Molecular Biology of the Cell
Vol. 12, 685–698, March 2001

Segregation of Heterotrimeric G Proteins in Cell Surface Microdomains

G_q Binds Caveolin to Concentrate in Caveolae, whereas G_i and G_s Target Lipid Rafts by Default

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Submitted December 11, 2000; Accepted January 17, 2001
Monitoring Editor: Monty Krieger

Select lipid-anchored proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins and nonreceptor tyrosine kinases may preferentially partition into sphingomyelin-rich and cholesterol-rich plasmalemmal microdomains, thereby acquiring resistance to detergent extraction. Two such domains, caveolae and lipid rafts, are morphologically and biochemically distinct, contain many signaling molecules, and may function in compartmentalizing cell surface signaling. Subfractionation and confocal immunofluorescence microscopy reveal that, in lung tissue and in cultured endothelial and epithelial cells, heterotrimeric G proteins (G_i, G_q, G_s, and $G_{\beta\gamma}$) target discrete cell surface microdomains. G_q specifically concentrates in caveolae, whereas G_i and G_s concentrate much more in lipid rafts marked by GPI-anchored proteins (5′ nucleotidase and folate receptor). G_q, apparently without $G_{\beta\gamma}$ subunits, stably associates with plasmalemmal and cytosolic caveolin. G_i and G_s interact with $G_{\beta\gamma}$ subunits but not caveolin. G_i and G_s, unlike G_q, readily move out of caveolae. Thus, caveolin may function as a scaffold to trap, concentrate, and stabilize G_q preferentially within caveolae over lipid rafts. In N2a cells lacking caveolae and caveolin, G_q, G_i, and G_s all concentrate in lipid rafts as a complex with $G_{\beta\gamma}$. Without effective physiological interaction with caveolin, G proteins tend by default to segregate in lipid rafts. The ramifications of the segregated microdomain distribution and the G_q-caveolin complex without $G_{\beta\gamma}$ for trafficking, signaling, and mechanotransduction are discussed.

INTRODUCTION

In cellular membranes, cholesterol, glycolipids, and select lipid-anchored proteins appear to organize into domains that are resistant to nonionic detergent solubilization. Although two such microdomains, caveolae and lipid rafts, share various biochemical properties, including a requirement for cholesterol (Rothberg *et al.*, 1990; Schnitzer *et al.*, 1994; Murata *et al.*, 1995; Monier *et al.*, 1996; Schroeder *et al.*, 1998) and similar low buoyant densities (Gorodinsky and Harris, 1995; Schnitzer *et al.*, 1995b), they are actually distinct morphologically, biochemically, and functionally (Schnitzer *et al.*, 1995b; Liu *et al.*, 1997). Lipid rafts are distinguished as flat domains rich in glycosylphosphatidylinositol (GPI)-anchored proteins that rely primarily, maybe even solely, on lipid-lipid interactions for their formation and detergent resistance (Ahmed *et al.*, 1997; Schroeder *et al.*, 1998; Brown and London, 2000). In contrast, caveolae are smooth, flask-shaped, cell-surface invaginations (Schnitzer *et al.*, 1995b) that appear to depend on caveolin oligomerization for their formation (Fra *et al.*, 1995; Monier *et al.*, 1996; Lipardi *et al.*, 1998). Caveolin is palmitoylated (Dietzen *et al.*, 1995; Monier *et al.*, 1996), and it binds cholesterol (Monier *et al.*, 1996), which appears to be required for its role in maintaining caveolar structure (Schnitzer *et al.*, 1994). Caveolae and lipid rafts may sometimes associate with each other, but they appear to exist predominately at the cell surface as independent structures (Schnitzer *et al.*, 1995b). Lipid rafts may exist in cells without caveolae (Fra *et al.*, 1994; Gorodinsky and Harris, 1995). Many subfractionation techniques that sort based on detergent-resistance and/or low buoyant densities (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Lisanti *et al.*, 1994; Smart *et al.*, 1995) tend to co-isolate caveolae and lipid rafts (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1999). However, more sophisticated subfractionation techniques can isolate caveolae and lipid rafts separately from the same plasma membranes (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1999). Unlike caveolae, with their distinct, readily observed, invaginated morphology, lipid rafts are more difficult to observe, and their existence is still the subject of some debate (Mayor

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and Maxfield, 1995; Kenworthy and Edidin, 1998; Kenworthy *et al.*, 2000).

Caveolae function in mediating endocytosis and transcytosis of select macromolecules (Schnitzer *et al.*, 1994; Schnitzer *et al.*, 1995a; Oh *et al.*, 1998; McIntosh and Schnitzer, 1999). Caveolae are also rich in many signaling molecules, including platelet-derived growth factor receptor (PDGF-R) (Liu *et al.*, 1996; Liu *et al.*, 1997), endothelial nitric oxide synthase (eNOS) (Feron *et al.*, 1996; Garcia-Cardena *et al.*, 1996; Rizzo *et al.*, 1998a), and nonreceptor tyrosine kinases (NRTK) (Liu *et al.*, 1997), many of which may interact directly with caveolin via its scaffolding domain (Li *et al.*, 1996; Garcia-Cardena *et al.*, 1997). The removal of cholesterol from the plasma membrane by cholesterol binding agents such as filipin affects caveolin organization (Rothberg *et al.*, 1990; Rothberg *et al.*, 1992). These compounds disassemble caveolae which disperses caveolar molecules to a more random distribution over the cell surface, thereby disrupting both transport and signaling functions (Schnitzer *et al.*, 1994; Liu *et al.*, 1997). For example, filipin treatment prevents both PDGF-induced downstream signaling past initial receptor autophosphorylation (Liu *et al.*, 1997) and mechanical stress-induced protein tyrosine phosphorylation and activation of the Ras/Raf/MAP kinase pathway (Rizzo *et al.*, 1998b). Like caveolae, lipid rafts contain various signaling molecules, including NRTK, immunoglobulin E receptor, T cell receptor (TCR), and GPI-anchored proteins (Stefanova *et al.*, 1991; Shenoy-Scaria *et al.*, 1992; Field *et al.*, 1995; Gorodinsky and Harris, 1995; Liu *et al.*, 1997; Montixi *et al.*, 1998; Xavier *et al.*, 1998). Lipid raft structure is also affected by cholesterol binding agents which cause the dispersal of raft molecular constituents throughout the plasma membrane (Rothberg *et al.*, 1990). Filipin treatment of cultured lymphocytes inhibits TCR-mediated Ca^{2+} mobilization and protein tyrosine phosphorylation (Xavier *et al.*, 1998). Thus, the compartmentalization of key signaling molecules in caveolae and lipid rafts appears necessary to provide rapid, efficient, and specific propagation of extracellular stimuli to intracellular targets.

Heterotrimeric G proteins, which are composed of three distinct subunits, α, β, and γ, mediate intracellular signaling by various receptors induced by specific ligands (Morris and Malbon, 1999). Although there is evidence that various G proteins concentrate in detergent-resistant membrane domains (Moffett *et al.*, 2000), it is unclear whether they localize to caveolae and interact with caveolin. Some groups report significant enrichment of G_i, G_s, G_o, and $G_{\beta\gamma}$ in caveolin-rich fractions (detergent-resistant buoyant membranes or low density, sonication-released vesicles) (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Lisanti *et al.*, 1994; Li *et al.*, 1995; Smart *et al.*, 1995). Others have observed G proteins (G_i, G_s, and $G_{\beta\gamma}$) present, but not enriched, in isolated caveolae (Schnitzer *et al.*, 1995a). In contrast, another group (Stan *et al.*, 1997) concluded that caveolae cannot function as signaling compartments based on their inability to detect G proteins (and other signaling molecules such as eNOS) in immuno-isolated caveolae. However, more recent work (Oh and Schnitzer, 1999) demonstrates that this past failure in detection was an artifact of the methodology and that G proteins and other signaling molecules are indeed at least present in immuno-isolated caveolin-coated caveolae. Like other caveolar signaling molecules, G proteins may interact with caveolin. In binding assays using recombinant proteins, one group observed a direct interaction between G_i and G_o subunits and the caveolin scaffolding domain (Li *et al.*, 1995). This interaction may be regulatory, because synthetic peptides corresponding to the scaffolding domain inhibit GTPase activities of G_i and G_o (Li *et al.*, 1995) and GTPγS binding to G_o (Li *et al.*, 1995). More recently, however, another group (Huang *et al.*, 1997) detected little G_i and G_s within caveolae or coated pits and did not observe either G protein-caveolin interactions or an inhibitory effect of caveolin or its scaffolding domain peptide on GTPase activity or GTPγS binding.

In this study, we focus in detail on the distribution of G proteins at the cell surface in vivo using dual immunofluorescence microscopy and subcellular fractionation techniques that allow reliable and consistent purification of caveolae and lipid rafts separately from each other and from complicating, contaminating membranes (i.e. Golgi) (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1999). We show that G_i and G_s at the cell surface preferentially target lipid rafts whereas G_q concentrates in caveolae through its specific interaction with caveolin. This differential distribution may help explain the basis of many of the discrepancies between studies reported in the literature.

MATERIALS AND METHODS

Materials

Antibodies against caveolin were purchased from Transduction Labs (Lexington, KY) (rabbit polyclonal (pAb) and mouse monoclonal (clone # 2234)), Zymed Laboratory (South San Francisco, CA) (mouse monoclonal (clone #Z034)), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (rabbit pAb). Polyclonal antibodies recognizing common subunits of G_q ($G_{q/11}$, cat# sc-392), G_i ($G_{i/o/t/z}$, sc-386), G_s ($G_{s/olf}$, sc-383), and G_β ($G_{\beta1-4}$, sc-261), as well as specifically G_q (sc-393), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Both $G_{q/11}$-common and G_q-specific antibodies gave similar results. Polyclonal antibodies to G_s, G_i, and G_q were a gift from Dr. David Manning (University of Pennsylvania, Philadelphia, PA). Antibodies to 5′NT were a gift from Dr. Paul Luzio (University of Cambridge). The folate receptor antibodies, MOV18 and MOV19, were a gift from Dr. John Ghrayeb (Centocor). Texas Red anti-mouse IgG and Bodipy anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). M-450 Dynabeads (anti-mouse conjugated magnetic beads) and M-280 Dynabeads (anti-rabbit conjugated magnetic beads) were purchased from Dynal (New Hyde Park, NY). The N2a mouse neuroblastoma cells were obtained from the American Type Culture Collection. All other reagents/supplies were obtained as in our past work (Schnitzer *et al.*, 1995a; Schnitzer *et al.*, 1995b; Schnitzer *et al.*, 1995c; Schnitzer *et al.*, 1996).

Isolation of Plasma Membrane- and Caveolae-enriched Fractions from Rat Lung Homogenates

Homogenates of rat lung tissues were subjected to Percoll gradient centrifugation to isolate a plasmalemmal fraction (PM) as described (Smart *et al.*, 1995; Oh and Schnitzer, 1998; Oh and Schnitzer, 1999). Briefly, rat lungs were flushed free of blood, homogenized in buffer (0.25 M sucrose/1 mM EDTA/20 mM Tricine, pH 7.8), filtered through a Nytex filter, and subjected to centrifugation (1000 × *g*) for 10 min. The post-nuclear supernatant was mixed with Percoll and subjected to centrifugation (84,000 × *g*) for 45 min. A single membranous band, readily visible ~ 2/3 from bottom of the tube (PM), was collected. PM is enriched in plasmalemmal markers such as caveolin, angiotensin-converting enzyme (ACE), and 5′ nucleotidase (5′NT), but it also contains endosomal and Golgi membrane

markers, such as ϵ-COP, and possibly other intracellular membranes (Oh and Schnitzer, 1999). A caveolin-rich low density fraction (AC) was separated from PM by sonication, followed by Optiprep density centrifugation. AC is significantly enriched in caveolin (and thus caveolae) but also contains lipid rafts, Golgi/endosomal, and possibly other membranes (Oh and Schnitzer, 1999).

Isolation of Luminal Endothelial Cell Plasma Membranes, Caveolae, and Lipid Rafts

The luminal endothelial cell plasma membranes and their caveolae were isolated directly from rat lung tissue using an in situ silica-coating procedure, as described (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1998). Briefly, rat lungs were perfused via the pulmonary artery with a colloidal silica solution to coat the endothelial cell luminal surface and allow selective isolation of the luminal endothelial cell plasma membranes (P) from the lung homogenate (H) by density centrifugation. P is enriched in the endothelial cell surface proteins caveolin, 5′NT, and ACE but is markedly depleted in markers for other cell types or intracellular organelles such as ϵ-COP (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1998). The caveolae (V) were separated from P by homogenization and isolated from the silica-coated membrane pellet stripped of caveolae (P-V) by sucrose density centrifugation in a low buoyant density fraction (Schnitzer *et al.*, 1995b; Schnitzer *et al.*, 1995c). V contains a homogeneous population of morphologically distinct caveolar vesicles enriched in caveolin, while being markedly depleted in noncaveolar proteins including ACE, β-actin, 5′NT, and uPAR (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1999). To isolate lipid rafts (LR), P-V was incubated in 2 M K_2HPO_4, to separate membranes from silica coating before their homogenization in 1% Triton X-100 at 4°C, followed by sucrose gradient centrifugation, as described previously (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1998). LR was collected as a visible, membranous band between 10 and 15% sucrose, and it is enriched in GPI-anchored proteins but essentially devoid of caveolin.

Immuno-affinity Isolation of Caveolae

Magnetic immuno-isolations were performed as described (Oh and Schnitzer, 1999). Briefly, M450 Dynal beads conjugated to caveolin mAb (clone 2234) (2×10^7 M450 beads and 25 μg IgG) were incubated for 1 h at 4°C with 25 μg of the starting membrane fraction (SM), then washed and magnetically separated to isolate two fractions: material bound to the beads (B) versus unbound material (U). Testing V by this method shows nearly complete binding of the membranes and proteins in V to the beads, indicating effective quantitative isolation of a reasonably homogeneous fraction of caveolae (Oh and Schnitzer, 1999).

Immunofluorescence Microscopy

Bovine lung microvascular endothelial cells (BLMVEC), monkey kidney epithelial cells (MA104), or N2a mouse neuroblastoma cells were grown on coverslips for dual immunofluorescence confocal microscopy as described in our past work (Oh *et al.*, 1998). Briefly, cells were fixed with methanol, blocked with 2% goat serum, then stained with antibodies to caveolin (clone Z034), 5′NT, folate receptor plus antibodies to specific G protein subunits (1:250 dilution). The bound primary antibody was detected with a reporter IgG conjugated to Texas Red (anti-mouse IgG) or Bodipy (anti-rabbit IgG) (Molecular Probes, Eugene, OR). The immunofluorescence signal was visualized and photographed using a confocal fluorescence microscope (Perkin Elmer-Cetus Wallac, Gaithersburg, MD) The ratio of overlapping signals was quantified using Metamorph Software (Universal Imaging, Chesterfield, PA) and was confirmed by drawing a line through a digital image of the cells and by counting the total number of red, green, or overlapping (yellow) signals contacting the line and calculating the ratio of yellow to green or red pixels, as in past work (Liu *et al.*, 1997)(our unpublished results). At least five lines were used in each calculation, and each line had at least 50 signals associated with it. Methanol fixation was used after a comparative evaluation of fixatives showed the equivalence of methanol and glutaraldehyde in preventing subsequent sequestration of GPI-anchored proteins by antibodies.

Immunoprecipitation of Caveolin and G Protein Complexes

Purified luminal endothelial cell membranes (P) (100 μg of total protein) or N2a cells that had been scraped from the plate, washed, and pelleted by centrifugation were solubilized for 1 h at 4°C with 20 mM CHAPS in TBS (50 mM Tris pH 7.6, 135 mM NaCl), then incubated for 1–2 h at 4°C with magnetic beads coated with antibodies to either caveolin or specific G-protein subunits, as in our past work (Rizzo *et al.*, 1998a). Magnetic separation was used to isolate two fractions—insoluble material bound to the beads (I) and soluble material not bound to the beads (S). Western analysis was performed as described using caveolin or G protein antibodies (Rizzo *et al.*, 1998b)

Preparation of Rat Lung Cytosols and Immunoprecipitation of Caveolin and G Proteins

Sprague Dawley rat lungs were perfused with Ringer's solution, followed by sucrose/HEPES solution containing protease inhibitors, as in past work (Schnitzer *et al.*, 1995b; Oh *et al.*, 1998). The lungs were minced, homogenized in 5 ml cytosolic buffer (25 mM KCl, 2.5 mM $Mg(C_2H_3O_2)_2$, 5 mM EGTA, 150 mM $KC_2H_3O_2$, 25 mM HEPES, pH7.4), and filtered through Nytex (53 μ then 33 μ). The filtered material was subjected to centrifugation at $100{,}000 \times g$ for 60 min at 4°C in a SW55 rotor using an Optima Max-E Ultracentrifuge (Beckman Coulter). The supernatant was subsequently respun at $300{,}000 \times g$ for 60 min at 4°C to generate membrane-free cytosol. Our testing of this material in sucrose gradients shows no detectable floating membranes or caveolin in the floating fractions. Fifty microliters cytosol (5 mg/ml) was incubated for 60 min at room temperature with M450 Dynal magnetic beads coated with antibodies (2×10^7 beads per 25 μg antibodies) either to caveolin or to specific G protein subunits, as described (Oh and Schnitzer, 1999). After magnetic separation of the bound material, the beads were split into four equal aliquots, and each was subjected to Western analysis.

RESULTS

Select G Proteins at the Cell Surface in Caveolae

To assess the G protein content in the plasma membrane and caveolae, we isolated luminal endothelial cell plasma membranes (P) from rat lung homogenates (H) using the colloidal silica coating technique (Schnitzer *et al.*, 1995b; Schnitzer *et al.*, 1995c; Oh and Schnitzer, 1998). The caveolae were stripped from the plasma membrane by homogenization, then isolated by sucrose density centrifugation to yield isolated caveolae (V) well separated from the pellet containing resedimented silica-coated membranes stripped of caveolae (P-V) (Schnitzer *et al.*, 1995b; Schnitzer *et al.*, 1995c; Oh and Schnitzer, 1998). Western analysis of 5 μg of each fraction revealed significant enrichment in P relative to H for G_q, G_i, G_s, and G_β (Figure 1A). In addition, all were found, to varying degrees, in caveolae (V). Only G_q was enriched along with caveolin in V compared with both P and P-V. G_i, G_s, and G_β, although present, were not concentrated in V. Their level in isolated caveolae did not correlate with the increased caveolin concentration but, rather, was equivalent to the rest of the plasma membrane, consistent with the

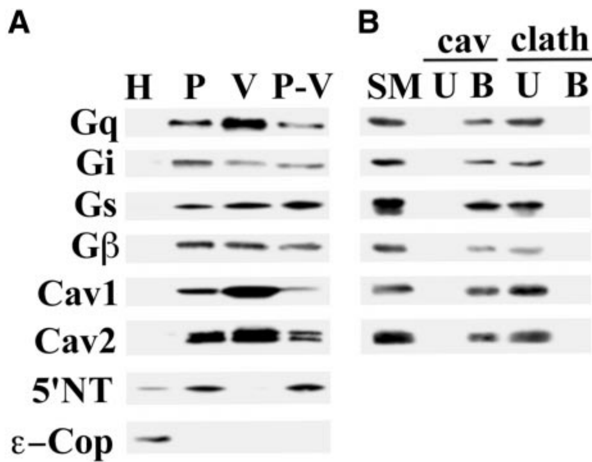

Figure 1. Distribution of G proteins in isolated membranes and caveolin-coated caveolae. (A) Subcellular fractionation was performed on rat lung tissues to obtain whole lung homogenates (H), silica-coated luminal endothelial cell plasma membranes (P), caveolae (V), and repelleted silica-coated membranes stripped of caveolae (P-V). Western analysis of 5 μg of each these fractions is shown using antibodies to G_i, G_s, G_q, and G_β, 5′ NT, ϵ-Cop, and caveolin-1 and 2. (B) The caveolae fraction (V) was subjected to immunoaffinity isolation using magnetic beads attached to antibodies to caveolin-1 (cav) or clathrin heavy chain (clath). Five micrograms of the V fraction starting material (SM) and the entire amount of antibody bound (B) or unbound (U) material was subjected to Western analysis using antibodies to the indicated proteins.

substantial signal for G_i, G_s, and G_β but not caveolin remaining in P-V. This is in agreement with past work demonstrating the presence of G_i, G_s, and G_β in both V and P-V fractions with very little detected in the Triton-soluble membrane fraction (Schnitzer *et al.*, 1995a). In contrast, G_q appeared to follow caveolin, to concentrate in V, and to be markedly depleted, though not absent, in P-V.

Because G proteins are also extensively present in Golgi membranes (Denker *et al.*, 1996), we assessed the quality of each fraction by Western analysis using antibodies against caveolin-1 and -2, the GPI-anchored protein, 5′NT, and the intracellular endosomal and Golgi marker, ϵ-COP. P displayed ample enrichment for 5′NT, caveolin-1, and -2 relative to H, while being markedly depleted in ϵ-COP (Figure 1A). V was enriched in caveolin-1 and –2, whereas 5′NT and ϵ-COP appeared absent.

To confirm further that the G proteins detected in V are indeed in caveolin-coated caveolae and not from any possible contaminating membranes, we performed immuno-isolation on V using caveolin-1α antibody-conjugated magnetic beads, as described previously (Oh and Schnitzer, 1999). The caveolin antibody bound (B) and unbound (U) fractions were subjected to Western analysis using G_q, G_i, G_s, and Gβ antibodies. As shown in Figure 1B, all of the G proteins detected in V were found in the caveolin-coated caveolae bound to the magnetic beads (B), with no signal detected in U, thereby confirming their existence in caveolin-1-coated caveolae. Both caveolin-1 (α and β) and -2 were found exclusively in B. Control immuno-isolations using an antibody to clathrin gave no signal in B with all of the G protein and caveolin remaining in U. These data are consistent with past reports (Oh and Schnitzer, 1999) in which essentially all of the material from V fractionates into B, indicating that V contains a rather homogeneous population of caveolin-coated caveolae.

Immunomicroscopy of Plasmalemmal G Proteins in Cultured Cells

One reason for G_i and G_s lacking enrichment in V may be that they are only weakly associated and become separated from the caveolae during the membrane subfractionation procedure. To avoid this possibility and to determine whether select G proteins also concentrate in caveolae of cultured cells, we examined their plasma membrane distribution in BLMVEC by dual immunofluorescence confocal microscopy, using antibodies to caveolin and either G_i, G_s, G_q, or G_β (Figure 2). Consistent with past reports (Liu *et al.*, 1997; Oh *et al.*, 1998), caveolin-1 antibodies revealed a punctate staining pattern marking the caveolae on the cell surface (Figure 2, left panels, red labeling). Likewise, all G proteins displayed significant punctate cell surface staining, indicating their preferential targeting and elevated concentration within discrete plasmalemmal microdomains (Figure 2, middle panels; green labeling). When the images were overlaid, G_q showed the greatest degree of colocalization with caveolin (mean of 88%; Figure 2, right panels; yellow signal), whereas the signal for G_i (18 $\pm$ 6%), and G_s (32 $\pm$ 9%) showed considerably less overlap with caveolin. G_β was a composite of the G_q, G_i and G_s signals, exhibiting marked colocalization with caveolin as well as significant nonoverlapping signals (our unpublished results). Similar results were obtained using other cell types including MA104 (see Figure 4 and our unpublished results). These data are in agreement with the membrane subfractionation data demonstrating substantial enrichment of G_q, but not G_i, G_s, and $G_{\beta\gamma}$ in caveolae of lung microvascular endothelium in tissue. Using techniques that detected caveolar localization of dynamin, eNOS, VAMP, and other proteins (Oh *et al.*, 1998; Rizzo *et al.*, 1998a; McIntosh and Schnitzer, 1999), we also attempted to localize G proteins by performing electron microscopy on immunogold labeled ultra-thin cryo-sections of rat lung tissue. We detected ample signal for caveolin coating the bulb of caveolae but no signal for G proteins (our unpublished results), suggesting that the antibodies are not suitable for such electron microscopy studies.

Segregation of G Proteins between Isolated Caveolae and Lipid Rafts

Because G_i and G_s were primarily concentrated in discrete, caveolin-free microdomains, as detected by immunofluorescence microscopy, we tested whether select G proteins differentially targeted lipid rafts. Caveolae and lipid rafts were isolated separately from each other from the same plasma membranes, namely P, as described previously (Schnitzer *et al.*, 1995b). Western analysis under equivalent protein conditions revealed that G_i and G_s were indeed enriched in the lipid rafts (LR), compared with caveolae (V) (Figure 3). Densitometric analysis revealed a 2- to 4-fold enrichment of G_i and G_s in lipid rafts. Note that the signal for these G proteins was always equivalent in P, V, and P-V, so that LR was the first subfraction of P to show any significant enrichment. Conversely, G_q (24-fold enrichment in V over LR) and

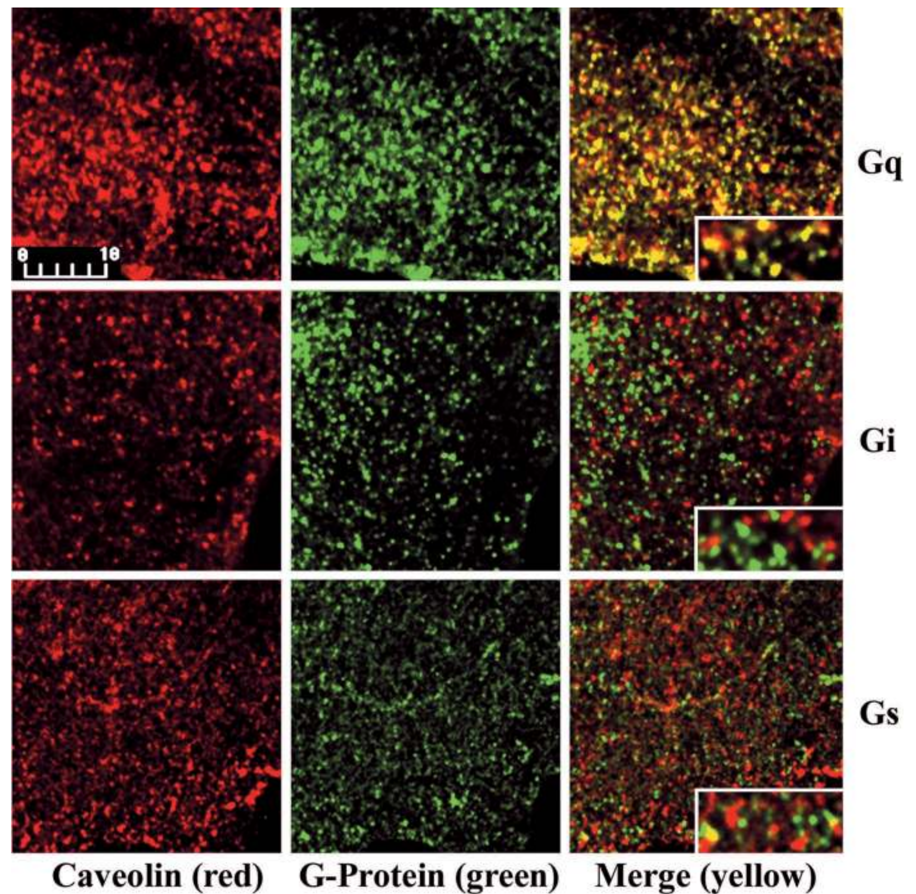

Figure 2. Immunofluorescence microscopy of G protein and caveolin-1 colocalization. Bovine lung microvascular endothelial cells (BLMVEC) were fixed and incubated with antibodies, either to caveolin (left panel, red labeling) or to the indicated G proteins (middle panel, green labeling). Signal overlap for caveolin and G protein generates yellow in the overlay of the two images (right panel).

caveolin (35-fold) were highly concentrated in caveolae and minimally or not detected in LR. Consistent with previous reports (Schnitzer *et al.*, 1995b), the GPI-anchored protein and lipid raft marker, 5′NT, was concentrated in LR (34-fold more concentrated in LR versus V) (Figure 3). These data demonstrate that G proteins can be differentially distributed at the cell surface between caveolae and lipid rafts with G_q selectively segregating and concentrating in caveolae, while G_i and G_s preferentially target lipid rafts.

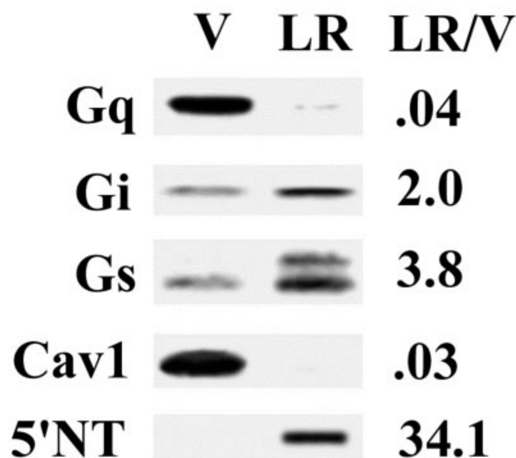

Figure 3. Differential distribution of G proteins in lipid rafts. Caveolae (V) and lipid rafts (LR) were isolated from the silica-coated endothelial cell plasma membranes (P) derived from lung. Western analysis of V and LR (5 μg each) is shown using antibodies to the indicated G proteins, caveolin-1, and the lipid raft marker, 5′NT.

G_i and G_s Target Lipid Rafts in Cultured Cells

To extend the subfractionation data demonstrating the selective concentration of G_i and G_s in, and apparent exclusion of G_q from, lipid rafts, we performed dual immunofluorescence confocal microscopy on cultured endothelial and epithelial cells using antibodies to caveolin-1, G_i, G_s, G_q, and the lipid raft markers, folate receptor, and 5′NT. Figure 4 shows an overlay of G protein (red label) and folate receptor (green label) images (see inset), displaying little overlap of G_q with folate receptor on the surface of MA104 cells (Figure 4A, 17 ± 6%, yellow label), whereas G_s (Figure 4E, 80 ± 5%) and G_i (Figure 4C, 61 ± 10%) exhibited significant overlap with folate receptor. Studies using BLMVEC colabeled with 5′NT and G proteins gave similar results (our unpublished results). Figure 4, panels B, D, and F, shows an overlay of caveolin (green label) and G protein (red/orange label)

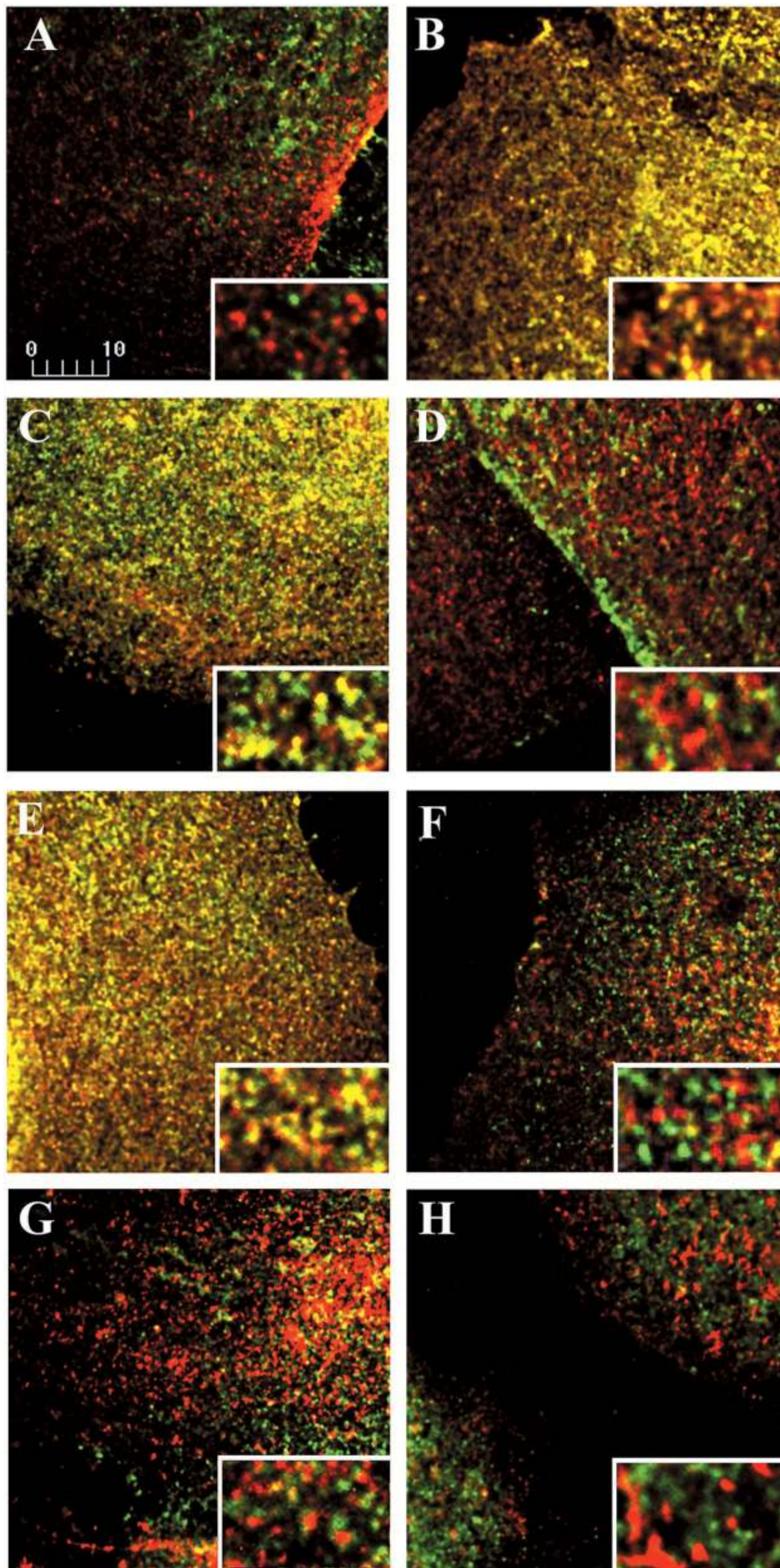

Figure 4. Immunofluorescence microscopy of lipid rafts for colocalization with G proteins and caveolin. MA104 cells (panels A-G) were grown on coverslips, fixed, and incubated with monoclonal antibodies to either folate receptor (panels A, C, E, green labeling) or caveolin (panels B, D, F; green labeling), and polyclonal antibodies to G_q (panels A and B, red/orange), G_i (panels C and D, red/orange), G_s (panel E and F, red/orange), and caveolin (G, red/orange). (Panel H) BLMVEC labeled with 5′NT antibodies (green) and caveolin antibodies (red/orange). Images were overlaid, and the signal overlap is indicated in yellow. Inset: higher magnification of a representative area of the image showing overlapping signals. Magnification of insets, 3×.

staining in MA104 cells. Similar to the BLMVEC (Figure 2), G_q exhibited significant overlap with caveolin-containing caveolae (88 ± 8%, yellow label), while G_i and G_s demonstrated much less colocalization with caveolin, at 17 ± 8% and 31 ± 9% (yellow label, see inset), respectively. We also observed little colocalization of caveolin with folate receptor (15 ± 3% in MA104) and 5′NT (3 ± 2% in BLMVEC), on the cell surface, consistent with caveolae and lipid rafts being distinct membrane domains (Figure 4G, H) (Schnitzer *et al.*, 1995b). These data support the above subfractionation and caveolin colocalization data demonstrating the rather selective segregation of G_q to caveolae and G_i and G_s to lipid rafts at the cell surface.

G Proteins in Other Caveolin-enriched Fractions

The above data are quite consistent with past studies showing the presence but not enrichment of G_s and G_i in caveolae (Liu *et al.*, 1996; Oh and Schnitzer, 1999). In contrast to the data presented here, other past studies reported that G_i, G_s, and G_β are enriched in caveolar membrane fractions (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Lisanti *et al.*, 1994; Li *et al.*, 1995; Smart *et al.*, 1995; Song *et al.*, 1997). Many factors could contribute to this difference, including the cell type and the membrane subfractionation technique used. Therefore, we examined G protein distribution utilizing a popular, alternative, detergent-free subfractionation method used in many of these studies. We isolated plasma membranes from rat lung tissue using Percoll gradient centrifugation (PM). A caveolin-enriched fraction (AC) was isolated from PM by sonication followed by density centrifugation. Western analysis revealed significant enrichment in AC for caveolin, G_q, G_i, G_s, and G_β as compared with PM (see Figure 5), thereby confirming the results reported in past studies.

Because AC has been shown to contain noncaveolar markers, including ε-COP and 5′NT (Figure 5A and past work (Oh and Schnitzer, 1999)), we subjected AC to immunoisolation using caveolin antibodies. This procedure isolates caveolin-coated vesicles away from other possible contaminating membranes in AC (Oh and Schnitzer, 1999), including lipid rafts and Golgi membranes, a site known to be rich in G proteins (Denker *et al.*, 1996). Caveolin antibody bound (B) and unbound (U) fractions were examined by Western analysis and, although ample signals for each of the G proteins tested (G_q, G_i, G_s and G_β) were seen in the starting material (AC), only G_q was enriched in the caveolae bound to the caveolin antibody-conjugated beads (B) (Figure 5A). The signal from the bound versus unbound fractions was quantified densitometrically, and the ratio was calculated for each G protein and caveolin (Figure 5B). As expected, caveolin was enriched 5-fold in B (Oh and Schnitzer, 1999), whereas ε-COP apparently remained completely in U. In addition, G_q was enriched 3-fold while G_i, G_s, and G_β exhibited no enrichment in B. These findings are consistent with the data obtained from Western analysis of the H, P, V, P-V, and LR subfraction analysis presented above. Thus, an alternative analysis not utilizing the silica-coating procedure also detected the enrichment of G_q, but not G_i, G_s, and G_β, in caveolae. It appears that the uniform enrichment observed in AC compared with PM for each of the G proteins may reflect their high concentration in either caveolae, lipid rafts, and/or Golgi membranes, all of which are present to a great extent in this heterogeneous low density membrane fraction.

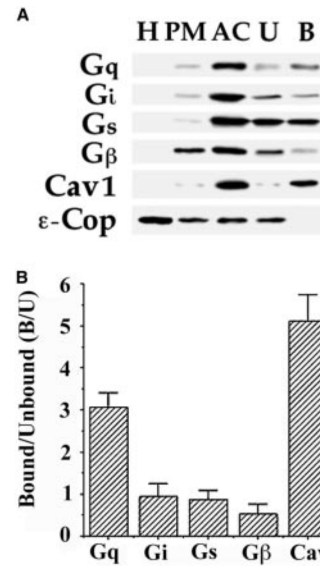

Figure 5. Western analysis of sonicated low-density caveolin-rich fractions and immuno-isolated caveolae. (A) Rat lung tissue homogenates were fractionated on a Percoll gradient to isolate plasma membranes (lane PM), followed by sonication and sucrose gradient centrifugation to isolate a caveolin-rich fraction (lane AC). Subsequently, 25 μg of AC was subjected to immuno-affinity isolation using caveolin-1 antibody conjugated magnetic beads separate caveolin-coated vesicles bound to the beads (B) from noncaveolar unbound material (U) in the supernatant. Western analysis was performed on 5 μg of each fraction using antibodies to the indicated proteins. (B) The level of specific proteins in the bound versus the unbound fraction was quantified, and the ratiowas plotted for caveolin and for each of the indicated G proteins.

G_q in Lipid Rafts of Cells without Caveolae

To determine the cell surface distribution of G_q in the absence of caveolae, we examined the localization of G_q in N2a neuroblastoma cells, which lack detectable caveolin and caveolae (Gorodinsky and Harris, 1995). We isolated Triton-insoluble membrane fractions (TIM) from N2a cells for Western analysis. G_q, G_i, and G_s were all detected in TIM (our unpublished results). Because TIM may contain intracellular membrane contaminants in addition to lipid rafts, we performed dual immunofluorescence microscopy on these cells with antibodies to G_q, G_i, and G_s, as well as caveolin and the lipid raft marker, 5′NT. Figure 6 shows that G_q and G_i exhibited a punctate cell surface staining distribution with significant signal overlap with 5′NT (73 ± 3% for G_q, 76 ± 9% for G_i), indicating their presence primarily in lipid rafts at the cell surface. A similar distribution was observed for G_s (79 ± 3%, our unpublished results). As expected (Gorodinsky and Harris, 1995), the caveolin antibody only gave a very faint diffuse background signal (our unpublished results). Thus, in the absence of caveolin and caveolae, G_q can target lipid rafts.

G_q Specifically Forms a Complex with Caveolin But Not $G_{\beta\gamma}$

Caveolin is a structural protein of caveolae but not of lipid rafts. Because G_q appears to be specifically restricted to

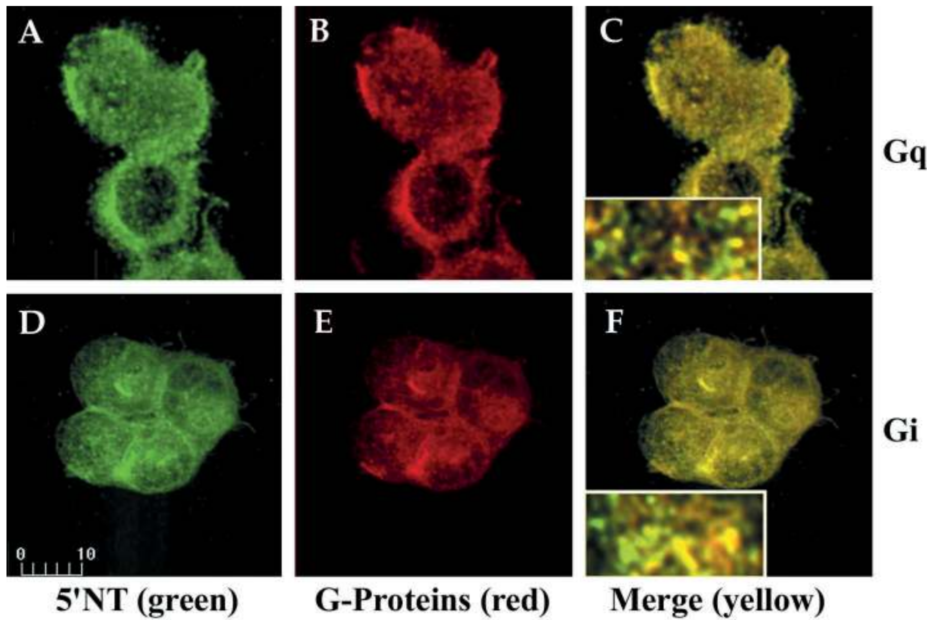

Figure 6. Immunofluorescence microscopy of G_q and G_i concentrated in lipid rafts of N2a cells lacking caveolin and caveolae. N2a cells were fixed and incubated with monoclonal antibodies to 5′NT (panels A, D, green labeling) and polyclonal antibodies to G_q (panel B, red) and G_i (panel E, red). Images were overlaid (panels C and F), and the signal overlap is indicated in yellow. Inset: higher magnification of a representative area of the image showing overlapping signals.

caveolae and not lipid rafts in cells with both microdomains, we investigated whether the basis for this preferential distribution involved an interaction with caveolin, which is known to associate with a variety of signaling molecules. Using a coimmunoprecipitation assay developed to explore eNOS interactions with caveolin (Rizzo *et al.*, 1998a), we mildly solubilized silica-coated plasma membranes (P) from rat lung and then immunoprecipitated with caveolin-1 or G-protein antibodies. The starting material (SM), immunoprecipitated proteins (I), and nonprecipitated, soluble fraction (S) were each examined by Western analysis using antibodies against G_i, G_s, G_q, G_β, and caveolin. SM represents an equivalent amount of the solubilized protein fraction used in each immunoprecipitation experiment. When the immunoprecipitates were probed with the same antibody used for the precipitation, G_q, G_s, and caveolin were found nearly completely in I with a signal intensity comparable to that seen in SM (Figure 7). Little or no signal was found in S, indicating reasonably quantitative immunoprecipitation (>95% immunoprecipitated). G_i and G_β were not as efficiently precipitated, with the signal divided approximately equally between I and S. When the immunoprecipitation was performed using caveolin antibodies, G_q was the only G-protein subunit detected in the immunoprecipitated caveolin complexes. Nearly all of the G_q and caveolin signal was in I, with little to none remaining in S. In contrast, neither G_i, G_s, nor G_β was detectably associated with caveolin-1 in this assay and remained in S. Likewise, when the immunoprecipitation was performed using each of the G-protein antibodies, only G_q, but not G_i, G_s, nor G_β, associated with caveolin. Notably, G_i and G_s, but not G_q or caveolin, appeared to associate with $G_{\beta\gamma}$ subunits. Western analysis of the G_β immunoprecipitated complex detected the presence of both G_i and G_s but not caveolin nor G_q. Given that G_i and G_s both interact with G_β in this assay, one can conclude that G_q does not associate equivalently, or at least not as avidly, with $G_{\beta\gamma}$ as do the others. Taken together, these data indicate that, in the plasma membrane under physiological conditions found in tissue, caveolin associates differentially with G proteins with a substantial preference for G_q but apparently not G_i, G_s, nor $G_{\beta1-4}$. The specificity of this interaction provides a clear mechanism to concentrate G_q preferentially within caveolae at the cell surface.

We (K.R. Solomon and J.E. Schnitzer, unpublished data) and others (Uittenbogaard *et al.*, 1998) have been able to detect the presence of soluble caveolin in cytosol from tissue or cultured cells. To investigate the possibility of G_q-caveolin complexes outside of membranes, we performed coimmunoprecipitation assays on membrane-free cytosols pre-

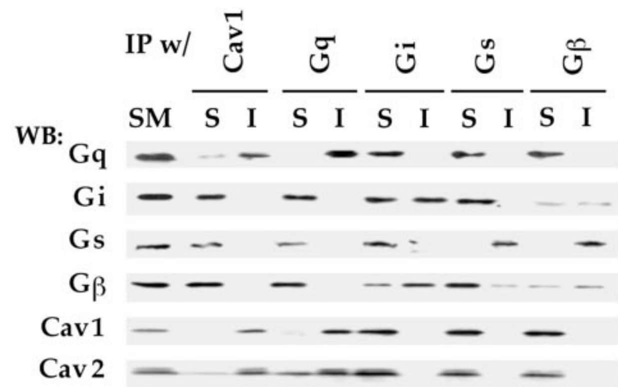

Figure 7. Coimmunoprecipitation of G proteins with membrane-bound caveolin. Proteins solubilized from plasma membranes (P) were subjected to immunoprecipitation (IP) using antibodies to the indicated proteins bound to magnetic beads. The starting material (SM) (5 μg) solubilized from P and equal volumes of the material bound to the beads (I) or unbound in the supernatant (S) were subjected to Western analysis using antibodies to the indicated proteins.

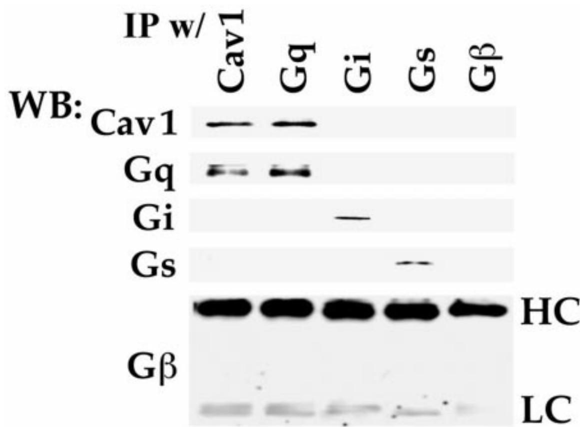

Figure 8. Coimmunoprecipitation of G_q with soluble caveolin. Cytosols were prepared from rat lung homogenates and subjected to immunoprecipitation (IP) with magnetic beads coated with caveolin-1 or G protein antibodies as indicated. Equal volumes of the material bound to the beads was then subjected to Western analysis (WB), using antibodies to caveolin-1 or the indicated G proteins. HC, immunoglobulin heavy chain; LC, immunoglobulin light chain.

pared from rat lung homogenates. Although it is clear that these molecules are found in small quantities in the cytosol relative to membranes, we were still able to detect the presence of caveolin, G_q, G_i, and G_s but not $G_{\beta\gamma}$ subunits in the cytosolic fraction (Figure 8). When the immunoprecipitation was performed using caveolin antibodies, only G_q, and not G_s, G_i, or G_β, was found in the immunoprecipitated caveolin complexes (Figure 8). Likewise, when the various G protein antibodies were used, caveolin was found only in G_q complexes and not in association with G_s, G_i, or G_β. In contrast to the immunoprecipitation assay performed on solubilized membranes, cytosolic G_i and G_s was not associated with $G_{\beta\gamma}$ subunits. This is consistent with GPR and $G_{\alpha\beta\gamma}$ complexes being completely membrane-associated (Morris and Malbon, 1999). Thus, in the cytosol and in the absence of detergent, G_q, but not G_i, G_s, or G_β, remained specifically bound to caveolin, even when not embedded in the lipid membrane.

Last, we investigated whether G_q, in the absence of caveolin, would interact with $G_{\beta\gamma}$. We again used N2a cells in this case to immunoprecipitate G_q, G_i, and $G_{\beta\gamma}$ for Western analysis of the SM, I, and S, as above. Figure 9 shows that both G_q and G_i associated with $G_{\beta\gamma}$ in N2a cells. Thus, in cells lacking caveolin expression, G_q can form a stable complex with $G_{\beta\gamma}$. Taken together, these data suggest that G_q, but not G_i nor G_s, preferentially associates with caveolin over $G_{\beta\gamma}$.

Dissociation of G Proteins from Caveolae

The preferential interaction of caveolin with G_q suggests that caveolin, acting as a scaffolding protein, retains and thereby concentrates G_q in caveolae. G_i, G_s, and G_β may still have a preference for the specialized lipid milieu of the caveolae but may be freer to diffuse into and out of caveolae and thus do not attain or maintain enrichment. To examine this possibility more closely, we studied the relative dissociation rates of G proteins from caveolae. In past work (Oh and Schnitzer, 1999), we observed dissociation of signaling molecules, including G_s, from caveolae after extended incubation in detergent-free buffer. We incubated an aliquot of V in MBS for 4 h at 4°C, then separated the pelleted caveolar membranes (R) from any dissociated proteins remaining in solution (D) by centrifugation. The starting caveolar membranes (SM), D, and R were examined by Western analysis for caveolin and G protein subunits (Figure 10). The caveolar membranes sedimented readily, as indicated by equivalent caveolin signals in SM and R with little to no signal detected in D. Likewise, G_q was also found exclusively in R with no signal in D. This is consistent with G_q remaining bound to caveolin. In contrast, G_i, G_s and G_β, although still present in

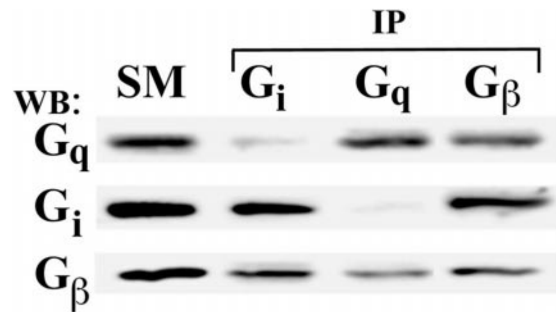

Figure 9. Coimmunoprecipitation of $G_{\alpha\beta\gamma}$ complexes in N2a cells lacking caveolae. Proteins solubilized from N2a cells were subjected to immunoprecipitation (IP) using antibodies to the indicated proteins bound to magnetic beads. The starting material (SM) (5 μg) and equal volumes of the material bound to the beads (I) or unbound in the supernatant (S) were subjected to Western analysis using antibodies to G_q.

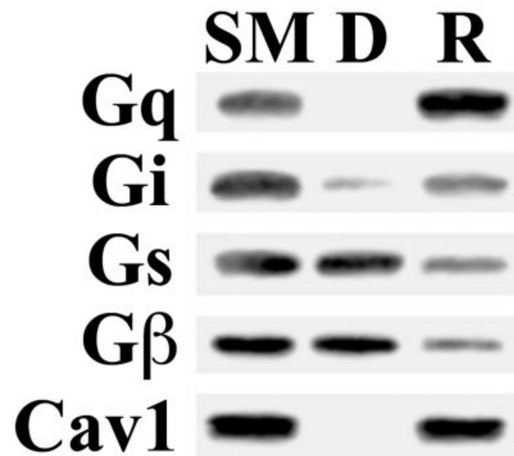

Figure 10. Dissociation of G proteins from caveolae. Isolated caveolae (V) (20 μg) were diluted in MBS and gently mixed at 4°C for 4 h followed by centrifugation to separate the repelleted caveolar membranes (R) from the soluble phase containing any dissociated proteins (D). An equal volume of starting caveolae (lane SM), R, and D were subjected to Western analysis using antibodies to the indicated proteins.

R, were readily detected in D, indicating that they can dissociate from the caveolar membrane. This is consistent with our previous observation that, over time, G_s can dissociate from the caveolar membrane fraction to a soluble, nonsedimenting fraction (Oh and Schnitzer, 1999). Note as previously reported (Oh and Schnitzer, 1999), little to no dissociation was detected with shorter 1-h incubations; in this case all G-protein subunits and caveolin sedimented with the membranes found in R and were not in D (our unpublished results). It appears that the preferential interaction of G_q with caveolin stabilizes this subunit to remain bound in the caveolar membrane, whereas those G-protein subunits not avidly associated with caveolin can move more freely out of caveolae.

DISCUSSION

Heterotrimeric G proteins are important cell surface molecules located on the inner leaflet of the lipid bilayer and are required to link select receptor-ligand interactions to intracellular signaling cascades (Morris and Malbon, 1999). There are multiple isoforms of each type of G-protein subunit. G_α contains at least 20 isoforms divided between four subfamilies, $G_{i/o/t/z}$, $G_{s/olf}$, $G_{q/11}$, and $G_{12/13}$, while G_β and G_γ contain at least five isoforms each (Morris and Malbon, 1999). Here, we report a detailed analysis of the localization of several G_α-isoforms and G_β-subunits in the plasma membrane, revealing that G proteins can target caveolae and lipid rafts differently. Cell surface G_q preferentially targets caveolae via its specific ability to associate with caveolin. Conversely, without equivalent effective association with caveolin, G_i, G_s, and G_β, apparently by default, tend to concentrate preferentially within lipid rafts. The differential microdomain distribution was verified by four independent lines of evidence: 1) G_q is enriched, whereas G_i, G_s, and G_β are present but not enriched, in caveolae purified from rat lung tissue using two different subfractionation methodologies yielding homogeneous populations of caveolin-coated caveolae; 2) G_q extensively colocalizes with caveolin-marked caveolae, whereas G_i and G_s primarily reside in lipid rafts rich in GPI-anchored proteins (5′NT and folate receptor) at the surface of cultured endothelial and epithelial cells as visualized by dual immunofluorescence confocal microscopy; 3) G_q, but not G_i, G_s, or G_β, specifically coimmunoprecipitates with caveolin, both from the membrane in caveolae and when free in the cytosol; and 4) G_i, G_s, and G_β, but not G_q, dissociate and readily move out of caveolae. Last, not all cells have caveolae. In cells lacking caveolin, we find that all three G proteins (G_q, G_i, and G_s) concentrate in lipid rafts as a complex with $G_{\beta\gamma}$ subunits. Thus, caveolin forms an oligomeric coat structure around the bulb of caveolae, which appears to act as a multimeric binding scaffold that traps, concentrates, and stabilizes G_q within caveolae.

G_α-subunits interact with membrane lipids via saturated acyl chains, typically myristate and/or palmitate, covalently attached at the amino terminus (Morris and Malbon, 1999). Our data reveal that G proteins at the cell surface can preferentially target and concentrate in lipid rafts of various intact cells grown in culture and in vivo in tissue. How and why they target the special lipid milieu of lipid rafts is less clear. One possibility is that certain GPI-anchored proteins, such as CD59, CD48, and Thy1 can somehow associate with select G_α subunits (Solomon *et al.*, 1996), possibly through an unknown bridging molecule. Alternatively, G-protein subunits may target lipid rafts directly via their lipid chain modification. Lipid rafts are thought to arise when highly saturated sphingolipids, in the presence of cholesterol, self-assemble to create detergent-resistant domains within the plasma membrane (Schroeder *et al.*, 1998; Brown and London, 2000). Proteins modified with saturated acyl chains, such as GPI and palmitate, have a higher affinity for this environment due to their ability to pack well into the highly ordered lipid milieu of lipid rafts (Melkonian *et al.*, 1999) and thus may selectively partition into these domains, thereby acquiring detergent resistance (Schroeder *et al.*, 1998; Moffett *et al.*, 2000). Other proteins modified by prenyl groups, which have a bulky, branched structure, do not pack well and appear to be excluded from rafts (Melkonian *et al.*, 1999). Yet differences have been observed in the relative detergent insolubility between GPI-linked proteins (Schnitzer *et al.*, 1995b; Melkonian *et al.*, 1999), which, with a similar lipid anchor should theoretically partition to lipid rafts in equal proportions, suggesting that there may be additional contributing factors that determine membrane targeting.

Our data suggest that another factor affecting targeting of lipid-anchored proteins may be their ability to interact stably with caveolin. Although many labs have found G proteins in detergent-resistant membrane fractions (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Lisanti *et al.*, 1994; Li *et al.*, 1995; Schnitzer *et al.*, 1995a; Smart *et al.*, 1995; Solomon *et al.*, 1996), we present here an example of preferential segregation between two distinct subtypes, caveolae and lipid rafts. Like GPI-anchored proteins, G-protein subunits are modified with saturated fatty acids that, in the absence of an interaction with caveolin (or other caveolar protein) to tightly hold them in caveolae, may cause them to target by default lipid rafts. This seems to be the case for cells that do not express caveolin where G_q also targets lipid rafts similarly to G_s and G_i. Of course, G_i and G_s may segregate to lipid rafts via direct binding to a yet unknown protein. Last, an interesting consequence of differential segregation is that it may provide a basis for compartmentalization of upstream and downstream signaling molecules on the cell surface—that is, the G protein coupled receptors (GPCR) may be targeted to specific microdomains based on their association with G proteins found there. Of course, there may be an as-yet-undiscovered GPCR component that targets the receptor to specific membrane microdomains, which may then draw its G-protein effectors to that region.

Although G_i and G_s appear to be concentrated primarily in lipid rafts, our data show that they may still be present to some degree in caveolae. G_s appears to localize to a more substantive subset of caveolae than G_i (see Figure 2) and thus may actually be associated specifically with a small subpopulation of caveolae. This subset-specific enrichment may not be detected in the general population of caveolae isolated by subfractionation. Unfortunately, our attempts to perform subset analysis by immuno-isolating caveolae in V using various G-protein antibodies have failed because the available antibodies do not efficiently immuno-isolate caveolae. Given that we can detect complexes of G_i and G_s with $G_{\beta\gamma}$ subunits but not caveolin by coimmunoprecipitation, G_s may be sequestered in caveolae indirectly as part of a GPCR complex. G_s-containing GPCR complexes may enter caveo-

lae via another mechanism, for instance, as part of an endocytic pathway functioning in GPCR internalization. Alternatively, the apparent overlap with caveolae may reflect instances in which caveolae and lipid rafts are closely associated with, yet still distinct from each other in the plasma membrane as first described in our past work (Schnitzer *et al.*, 1995b). Caveolae can be attached by their necks to flat, detergent-resistant microdomains that form an annular region surrounding the caveolar ostia and are rich in GPI-anchored proteins (Schnitzer *et al.*, 1995b). Molecules in lipid rafts located in this region immediately adjacent to caveolae would, at the level of resolution obtainable by immunofluorescence microscopy, appear to overlap with the caveolin punctate signals. Such localization of G_s near the neck of the caveolae may be an intermediate state in a normal translocation process for G proteins into caveolae, for example, as part of a recycling signaling pathway. Further investigation of these possibilities will require careful examination of the membrane localization by immunogold electron microscopy with other G-protein antibodies (our attempts at immunolabeling ultrathin frozen tissue sections with currently available antibodies failed), the development of new antibodies, and/or additional refinements to the purification procedure that would allow individual subsets of caveolae to be isolated. Note that the interaction of G_q with caveolin in caveolae likely places G_q not at or near the neck, but rather at the bulb of caveolae.

Because G_i and G_s do not form strong interactions with caveolin as does G_q, and because they can dissociate from caveolae into solution and thus presumably out into the noncaveolar membrane, it is possible that their presence in caveolar membrane subfractions was underestimated due to loss during the isolation procedure. Yet the isolated lipid rafts, but not caveolae, were enriched in G_s and G_i. This concern is also minimized by the confirmation using immunomicroscopy of intact cells that G_q colocalizes with caveolin to a much greater degree than G_i and G_s. If differential dissociation from membranes or other movement during subfractionation contributes to low G_i and G_s signals in caveolae, then significant differences between caveolae and lipid rafts are likely to exist, possibly in the lipid milieu responsible for G protein targeting to these domains. Although caveolae and lipid rafts may have common lipids, such as cholesterol and sphingolipids, the full complement of lipids in each domain is presently unknown. Any lipid differences may explain the differential G-protein partitioning, which may be quite dynamic within each microdomain. The experiments presented herein represent only a snapshot of protein distribution at equilibrium. Because the lipid composition of caveolae may be similar but not necessarily identical to lipid rafts (Brown and Rose, 1992; Liu *et al.*, 1997), Gi and G_s may actually be moving in and out of caveolae and back to the rest of the plasma membrane at rates faster than in lipid rafts.

It is noteworthy that G_q appears to have a higher affinity for caveolin than for $G_{\beta\gamma}$. In our assays, we cannot detect an interaction between G_q and $G_{\beta\gamma}$ in caveolae in situ or in cytosolic caveolin:G_q complexes. Yet G_q-$G_{\beta\gamma}$ complexes were readily detected in N2a cells, which lack caveolin expression. G_q-$G_{\beta\gamma}$ interactions have also been detected in detergent extracts of *S. frugiperda* cells overexpressing various G proteins (Fletcher *et al.*, 1998), again showing that G_q can stably interact with $G_{\beta\gamma}$ under conditions where caveolin is absent. When caveolin is expressed, G_q appears to preferentially form a complex with it rather than any $G_{\beta\gamma}$ subunits, causing G_q to selectively partition to caveolae. In cells lacking caveolin and caveolae, G_q may, by default, stably associate with $G_{\beta\gamma}$ and partition to lipid rafts. Interestingly, the lack of G_q association with $G_{\beta\gamma}$ in caveolae suggests that G_q may reside in caveolae not as part of a $G_{\alpha\beta\gamma}$ complex or perhaps even a GPCR complex. The exclusive nature of the G_q-caveolin complex formation, i.e. G_q-$G_{\beta\gamma}$ complexes were not detected at all in the presence of caveolin, suggests that $G_{\beta\gamma}$ and caveolin probably bind to the same region of G_q. Thus, G_q activity may be negatively regulated independently of $G_{\beta\gamma}$ by caveolin in a manner analogous to other caveolin-bound signaling molecules, such as eNOS (Garcia-Cardena *et al.*, 1997; Ju *et al.*, 1997; Ghosh *et al.*, 1998; Rizzo *et al.*, 1998a). In a sense, caveolin may be functioning similarly to GPCR and/or $G_{\beta\gamma}$. Finally, this G_q-caveolin complex also may have a unique role in caveolae, perhaps functioning in mediating acute cellular responses to mechanical stress (see below) or in regulating the trafficking of caveolae and perhaps select signaling molecules. For instance, we find that the G_q-coupled receptor for endothelin (ET_B) is concentrated in endothelial caveolae, and endothelin stimulates both the budding of caveolae and internalization of the ET_B (Oh *et al.*, 2000).

In addition to its possible role in compartmentalized signaling, caveolae in vascular endothelium play a key role in sensing and responding to mechanical stressors, such as fluid shear and pressure, acting externally on the cell surface (Rizzo *et al.*, 1998b; Rizzo and Schnitzer, 1999). Caveolae can function as mechanosensitive organelles and may contain many of the signaling molecules, including eNOS and heterotrimeric G proteins, that mediate acute responses to mechanical stress (Rizzo *et al.*, 1998a; Rizzo and Schnitzer, 1999). G_q is activated in cultured endothelial cells in response to fluid shear (Gudi *et al.*, 1996; Gudi *et al.*, 1998). We have proposed that caveolae may be mechanosensing organelles and that caveolin may be a mechanosensor on the surface of vascular endothelial cells (Rizzo *et al.*, 1998b; Rizzo and Schnitzer, 1999), whereas Frangos, Gudi, and colleagues propose that a G protein may constitute the mechanosensor (Gudi *et al.*, 1998). Given that G_q is concentrated specifically in caveolae, and because G_q interacts avidly with caveolin, the cell surface mechanosensor may actually be the G_q-caveolin complex rather than G_q alone (found minimally in caveolae). We suggest that caveolin oligomers may act as loaded tension-bearing coiled springs responding acutely to changes in membrane tension (Rizzo *et al.*, 1998b; Rizzo and Schnitzer, 1999). Recently, we found that mechanical stressors may place a strain on caveolae, resulting in conformational changes in caveolin and its oligomers (Oh and Schnitzer, 2000) to cause the release of key signaling molecules such as eNOS (Rizzo *et al.*, 1998a) and G_q (Oh and Schnitzer, 2000). Likewise, caveolin oligomers may inhibit G_q activation until stressor changes induce G_q release for activation and downstream signaling. In some ways, caveolin oligomers may function analogously to the $G_{\beta\gamma}$ subunit and/or the GPCR by replacing ligand induction with stress induction.

In the end, the data presented here may help to clarify the relationship between caveolae and lipid rafts as specific

distinct plasma membrane microdomains. Because both caveolae and lipid rafts may have somewhat similar lipid compositions and are thus resistant to solubilization by Triton X-100, there has been a propensity in the field to equate caveolae with lipid rafts. However, there is evidence indicating that caveolae constitute a separate microdomain from lipid rafts. First, detergent-insoluble membranes can be isolated from cells that lack caveolae and caveolin expression (Fra *et al.*, 1994; Gorodinsky and Harris, 1995). Second, electron microscopy studies on homogeneous caveolae preparations reveal that caveolae have a distinct morphology as compared with the other membranes found in the detergent-insoluble fraction (Schnitzer *et al.*, 1995b). Third, new techniques have been devised that allow lipid rafts to be purified away from caveolae, permitting each of these fractions to be studied independently (Schnitzer *et al.*, 1995b; Oh and Schnitzer, 1998). These studies have revealed that molecules previously detected in detergent-insoluble membranes and thus assumed to reside in caveolae, such as several GPI-anchored proteins, actually segregate to noncaveolar lipid rafts. Moreover, structural proteins of caveolae such as caveolin (Rothberg *et al.*, 1992) and dynamin (Oh *et al.*, 1998) are present in caveolae but not in lipid rafts. Here, we have extended these observations to another set of molecules, select heterotrimeric G proteins. We have demonstrated both biochemically and by immunofluorescence microscopy that various G protein α-subunits can differentially segregate to distinct plasma membrane microdomains—G_q to caveolae, and G_i and G_s to lipid rafts. The molecular mechanism for this segregation appears to be a replacement of G_q interaction with its $G_{\beta\gamma}$-subunit by a physiologically effective and stable association of G_q with caveolin. In cells without both microdomains and without caveolin expression, all three G proteins form a complex with $G_{\beta\gamma}$ and target lipid rafts. G proteins tend to exist not randomly on cell surfaces, but rather concentrated in specialized distinct microdomains.

ACKNOWLEDGMENTS

We thank Dr. David Manning for the G protein antibodies, Dr. Paul Luzio for the 5′NT antibodies, and Dr. John Ghrayeb for the folate receptor antibodies. We thank Lucy A. Carver for writing assistance. This work was supported in part by National Institutes of Health grants HL-52766 and HL-58216. This work was presented in part at the FASEB meeting in San Diego, CA (April, 2000) and American Society for Cell Biology in Washington, D.C. (December, 1999).

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