered in irregular structures of the plasma membrane that have not been identified morphologically (9). Recently Oh and Schnitzer (37) reported that, in lung tissue and in endothelial and epithelial...
cultured cell lines, the heterotrimERIC G-proteins G_i and G_o appear to be associated primarily with a lipid raft fraction distinct from caveolae, whereas G_o is concentrated in caveolae (37). Future studies will be necessary to address whether PAT activity is associated with one or both types of plasma membrane subdomains.

PAT activity partially redistributes to membranes of higher density when cholesterol is depleted, suggesting that its enrichment in rafts is dependent upon cholesterol. This is not surprising, because cholesterol is required for the integrity of lipid rafts. PAT activity can only be solubilized from bovine brain in a nonaggregated form with high concentrations of Triton X-100 (38) or the ZWITTERGENT Z3-12.2 This resistance to detergent extraction is consistent with its localization in lipid rafts. PAT activity is stable in a detergent extract, but the preparation rapidly loses activity when fractionated through several chromatographic steps. The observation that PAT is inhibited when cholesterol is depleted suggests that cholesterol may be an important stabilizing factor for PAT. Chromatographic separation may remove lipids required for PAT activity. We have attempted to stabilize partially purified PAT with exogenously added cholesterol but have not been successful.2 Further investigation of the lipid requirements for PAT activity is warranted as specific lipids may be required to purify this elusive enzyme.

The lipid environment of rafts may impact the lipid substrate availability for PAT.RAFT membrane lipids are believed to exist in a state similar to the liquid-ordered phase described in model membranes (39, 40). In this state, lipid acyl chains are tightly packed, highly ordered, and extended, an environment that favors saturated acyl chains. Proteins modified with saturated acyl chains would be expected to partition favorably into liquid-ordered phase domains. In support of this model, proteins modified with saturated but not unsaturated fatty acids associate with detergent-resistant membranes in cells (23) and in model membranes (41). In vitro, PAT uses acyl-CoAs with saturated or unsaturated acyl chains as substrates (25, 42). However, the finding that raft proteins are modified with saturated fatty acids suggests that the pool of acyl-CoAs available to PAT may be enriched in species with saturated acyl chains.

In addition to its ability to recruit G_i directly into rafts, the localization of PAT in these domains may have implications for regulation of G_o by palmitoylation. Palmitoylation is dynamic, thus PAT must be available for regeneration of palmitoylated G_o after palmitate turnover. The co-distribution of PAT and its substrates in lipid rafts would facilitate palmitoylation. Agonists stimulate palmitate turnover on G_o and G_o, and this may have a regulatory role in desensitization of G-protein-activated G-proteins, including G_o, in low density membranes. In a cell-free system, PAT can recruit exogenously added G-protein to these domains, suggesting that PAT is responsible at least in part for the localization of G-proteins in lipid rafts.

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