Organization of G Proteins and Adenylyl Cyclase at the Plasma Membrane

Chunfa Huang,* John R. Hepler*[†], Linda T. Chen,* Alfred G. Gilman,*
Richard G.W. Anderson,[‡] and Susanne M. Mumby*[¶]

*Department of Pharmacology, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9041; and [†]Department of Cell Biology and Neuroscience, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9039

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There is mounting evidence for the organization and compartmentation of signaling molecules at the plasma membrane. We find that hormone-sensitive adenylyl cyclase activity is enriched in a subset of regulatory G protein-containing fractions of the plasma membrane. These subfractions resemble, in low buoyant density, structures of the plasma membrane termed caveolae. Immunofluorescence experiments revealed a punctate pattern of G protein α and β subunits, consistent with concentration of these proteins at distinct sites on the plasma membrane. Partial coincidence of localization of G protein α subunits with caveolin (a marker for caveolae) was observed by double immunofluorescence. Results of immunogold electron microscopy suggest that some G protein is associated with invaginated caveolae, but most of the protein resides in irregular structures of the plasma membrane that could not be identified morphologically. Because regulated adenylyl cyclase activity is present in low-density subfractions of plasma membrane from a cell type (S49 lymphoma) that does not express caveolin, this protein is not required for organization of the adenylyl cyclase system. The data suggest that hormone-sensitive adenylyl cyclase systems are localized in a specialized subdomain of the plasma membrane that may optimize the efficiency and fidelity of signal transduction.

INTRODUCTION

Heterotrimeric regulatory G proteins are associated with the inner face of the plasma membrane, where they are positioned to be activated by membrane-spanning, heptahelical receptors and to regulate a variety of intracellular effectors. A common view of the ligand-driven, protein-protein interactions that characterize G protein-coupled transmembrane-signaling

systems includes random collisions between proteins that diffuse freely in the plane of the plasma membrane. However, there is mounting evidence for a higher level of organization and compartmentation of these signal-transducing molecules. These suggestions are based on demonstrations of restricted mobilities of certain receptors and G proteins, the possibility of interactions of signaling components with the cytoskeleton, and failure to replicate the high degree of specificity of signaling observed *in vivo* by reconstitution of purified proteins *in vitro* [for review, see Neubig (1994)]. Thus, G proteins may be restricted to particular compartments or specializations of the plasma membrane.

We (Chang *et al.*, 1994; Smart *et al.*, 1995b), and others (Sargiacomo *et al.*, 1993; Schnitzer *et al.*, 1995) have presented evidence that G proteins can be found in plasma membrane specializations called caveolae.

[¶] Corresponding author.

[†] Current address: Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.

¹ Abbreviations used: α_i , α_o , and α_s , α subunits of heterotrimeric G proteins G_i , G_o , and G_s , respectively; β , β subunit of heterotrimeric G protein; CH₆, carboxy-terminal hexahistidine tag; FL-caveolin, full-length caveolin; γ , gamma subunit of heterotrimeric G protein; GST, glutathione S-transferase; MDCK, Madin Darby canine kidney; NH₆, amino-terminal hexahistidine tag; NT-caveolin, amino-terminal 101 amino acids of caveolin.

Although these structures are most often identified morphologically in cross-section as flask-shaped invaginations of the plasma membrane, they also can exist in a flattened state. Caveolae can be opened or closed to the external milieu and play a role in transport processes such as transcytosis in endothelial cells and potocytosis in epithelial cells. A growing body of biochemical and morphological evidence also indicates that a variety of molecules that participate in signal transduction reactions are concentrated in caveolae [for review see Anderson (1993); Lisanti *et al.* (1994a); and Parton and Simons (1995)]. Furthermore, it has been reported by one group of investigators that G proteins interact directly with caveolin (S.W. Li *et al.*, 1995; Scherer *et al.*, 1996; Tang *et al.*, 1996, 1997), a 21-kDa membrane protein that has been localized by immunocytochemistry to the membrane coat of caveolae. However, others (Stan *et al.*, 1996) have recently questioned the specificity of subcellular fractionation procedures that have implied localization of G proteins (and several other molecules) in caveolae and suggest that this is not their predominant site of residence, at least in rat lung vasculature.

We have examined the organization of certain G protein subunits in the plasma membrane and, to a lesser extent, the localization of other components of a prototypical G protein-regulated signal transduction pathway, the hormone-sensitive adenylyl cyclase system. Receptors communicate with a pair of homologous G proteins, one of which (G_s) mediates stimulation of adenylyl cyclase, while the other (G_i) is responsible for inhibition. We provide additional evidence that these signaling molecules are localized to distinct domains, a portion of which colocalized with caveolin. Proper organization of these signaling proteins at the plasma membrane may optimize fidelity and efficiency of signal transduction in the intact cell.

MATERIALS AND METHODS

Cell Culture

Mammalian cells were cultured in DMEM (high glucose) supplemented with 10% fetal calf serum, 5 U per ml of penicillin, and 5 μ g/ml of streptomycin (unless otherwise noted). Madin Darby canine kidney (MDCK) cells were transfected with Lipofectamine and either empty pCB6⁺ vector (Brewer, 1994) (clone 1) or the α_o expression vector, α_o pCB6⁺ (clone 34). Cells were selected, cloned, and maintained in medium containing 500 μ g/ml Geneticin (G418 sulfate). MA104 cells were derived from rhesus monkey kidney (Roth *et al.*, 1987), and normal human fibroblasts were from skin biopsies. Murine lymphoma (S49) cells were grown suspended in medium supplemented with 10% heat-inactivated horse serum and no antibiotics. Fall army worm ovarian (Sf9) cells were propagated by suspension in IPL-41 medium supplemented with 10% heat-inactivated fetal calf serum as described (Tang *et al.*, 1991). All cell culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD) except for IPL-41 medium, which was from JRH Biosciences (Lenexa, KS).

Antibodies and Western Blotting

Caveolin antibodies (mouse monoclonal and affinity purified rabbit polyclonal) were purchased from Transduction Laboratories (Lexington, KY). The properties of G protein antibodies are summarized here and in Figure 1. B087 was made (in rabbit) against a synthetic peptide representing the last 10 amino acids of α_{i1} and α_{i2} (Linder *et al.*, 1993); its reactivity by immunoblotting was $\alpha_{i1} = \alpha_{i2} \gg \alpha_{i3}, \alpha_o$ (Figure 1A). A569 was made (in rabbit) against a "common α " peptide representing amino acids 40–54 of α_i (Mumby *et al.*, 1986); its reactivity by immunoblotting is $\alpha_o > \alpha_{i1} = \alpha_{i2} = \alpha_{i3} \gg \alpha_\sigma$ (Figure 1A). B600 (Linder *et al.*, 1993) and T20 (Santa Cruz Biotechnology, Santa Cruz, CA) were made in rabbit against a peptide representing the highly homologous carboxyl terminus of β subunit isoforms. Antiserum 584 is specific for α_s (Mumby and Gilman, 1991). 2A and R4 are mouse monoclonal antibodies that were generated against α_o or α_{i1} proteins, respectively, and each was specific for the immunogen without cross-reaction with other α subunits when analyzed by western immunoblotting (X. Li *et al.*, 1995).

For many experiments G protein-specific antibodies were affinity purified (Mumby *et al.*, 1988). All antibody preparations employed for immunolocalization were tested for specificity of reactivity by immunoblotting of purified recombinant α subunits (Lee *et al.*, 1994) and crude membrane fractions prepared from cultured cells (Mumby *et al.*, 1990). Samples were treated with *N*-ethylmaleimide (50 mM) (Sternweis and Robishaw, 1984) and were resolved by SDS-PAGE (Laemmli, 1970). Immunoblotting of proteins transferred to nitrocellulose (Towbin *et al.*, 1979) was performed with enhanced chemiluminescence reagents from Amersham (Arlington Heights, IL).

Immunofluorescence

To obtain plasma membranes, MA104 cells were grown on poly-L-lysine-coated coverslips. Coverslips were soaked in sterile poly-L-lysine (0.5 mg/ml) in 0.1 M sodium borate, pH 8, for 30 min or longer and then rinsed twice with sterile water before cells were plated. Fibroblasts and MDCK cells required no coating of the coverslips. Cells on coverslips were sonicated with a Vibra Cell sonicator (Sonics and Materials, Danbury, CT) set to 40% output/10 J for MA104 cells and fibroblasts and 80% output/15 J for MDCK cells (Muntz *et al.*, 1992). Fixation was performed with 4 or 10% paraformaldehyde with similar results. Coverslips were processed for immunofluorescence using secondary antibodies (20 μ g/ml) labeled with Oregon Green (Molecular Probes, Eugene, OR) or Texas Red (Zymed, South San Francisco, CA) (Muntz *et al.*, 1992). Labeled membranes were viewed and photographed using a Zeiss epifluorescence photo microscope III R5 with a 100 W DC mercury lamp (Carl Zeiss, Thornwood, NY).

Fractionation

Triton X-100 extraction and sucrose gradient centrifugation of cells were performed as described by Lisanti and colleagues (S.W. Li *et al.*, 1995). Detergent-free caveolae were prepared on OptiPrep gradients (fibroblast, MDCK, and MA104 cells) by the method of Smart *et al.* (1995b). S49 cells (harvested at $2-4 \times 10^6$ cells/ml) were disrupted by nitrogen cavitation (3×10^7 cells/ml), and plasma membranes were collected from the interfacial areas of 20/30% and 30/40% sucrose step gradients as described (Ross *et al.*, 1977). Two milligrams of S49 cell membranes were suspended in 23% OptiPrep and applied below a linear OptiPrep gradient as described (Smart *et al.*, 1995b).

Adenylyl Cyclase Assay

Enzyme activity was quantified as described (Salomon *et al.*, 1974; Smigel, 1986). Samples were assayed in a volume of 100 μ l (S49) or 200 μ l (fibroblasts) for 30 min at 30°C in the presence of 10 mM MgCl₂. [α -³²P]ATP was purchased from DuPont NEN (Boston,

MA). Five micrograms of S49 cell plasma membranes isolated from the sucrose gradients were assayed as a control for several experiments. These plasma membranes had basal adenylyl cyclase-specific activities of 0.03 ± 0.01 nmol/min/mg protein. Activity was raised four- to eightfold by 2 μ M isoproterenol and 15 μ M guanosine triphosphate (GTP) (0.12 ± 0.03 nmol/min/mg) or 50 μ M forskolin (0.23 ± 0.09 nmol/min/mg).

Electron Microscopy

Plasma membranes were isolated from the upper surface of cells by the method of Sanan and Anderson (1991). Immunogold labeling was performed on plasma membranes that had been fixed in 3% paraformaldehyde. Secondary antibodies (goat anti-mouse or anti-rabbit from Zymed Laboratories, Inc., South San Francisco, CA) were diluted to 50 μ g/ml. Rabbit anti-goat 10 nm gold conjugate (Sigma Chemical, St. Louis, MO) was used at 2×10^{11} particles per ml. Grids were viewed and photographed using a JEOL JEM-100CX electron microscope.

Construction of Transfer and Expression Vectors

The vector for stable expression of α_o in MDCK cells was synthesized by inserting cDNA encoding rat α_o (from clone 31 in pGEM2 provided by Dr. Randall Reed, Johns Hopkins, Baltimore, MD) (Jones and Reed, 1987) into the *Bgl*II and *Eco*RI sites of pCB6⁺ (Brewer, 1994). A full-length cDNA encoding rat VIP21/ α -caveolin-1 in pBluescript (Kurzchalia *et al.*, 1992) was kindly provided by Drs. D. Zacchetti and K. Simons (European Molecular Biological Laboratory, Heidelberg, Germany). α -Caveolin-1, tagged with six histidine residues at the amino terminus (NH₆-caveolin), was generated by synthesizing an oligonucleotide cassette for substitution insertion. This cassette encodes an initiator ATG followed by six histidines and the first seven amino acid residues of caveolin; *Not*I and *Acc*I sites were placed at its 5' and 3' ends, respectively. After annealing, the cassette was subcloned into the unique *Acc*I site near the extreme amino terminus of the caveolin-coding region and the 5' *Not*I site of pBluescript (Stratagene, La Jolla, CA). The resulting plasmid was linearized with *Xho*I, end-filled with Klenow fragment, and then digested with *Not*I to yield a fragment containing the full-length NH₆-caveolin-coding sequence. This fragment was subcloned into the *Not*I and *Sma*I sites of the Sf9 baculovirus expression vector pVL1392 (Summers and Smith, 1987) to yield a transfer vector encoding full-length α -caveolin-1 (Sf9 NH₆:FL-caveolin). FL-caveolin or the first 101 amino-terminal residues of caveolin (NT-caveolin) were also fused to the carboxyl terminus of glutathione S-transferase (GST) for expression in *Escherichia coli*. Three oligonucleotides were designed for use in polymerase chain reaction amplification of caveolin: Oligo 1, a sense oligonucleotide encoding an *Xba*I site in frame with the first thirty 5' coding bases of α -caveolin; oligo 2, an antisense oligonucleotide encoding bases 273–303, a glycine and six histidines, followed by a stop codon and a *Hind*III site; oligo 3, an antisense oligonucleotide encoding bases 507–534 and a hexahistidine tag at the carboxyl terminus followed by a stop codon and a *Hind*III site. Oligo 1 and oligo 2 were used to generate, by polymerase chain reaction amplification, a DNA fragment encoding NT-caveolin with a hexahistidine tag appended to the carboxyl terminus. Oligos 1 and 3 were used to generate a DNA fragment encoding full-length caveolin with a hexahistidine tag appended to the carboxyl terminus. These fragments were each digested with *Xba*I and *Hind*III and subcloned into the pGEX-2T (Pharmacia Biotechnology, Piscataway, NJ) vector in frame with GST to yield an *E. coli* expression vector that encoded full-length α -caveolin-1 fused to the carboxyl terminus of GST (GST-FL-caveolin:CH₆) or the amino-terminal 101 residues of α -caveolin-1 (GST-NT-caveolin:CH₆), each with a hexahistidine tag at the carboxyl terminus (CH₆). The nucleotide sequence of all caveolin constructs was confirmed by dye terminator sequencing using an Applied

Biosystems 373A automated sequencer (Perkin Elmer-Cetus, Foster City, CA).

Purification of Caveolin and GST-Caveolin Fusion Proteins

Sf9 NH₆:FL-caveolin was extracted from recombinant virus-infected Sf9 membranes (3.5 mg/ml protein) by addition of TX-100 to 1% (vol/vol) and isolated by Ni-NTA affinity chromatography (Qiagen, Santa Clarita, CA). The highly enriched NH₆:FL-caveolin recovered from the first column was purified to near homogeneity by Q-Sepharose anion exchange chromatography as described (Hepler *et al.*, 1996). GST-FL-caveolin:CH₆ and GST-NT-caveolin:CH₆ expressed in *E. coli* were purified by sequential Ni-NTA and glutathione-agarose affinity chromatography and were judged to be greater than 95% pure by SDS-PAGE and Coomassie blue staining. These proteins were used for experiments shown in Figures 8 and 9. GST-caveolin constructs without the CH₆ tag were also synthesized, but they yielded proteins from glutathione-agarose that appeared (by Western immunoblotting) to have been proteolyzed to varying extents and were thus considered poor candidates for further analysis.

Preparation of G Proteins and G Protein Binding to GST-NT-Caveolin Affinity Resin

Myristoylated α_o was synthesized in *E. coli* and purified as described (Linder and Mumby, 1994). To examine G protein binding to GST-caveolin:CH₆, protein affinity resins were prepared following a published procedure (S.W. Li *et al.*, 1995). Analysis of starting material (load samples), bound, and recovered GST and GST-NT-caveolin:CH₆ proteins indicated that essentially all of the applied GST fusion proteins bound to the glutathione-agarose (not shown). Purified G proteins were incubated with GST-agarose, GST-FL-caveolin:CH₆, or GST-NT-caveolin:CH₆-agarose overnight at 4°C to facilitate G protein interaction with caveolin. Unbound material, washes, and eluted proteins were analyzed by immunoblot analysis using G protein antibodies or goat anti-GST (Pharmacia, Piscataway, NJ). No G protein subunits, GST-NT-caveolin:CH₆, or GST remained bound to the agarose after glutathione elutions as assessed by immunoblot analysis of resin boiled in SDS-PAGE sample buffer (not shown).

Preparation and Analysis of Caveolin Peptide and Steady State GTP Hydrolysis Assays

A 20-amino acid peptide corresponding to α -caveolin-1 residues 82–101 (DGIWKASFTTFTVTKYWFYR) was synthesized in the Howard Hughes Medical Institute Biopolymer Facility at the University of Texas Southwestern Medical Center. The identity and purity of the peptide was confirmed by high pressure liquid chromatography and mass spectral analysis. This peptide was identical in sequence to the peptide designated caveolin-2 by Lisanti and co-workers (S.W. Li *et al.*, 1995). Peptide was solubilized in water and further resolved from low molecular weight impurities by size exclusion chromatography; recovered peptide was detected and quantified by measurement of the optical density at 280 nm. This peptide and the purified caveolin proteins were assayed for their effects on the steady-state rate of hydrolysis of [γ -³²P-GTP] (Hepler *et al.*, 1996). Guanosine triphosphatase (GTPase) reactions were carried out in a final assay volume of 50 μ l. Myristoylated α_o was incubated together with either G $\beta\gamma$ alone or together with Sf9 FL-NH₆:caveolin, GST-FL-caveolin:CH₆, GST-NT-caveolin:CH₆, or caveolin peptide for 15 min at 4°C in reaction buffer (20 mM HEPES, pH 8, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM MgSO₄, 0.05% C₁₂E₁₀). Duplicate reactions were started by addition of 30 μ l of reaction buffer containing 1.67 μ M [³²P]GTP (50 pmol GTP/assay final; 10,000–15,000 cpm/pmol) and incubating samples for 20 min at 30°C. Reactions were terminated by addition of 750 μ l of a Norit

A-activated charcoal suspension (5% in 50 mM NaH_2PO_4). Samples were centrifuged and 500 μl of supernatant fraction were assayed for the presence of inorganic [^{32}P]phosphate by liquid scintillation counting. These experiments were conducted twice with similar results obtained.

RESULTS

Antibody Specificity

The specificities of the antibody preparations employed for Western immunoblotting, immunofluorescence, and immunogold labeling of α_i and caveolin are summarized in Figure 1 and MATERIALS AND METHODS. The reactivity of peptide antibodies A569 and B087 with purified G protein α subunits is shown in Figure 1A; the specificity of monoclonal antibody R4 for α_{i1} has been demonstrated previously (X. Li *et al.*, 1995). The antibodies to G protein α subunits interact selectively with α_i in crude membrane preparations (Figure 1B), and they are thus useful reagents for immunolocalization experiments.

Immunofluorescence

We examined the distribution of α_i on the inner face of isolated plasma membranes (prepared by sonicating cells adherent to coverslips) by immunofluorescence. Antibodies to G protein α_i or β subunits revealed punctate patterns of immunofluorescent staining suggesting a concentration of these proteins at distinct sites on the plasma membrane (Figure 2). We compared the distribution of α_i with caveolin by conduct-

ing double immunofluorescence on plasma membranes prepared from three different cultured cell types: MDCK and MA104 cells [renal epithelial cells that have been used previously for isolation and functional characterization of caveolae (Anderson *et al.*, 1992; Sargiacomo *et al.*, 1993; Smart *et al.*, 1994, 1995a)] and primary human fibroblasts (which have excellent morphological characteristics and many invaginated caveolae). Although there are clearly areas where the patterns of fluorescence are coincidental, consistent with colocalization of the two proteins, the coincidence is not complete (Figure 3). The extent of colocalization appeared to be considerably less for fibroblasts than for the two epithelial cell lines. The punctate patterns for both proteins are composed of dots that are generally smaller and more variable in size than those observed with antibodies to clathrin, which is localized in coated pits (data not shown). G proteins did not appear colocalized appreciably with clathrin in double immunofluorescence experiments with antibodies to both proteins (not shown). The fluorescent dots that represent α_i often appear finer than those for caveolin, and they can be so closely juxtaposed that it is difficult to reproduce them faithfully for publication. Similar patterns were observed with different antibodies to α_i or caveolin; the pattern for α_i was indistinguishable with antibodies P960 (Casey *et al.*, 1990), A569, B087, and R4. Fluorescence was not observed if antibodies to the G proteins were first incubated with immunogen peptide (or relevant G

Figure 1. Specificity of antibody preparations. (A) Preparations of purified recombinant G protein α subunits (25 ng) were resolved by SDS-PAGE and analyzed by silver staining or Western immunoblotting. Only the region of the gel where G protein α subunits migrate is shown (7 cm long, 11% polyacrylamide gel). If the film is exposed to the blots for an extended time (not shown), a reaction with α_s can be observed with the A569 antibody preparation (but not with B087 or R4). (B) Crude membrane fractions (25 μg protein) were resolved on a 16-cm long, 9% polyacrylamide gel, and immunoblots are shown. Numbers at left indicate the position of prestained molecular mass standards in kilodaltons. The crude membrane preparations are from MDCK (vector control cells, lanes 1), MA104 (lanes 2), or fibroblasts (lanes 3). The α subunit reactivities of the antibodies are indicated in parentheses near the top of the panel. A569 detects only α_i in cell membranes because it reacts better with α_i than α_s and there is approximately 10-fold more α_i than α_s in most cells (and there is little or no α_o expressed in these cells). Mab R4 is specific for α_{i1} (X. Li *et al.*, 1995). Monoclonal antibody R4 does not react well for immunoblotting and is not sensitive enough, by this method, to detect α_{i1} in MDCK cells or fibroblasts; only MA104 membranes are shown for this antibody. The polyclonal caveolin antibody preparation reacts primarily with one protein band but also with other minor isoforms of caveolin in the 21–25 kDa region of the blot. Please note that the 40-kDa band labeled α_i (to the right of lane 3 of the caveolin blot, panel B) is residual signal remaining from a previous incubation of the blot with the α_i reactive A569 antibody preparation and is not from reactivity with the caveolin antibodies. Antibodies for Western immunoblotting were as follows: affinity-purified A569 and B087 at 100 ng/ml, the purified polyclonal caveolin antibodies at 500 ng/ml, culture medium from monoclonal antibody R4-producing cells diluted 1:25 (vol/vol).

Figure 2. Immunofluorescence of plasma membranes performed with antibodies specific for G protein subunits. *En face* views of the inner side of plasma membrane fragments were obtained by sonicating MA104 cells adherent to coverslips. Oregon green-conjugated secondary antibodies were used to visualize primary antibodies to detect: (A) α_i subunits with B087 antibodies (10 $\mu\text{g}/\text{ml}$) or (B) β subunits with T20 antibodies (1 $\mu\text{g}/\text{ml}$). The larger areas of the photographs that are devoid of fluorescent signal represent spaces where plasma membrane fragments are absent. Scale bar, 2 μm .

protein subunit), whereas the staining pattern was preserved if the antibodies were incubated with a nonreactive G protein subunit or peptide (not shown).

Subcellular Fractionation of G Proteins, Caveolin, and Adenylyl Cyclase Activity

Methods designed to isolate low-density membrane fractions from tissues or cultured cells have permitted enrichment of both G proteins and caveolin. MDCK cells, Triton X-100 extraction, and sucrose gradients have been employed frequently by others (Sargiacomo *et al.*, 1993; Lisanti *et al.*, 1994b; S.W. Li *et al.*, 1995; Song *et al.*, 1996) with results similar to those shown in Figure 4. Most of the cellular proteins (assayed by Ponceau S staining of the blot, not shown) remained in the region where the Triton X-100 extract was loaded at the bottom of the tube (fractions 8–11), while Triton X-100-insoluble membranes of low density floated up into the sucrose gradient (lower numbered fractions). Immunoblotting revealed that endogenous α_i and β subunits, exogenously expressed α_o protein, and the

bulk of the caveolin fractionated closely together. G proteins and caveolin (from MDCK cells) also fractionated together when this procedure was modified by replacement of detergent with sodium carbonate, pH 11 (Song *et al.*, 1996) (not shown).

An alternative method of fractionation involves floatation of plasma membrane-containing fractions (from a Percoll gradient) through a continuous gradient of OptiPrep (Smart *et al.*, 1995b). Endogenous G protein α_i subunits and β subunits, as well as exogenously expressed α_o protein, did not cofractionate completely with caveolin through the OptiPrep gradients. We routinely observed that a substantial portion of G protein migrated into lower density fractions than did caveolin. These observations were made for MDCK cells (Figure 5A–C), MA104 cells (not shown), and fibroblasts (not shown) and are consistent with the results of double immunofluorescence experiments shown in Figure 3.

We extended our fractionation studies to S49 lymphoma cells because they lack detectable caveolin (by immunoblotting) and have been utilized extensively

Figure 3.

to study G protein-mediated signal transduction. The lack of caveolin in these cells is consistent with reports from others who have studied lymphocytes (Fra *et al.*, 1994). Plasma membranes from these cells were fractionated by the OptiPrep method (Figure 5, D and E), with results similar to those obtained with MDCK cells (Figure 5, A-C). G protein subunits (α_i , both long and short isoforms of α_s , and β) were found in fractions that migrated up into the gradient and were resolved from the bulk of the protein (Figure 5E). Although antibodies of sufficient quality to detect β -adrenergic receptors and adenylyl cyclase in these fractions are not available, the highest specific activities of β -adrenergic receptor- and forskolin-stimulated cAMP synthesis were found in fractions 3–6 (Figure 5F). Thus, all components of a hormone-sensitive adenylyl cyclase system are present in membrane fractions with low densities and can be separated from the bulk of the plasma membrane protein prepared on sucrose gradients from cells that do not contain caveolin. Similar results were obtained for fibroblasts. The highest total activity of forskolin-stimulated adenylyl cyclase in fibroblast membranes was present in fractions 3–7 (Figure 6B), and the highest specific activity was found in fractions 3–6 (Figure 6C). Fibroblast G proteins were found (by Western immunoblotting, not shown) evenly spread, as in MDCK cells (Figure 5, B and C), over more fractions (1–8) than was the bulk of caveolin (not shown) and adenylyl cyclase activity (Figure 6B). Isoproterenol-stimulated adenylyl cyclase activity was not detected in membranes from fibroblasts. Depending on the cell type and variations between independent experiments, 50–80% of total adenylyl cyclase activity was found in fractions 1–6 whereas only 15–20% of total plasma membrane protein was present in these fractions.

Immunogold Electron Microscopy

We examined plasma membranes of fibroblasts and other cells to determine sites of localization of α_i and to evaluate the extent to which this protein is associated with morphologically recognizable caveolae.

Figure 3 (facing page). Double immunofluorescence of plasma membranes performed with antibodies specific for α_i and caveolin. Plasma membranes similar to those in Figure 2 were prepared from MDCK cells (A and B), MA104 cells (C and D), and fibroblasts (E and F). Oregon green-conjugated secondary antibodies were used to visualize α_i in the lefthand panels and Texas red-conjugated antibodies were used to detect caveolin in the righthand panels. The spillover of signal between the two fluorophores was insignificant (determined by single immunofluorescence, not shown). Affinity-purified B087 (10 μ g/ml) was used for panels A and C, R4 (1:100 dilution of culture medium from antibody-producing cells) for panel E, caveolin monoclonal antibody (5 μ g/ml) for B and D, and affinity-purified polyclonal antibody (10 μ g/ml) for panel F. Scale bar, 4 μ m.

Figure 4. Fractionation of endogenous and ectopically expressed G proteins and caveolin from TX-100 extracts of MDCK epithelial cells. Stably transfected MDCK cells that ectopically express α_o (panel A) were compared with G418-resistant control cells that had been stably transfected with the empty vector (panel B). Four milliliters of detergent extract, adjusted to 40% sucrose, was loaded at the bottom of a tube followed by a 7-ml linear gradient of 30–5% sucrose. After centrifugation, 0.8-ml fractions were collected from the top of the gradients, half of which was acetone-precipitated and analyzed by Western immunoblotting. Most of the cellular protein (assayed by Ponceau S staining of the blots, not shown) remains below the gradient in the five bottom fractions (numbered 10–14). Ectopically expressed α_o (A) consistently comigrates with endogenous α_i , β , and caveolin (cav) in detergent-resistant, low-density fractions centered around fraction 6 (A and B). α_i was detected by B087 antiserum (1:10,000 dilution), β by B600 antiserum (1:10,000), α_o by culture medium from Mab 2A-producing cells (1:500), and caveolin (cav) by the purified polyclonal antibodies (30 ng/ml). Not shown are results for α_s , which comigrates with the other G protein subunits from MDCK and MA104 cells.

Similar immunogold labeling patterns were obtained with peptide antibodies directed to two different regions of α_i (Figure 7B and C). This pattern differed from that obtained with an anti-caveolin antibody (Figure 7A). Both anti- α_i antibodies yielded clusters of gold particles that were most frequently associated with structures of irregular shape and size that we were unable to identify. Coated pits were not labeled by α_i antibodies (Figure 7, B and C) consistent with

Figure 5. OptiPrep gradient fractionation of detergent-free plasma membranes from MDCK epithelial or S49 lymphoma cells. Sonicated plasma membranes were brought to 23% OptiPrep in 4 ml and were placed at the bottom of the tube. A linear gradient of 20–10% OptiPrep was poured on top of the plasma membranes. After centrifugation, either 1 ml (panels A, B, C, and F) or 0.7-ml fractions (panels D and E) were taken from the top of the tube. These fractions were analyzed for total protein content by Bradford assay (panels A and D), G protein or caveolin content by Western immunoblotting (panels B, C, and E), or adenylyl cyclase activity (panel F). The specific activity of isoproterenol- (Iso-) and forskolin- (Fsk-) stimulated adenylyl cyclase in the individual fractions 1–6 and combined fractions 7–11 are shown in panel F. Antibody preparations utilized for immunoblotting were the same concentration as Figure 4 but, in addition, 584 antiserum was employed for detection of long and short isoforms of α_s at a dilution of 1:5,000.

our double immunofluorescence results with clathrin antibodies (not shown). Immunogold labeling of α_i was reduced dramatically (74–88% in two experiments) when the primary antibodies were combined with the immunogen peptide but not with an irrelevant peptide. Only about 10–20% of morphologically identifiable caveolae in fibroblasts were labeled by α_i antibodies (see solid arrows, Figure 7; Table 1). By contrast, essentially all of the morphologically identifiable (invaginated, doughnut-shaped) caveolae were labeled by the preparation of caveolin antibodies. Similar patterns of immunogold staining of α_i were also seen with plasma membranes of MDCK and MA104 cells, although fewer invaginated caveolae could be identified (not shown).

Invaginated caveolae are not evenly distributed on the fibroblast plasma membrane, and we sought to determine whether α_i is enriched in areas that have high densities of these membrane specializations.

Square micron areas of the plasma membrane that were obviously caveolae-rich or caveolae-poor were chosen arbitrarily for counting the total number of caveolae and the total number of gold particles observed after labeling with either α_i or caveolin antibodies. As expected, the caveolin antibody yielded total numbers of gold particles in the caveolae-rich areas that were 10- to 20-fold greater than those in the caveolae-poor areas (Table 2). By contrast, immunogold labeling with either of two α_i antibodies was greater in areas of plasma membrane that were relatively poor in invaginated caveolae.

Interaction of Caveolin with G Proteins

Caveolin has been proposed to act as a scaffold that localizes G proteins to caveolae and, further, as a regulator of the activity of these signal transducers (S.W. Li *et al.*, 1995; Scherer *et al.*, 1996; Tang *et al.*,

Table 1. Percentage of caveolin and G α immunogold localized to invaginated caveolae of fibroblasts

Antibodies	No. of invaginated caveolae		Caveolae with gold (%)
	With Gold	Total	
Caveolin	222–241	223–244	99–100
G α			
B087	26–70	346–395	7–18
A569	26–42	200–213	13–20

The quantification is presented as the range obtained by two investigators who independently identified invaginated caveolae (such as those designated by closed arrows in Figure 7) and counted gold particles within 10 nm of these structures. Prints from 25 micrographs were used for counting (six for caveolin, ten for B087, and nine for A569 antibodies).

1996, 1997). Li, S.W. *et al.* (1995) demonstrated qualitatively that recombinant G protein α subunits (α_o and α_{i2}) from extracts of transfected MDCK or baculovirus-infected Sf21 insect cells bind to GST-FL-caveolin or GST-NT-caveolin. Because these experiments were not performed with purified proteins, the interaction of α subunits with the caveolin constructs could have been indirect or require additional factors. If G proteins bind to caveolin directly, pure α subunits should bind to GST-caveolin attached to glutathione agarose beads. We investigated such binding with pure myristoylated α_o in the presence or absence of $\beta\gamma$. The α_o and $\beta\gamma$ appeared to be quantitatively recovered in the flow through and first wash of the GST-caveolin resins in a manner indistinguishable from GST without appended caveolin (Figure 8A for GST-NT-caveolin:CH $_6$; GST-FL-caveolin:CH $_6$ not shown). Only after prolonged exposure of the blot to film could we detect a hint of α_o in the glutathione elution from the GST-NT-caveolin:CH $_6$ agarose (Figure 8B), and we estimate that the eluted material represented much less than 1% of that added to the caveolin constructs. In experiments conducted similarly to those published (S.W. Li *et al.*, 1995), we were unable to detect binding of recombinantly expressed unpurified α_o or α_{i2} (from extracts of baculovirus-infected Sf9 cells) to FL-caveolin:CH $_6$ (data not shown). Thus, we were unable to demonstrate a substantial interaction between G protein subunits and caveolin in the presence or absence of other cellular components.

It has been reported that synthetic peptides corresponding to specific regions of caveolin (e.g., residues 82–101 of caveolin-1) modulate the basal GTPase activity of α_i and α_o and alter binding of GTP γ S to these proteins (S.W. Li *et al.*, 1995; Scherer *et al.*, 1996; Tang *et al.*, 1996, 1997). If these represent physiologically relevant reactions, intact caveolin should cause similar effects. We found that neither the caveolin-1 peptide

Figure 6. Adenylyl cyclase activity in OptiPrep gradient fractions prepared from fibroblasts. Plasma membranes were fractionated similarly to those shown in Figure 5. (A) Protein content of each 1-ml fraction. (B) cAMP produced in an assay tube containing 120 μ l of gradient fraction incubated with 25 μ M forskolin. (C) Specific activity of forskolin-stimulated adenylyl cyclase.

(reported to inhibit GTPase activity), Sf9 NH $_6$:FL-caveolin, GST-FL-, nor GST-NT-caveolin:CH $_6$ influenced the basal GTPase activity of α_o significantly in the presence or absence of $\beta\gamma$ (Figure 9); the inhibitory effect of $\beta\gamma$ on the GTPase activity of α_o is characteristic (Figure 9A, hatched bars). We also failed to observe effects of the peptide or proteins on binding of GTP γ S to α_o (not shown). Finally, we tested two other peptides that were said to modulate GTPase activity (Scherer *et al.*, 1996; Tang *et al.*, 1996). These peptides represent regions of caveolin-2 and caveolin-3 isoforms that correspond to the caveolin-1 sequence specified above. These two peptides were difficult to solubilize and gave irreproducible results. In summary, we cannot reproduce the published effects of caveolin peptides on the GTPase activity of α_o or on GTP γ S

Figure 7. Immunogold labeling of plasma membranes with caveolin or α_i antibodies. Electron micrographs show *en face* views of the

binding to the protein, and we also observe no effects of FL- or NT-caveolin on these parameters.

DISCUSSION

Individual cells express an imposing array of proteins that participate in G protein-mediated signal transduction reactions: a large number of receptors; a substantial variety of G protein α , β , and γ subunits; many distinct effectors that exist in multiple isoforms; and regulators such as members of the large families of receptor kinases, arrestins, regulators of G protein signaling (RGS) proteins, and probably others. Although the critical phenomena that result from the interactions between these components can and at times must be studied using purified proteins reconstituted *in vitro*, other aspects of G protein-mediated signaling are not yet observable in such well-defined systems. For example, the magnitude of stimulation of adenylyl cyclase activity by appropriate hormones is much greater in intact cells than in membranes; the specificity of receptor-G protein interactions in reconstituted systems appears to be less stringent than that observed in membranes or cells; requirements for specific combinations of isoforms of G protein subunits to observe appropriate responses to individual receptor agonists cannot yet be duplicated *in vitro* (Neubig, 1994). These and other phenomena suggest the possibility of or need for nonrandom, functional organization of these components in the plasma membrane to achieve the requisite speed, magnitude of response, functional specificity, and regulation that characterize these systems.

A substantial fraction of the total activity and the highest specific activity of isoproterenol-stimulated adenylyl cyclase activity in S49 cells was found in low-density subfractions of the plasma membrane with buoyant properties similar to those of caveolae (Figure 5F). Thus, G_s -coupled receptors, G_s itself, and adenylyl cyclase appear to be localized in similar domains — perhaps together to increase the efficiency and fidelity of signal transduction at distinct sites on the plasma membrane. In this regard, investigators have come to different conclusions about the distribu-

Figure 7 (cont). inner side of plasma membrane fragments that have been torn from the upper surface of cultured fibroblasts. The location of some of the morphologically identifiable caveolae (full or partial doughnut shapes indicated by closed arrows) and coated pits (flat or curved honeycomb patterns indicated by open arrows) are shown. (A) Caveolae are decorated well by gold particles when the polyclonal (panel A, 1 μ g/ml) or monoclonal (not shown) caveolin antibodies are utilized. (B) Morphologically identifiable caveolae are infrequently labeled by α_i reactive B087 antibodies (10 μ g/ml) or (C) A569 antibodies (10 μ g/ml). The letters at the upper left corner of each panel are placed over a small area that is devoid of plasma membrane or gold particles. Scale bar, 0.5 μ m.

Table 2. Quantification of caveolin and G_{iα} immunogold in caveolae-enriched and caveolae-poor areas of fibroblast plasma membranes

Antibodies	Caveolae-enriched area ^a		Caveolae-poor area ^b	
	Caveolae/μm ²	Gold/μm ²	Caveolae/μm ²	Gold/μm ²
Caveolin	9.1–11	78–83	0–0.7	3.6–7.1
G _{iα}				
B087	8.5–9.2	4.2–5.4	0.2–0.4	34–41
A569	8.7–9.7	9.9–15	0.0–0	31–38

The quantification is presented as the range of the average numbers obtained by two investigators who independently chose multiple square micrometer areas to count all invaginated caveolae and gold particles present. Each investigator counted 8 μm² for caveolin antibodies (from three micrographs). Fifteen square micrometers were counted for α_i antibodies, B087 (from eight micrographs) and A569 (from seven micrographs).

^a Caveolae-enriched areas have ≥ 5 invaginated caveolae per square micrometer.

^b Caveolae-poor areas have ≤ 2 invaginated caveolae per square micrometer.

tion of endogenous β-adrenergic receptors in the plasma membrane. Some have indicated that the distribution of unstimulated receptors was random and homogeneous (Kaveri *et al.*, 1987; Muntz *et al.*, 1988; Raposo *et al.*, 1989), while others have observed non-

random patterns (Zemick and Strader, 1988; Wang *et al.*, 1989). Cytochemical experiments have also suggested that adenylyl cyclase activity is associated with structures resembling caveolae (Wagner *et al.*, 1972; Slezak and Geller, 1984; Rechardt and Hervonen, 1985).

We and others (Wang *et al.*, 1989; Lewis *et al.*, 1991) have focused on G proteins and found them to be clustered at the inner surface of the plasma membrane, indicating their concentration at particular sites. Our membrane fractionation data indicate localization of G proteins in low-density subfractions of the plasma membrane and are consistent with colocalization of a substantial fraction of G protein subunits with caveolin. These experiments are at least semiquantitative, in that all of a particular G protein subunit in the cell can presumably be detected by immunoblotting after denaturation; however, the resolution of this technique is relatively low. Immunofluorescence experiments indicate that there is significant colocalization of G protein subunits and caveolin, but that it is incomplete and variable among cell types. This is consistent with recent reports demonstrating coimmunoprecipitation of G proteins or related signaling components with caveolin using cell extracts (Chun *et al.*, 1994), some of which included detergent-insoluble particles (de Weerd and Leeb-Lundberg, 1997; Feron *et al.*, 1997). Incomplete and/or cell type-dependent colocalization of G pro-

Figure 8. Assay for interaction between G protein subunits and the amino-terminal domain of caveolin. (A) Purified GST (upper panel) or GST-NT-caveolin containing caveolin:CH₆ residues 1–101 (lower panel) immobilized on glutathione agarose beads (3500 pmol) was incubated with purified myristoylated α₀ (410 pmol), bovine brain βγ (580 pmol), or α₀βγ heterotrimer (410 pmol) as indicated at the top of the figure. After allowing the proteins to interact overnight, the resin was washed six times, and bound GST and any associated proteins were eluted with 25 mM reduced glutathione. Equal portions of the load samples (L), protein that did not bind the affinity resin and was present in the flow through (FT), the first wash (W1), the last wash (W6), and glutathione eluates (E) were analyzed for the presence of G protein subunits by immunoblotting with specific G protein α and/or β antisera. Film was exposed to the blot for 2 min. (B) Samples from glutathione eluates from each binding condition described above were analyzed side-by-side for comparison and analyzed for the presence of G protein subunits by immunoblotting. Film was exposed to immunoblots for either 2 min or, to maximize detection of the chemiluminescence signals, for 15 h. There is a hint of signal for α₀ perceptible in the GST NT-caveolin eluate only after prolonged exposure of the blot to film. This signal represents an extremely small portion of that which was loaded on the resin. An ink dot was placed to the right of each of three bands detected after the 15-h exposure: two bands corresponding to β (one each for GST and GST-NT-caveolin:CH₆ elutions) and one for α₀ in the elution from GST NT-caveolin:CH₆.

Figure 9. Failure of purified full-length caveolin or caveolin derivatives to alter steady-state GTPase activity of α_o or G_o heterotrimer. (A) The steady state GTPase activity of purified myristoylated α_o (3 nM), myristoylated G_o heterotrimer ($\alpha_o + \beta\gamma$, 3 nM each), or $\beta\gamma$ (3 nM) was measured (hatched bars). The relative capacities of purified GST fusion protein containing residues 1–101 of caveolin:CH₆ (GST-NT-cave; 1 μ M), purified full-length caveolin:CH₆ fused to GST (GST-FL-cave; 1 μ M), full-length NH₆:caveolin purified from Sf9 cells (Sf9-FL-cave; 1 μ M), or a peptide corresponding to residues 82–101 of caveolin (cave peptide; 10 μ M) to alter the steady-state GTPase activity of myristoylated α_o (black bars) or G_o heterotrimer (gray bars, $\alpha_o + \beta\gamma$) were assayed but were found to be negligible. (B) The effects of increasing concentrations of caveolin peptide (residues 82–101) on the steady-state GTP hydrolysis of myristoylated α_o (0.6 nM) were assayed but were also found to be negligible.

teins and caveolin may explain the results of Stan *et al.* (1996), who concluded that G proteins are not enriched in caveolae. Although this may be true in the cells studied, those of rat lung vasculature, the techniques utilized for isolation of caveolae (perfusion of lung with cationized silica and the immunoisolation of

caveolin-containing membranes) may have altered the distribution of the proteins in question. Our electron microscopy studies suggest that most of the G protein α_i subunits reside in irregular structures that could not be identified. Although the resolution of the microscopic techniques is of course intrinsically high, quantitation can be lost. It is possible that populations of G protein subunits are not visualized in these experiments because of fixation artifacts or masking of epitopes. These caveats aside, we would conclude that morphologically distinct, invaginated caveolae are a minor location of α_i in the cells we studied by electron microscopy.

Caveolin *per se* is not required for localization of G proteins in low-density subfractions of the plasma membrane (Figure 5E). In this regard we must question a premise for colocalization of caveolin and G proteins — direct protein–protein interactions. We were unable to observe interactions between G protein α or $\beta\gamma$ subunits and caveolin, and we were unable to detect functional effects of caveolin or caveolin-based peptides on guanine nucleotide binding or hydrolysis of G protein subunits. Our use of recombinant myristoylated α_o purified from bacteria (protein that could lack important covalent adducts such as palmitate), may have prevented our detection of a substantial direct interaction between G proteins and caveolin. We are unable to explain why, in experiments conducted similarly to those published (S.W. Li *et al.*, 1995), we did not detect interaction between caveolin and unpurified α_o or α_{i2} from extracts of baculovirus-infected Sf9 cells (in which G proteins are properly palmitoylated) or effects of caveolin-based peptides on G protein function.

Our working hypothesis on localization is that G proteins in particular (and probably entire G protein-coupled signaling systems) are not randomly distributed in the plasma membrane but are physically and functionally segregated into distinct membrane domains. If caveolae are defined narrowly as caveolin-containing invaginations of the plasma membrane (Simons and Ikonen, 1997) then we conclude that these structures are not the predominant location of α_i [Table 1 and Stan *et al.*, (1996)]. If, instead, caveolae are defined more broadly as a membrane system that includes low-density subdomains of the plasma membrane (Anderson, 1998), then our data show that such structures are major sites for localization of G proteins and adenylyl cyclase. Using this definition, caveolae may be similar or identical to the protein-containing clusters of glycosphingolipids and cholesterol that Simons and Ikonen (1997) have described as mobile rafts within the fluid membrane bilayer. It would be of great interest to examine the signaling properties of cells in which these structures were specifically disrupted.

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