Purification and Characterization of Smooth Muscle Cell Caveolae

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Abstract. Plasmalemmal caveolae are a membrane specialization that mediates transcytosis across endothelial cells and the uptake of small molecules and ions by both epithelial and connective tissue cells. Recent findings suggest that caveolae may, in addition, be involved in signal transduction. To better understand the molecular composition of this membrane specialization, we have developed a biochemical method for purifying caveolae from chicken smooth muscle cells. Biochemical and morphological markers indicate that we can obtain ~1.5 mg of protein in the caveolae fraction from ~100 g of chicken gizzard. Gel electrophoresis shows that there are more than 30 proteins enriched in caveolae relative to the plasma membrane. Among these proteins are: caveolin, a structural molecule of the caveolae coat; multiple, glycosylphosphatidylinositol-anchored membrane proteins; both Go and Gq subunits of heterotrimeric GTP-binding protein; and the Ras-related GTP-binding protein, Rap1A/B. The method we have developed will facilitate future studies on the structure and function of caveolae.

There is increasing evidence that plasmalemmal caveolae are a membrane specialization capable of sealing off from the extracellular environment to create a unique, membrane bound compartment at the cell surface. The dynamics of caveolae opening and closing is best observed in endothelial cells (46, 47), where they appear to form plasmalemmal vesicles that move across the cell and fuse with the abluminal membrane. Each round of caveolae-mediated transcytosis transports a portion of molecules from the blood to the tissue space without merging with other endocytic pathways. Although in other cell types the budding event has not been seen with the electron microscope, biochemical studies have shown (17-19) that caveolae can sequester membrane bound ligands away from the extracellular space and facilitate their delivery to the cytoplasm of the cell. This process is called potocytosis (3).

What distinguishes potocytosis from other endocytic pathways is the use of glycosylphosphatidylinositol (GPI)-anchored membrane proteins to concentrate low molecular weight molecules and ions in closed caveolae (22, 41). Morphological (54) and biochemical (5, 7) methods have documented that a variety of different cell types contain highly clustered arrays of GPI-anchored membrane proteins and that a subset of these clusters is in caveolae (35, 43). The cytoplasmic surface of each caveolae has a characteristic striated coat that is composed of integral membrane proteins (35, 39). The integrity of this coat (39), as well as the clustering of the GPI-anchored proteins (6, 40), is dependent on the presence of cholesterol in the membrane. Therefore, the structure of caveolae is highly dependent on the lipid composition of the membrane.

Potocytosis may be a mechanism for delivering signaling molecules or ions to the cell (2, 3). An extension of this idea is that caveolae can sometimes store or process incoming and outgoing cellular messengers (1). These proposals are based on the finding that certain of the molecules associated with caveolae are known to participate either directly or indirectly in cell signaling. One example is caveolin, an integral membrane component of the caveolar coat (39). The phosphorylation of this protein by pp60

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1. Abbreviations used in this paper: CRD, cross-reacting determinant; GPI, glycosylphosphatidylinositol; IPG, inositolphosphoglycans; IP3, 1,4,5-tri phosphate; PI-PLC, phosphatidylinositol specific phospholipase C.
as an ATP-dependent calcium pump (11), have been recently localized to caveolae, which suggests a role for potocytosis in calcium signaling. Finally, non-receptor tyrosine kinases have been found associated with isolated GPI anchor clusters (48, 50) and caveolin-rich membrane fractions (43). These kinases are activated by the binding of antibodies to GPI-anchored proteins (44).

The eventual understanding of how caveola function in the cell will depend on the development of purification schemes that allow investigators to obtain large quantities of this organelle for biochemical analysis. Recently, Sargiacomo et al. (43) described an analytical method for obtaining caveolin-rich membrane fractions from cultured fibroblasts. We now report on the development of a method for purifying biochemical quantities of caveolae from chicken gizzard smooth muscle cells. In addition to being enriched in at least 30 different proteins, these caveolae appear to be a cell surface location for small and heterotrimeric GTP-binding proteins.

**Materials and Methods**

**Materials**

Triton X-100, β-mercaptoethanol, glyciner, crystalline BSA, leupeptin, sodium chloride, sodium azide, potassium iodide, sucrose, fish gelatin, EDTA, EGTA, MOPS, NP-40, soybean trypsin inhibitor, pepstatin A, N-ethylmaleimide, and benzamidine were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride was from Fisher Scientific Co. (Fair Lawn, NJ). PMSF was from Boehringer Mannheim GmbH (Germany). DTT and paraformaldehyde were from Fluka Chemical Corp. (Buchs, Switzerland). A monoclonal antibody that recognizes a common epitope [Cross Gai2 (BO87), anti-Ga (C-260), and anti-Gf (B-600). An affinity purified anti-caveolin IgG (20B) was kindly provided by Dr. J. Glenney (University of California, CA). Alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG was from DuPont New England Nuclear Co. (Boston, MA). Biotinylated horse anti-mouse IgG (2 μg/ml in buffer H) was from Vector Labs. (Burlingame, CA). Biotinylated horse anti-mouse IgG was from Jackson ImmunoResearch Laboratories (Westbury, NY) for 2 x 30 s at 27,000 rpm. The crude homogenate was centrifuged for 20 min at 5,000 g to obtain the starting material. This was filtered through four layers of gauze before being used to prepare caveolae.

**Methods**

**Purification of Caveolae.** The purification scheme is shown in Fig. 1. All steps were carried out at 4°C. A plasma membrane fraction from chicken gizzard was prepared by a modification of the methods reported by Lucceschi et al. (28) and Hubbard et al. (16). One hundred grams of gizzard was examined for reagents and tissues and minced in 0.25 M buffer A (10 mM HEPES, pH 7.5, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.5% sodium azide, and 0.02% sodium azide) before 50 μl of [125I]streptavidin (2 μCi/ml in buffer F, preadsorbed with 2 x 30 s in a Waring blender in a total of 1 L of buffer A. Finally the sample was homogenized in a polytron homogenizer (Brinkman Instruments Inc., Westbury, NY) for 2 x 30 s at 27,000 rpm. The crude homogenate was centrifuged for 20 min at 5,000 g to obtain the starting material. This was filtered through four layers of gauze before being used to prepare caveolae.

A small portion of the starting material was centrifuged at 100,000 g for 1 h and designated as the total membrane fraction (see Fig. 5). The remaining starting material was centrifuged for 10 min at 10,000 g to remove any aggregated material (Pellet A). Supernatant A was filtered through 12 layers of gauze, adjusted to 1 M KCl and stirred for 30 min. The mixture was centrifuged for 90 min at 30,000 g. Pellet B was resuspended in buffer B (5 mM MgCl2, pH 7.4) and nine-down strokes of a tight fitting dounce homogenizer. Two volumes of buffer C were added to the homogenate (final concentration of sucrose 1.42 M) and 35-ml aliquots were added to each centrifuge tube. Each sample was overlaid with 2 ml buffer B (sucrose concentration, 0.25 M) and centrifuged for 60 min at 82,000 g in a Beckman SW 28 rotor at 4°C. The 0.25/1.4 M interface (Membrane I) was collected with a blunt tipped Pasteur pipette and resuspended in buffer A by 10 strokes of a tight fitting dounce homogenizer. This suspension was mixed with an equal volume of 4 M KI (freshly prepared in buffer A) and stirred for 30 min. 36-ml aliquots of the sample were added to centrifuge tubes and centrifuged for 60 min at 100,000 g in a Beckman SW 28 rotor. A brownish, membrane pellicle formed at the top of 2 M KI (Membrane II), which was collected with a blunt-tipped Pasteur pipette and resuspended by 10 strokes of the tight dounce homogenizer in buffer D and stored at 4°C.

Membrane II, which corresponded to the plasma membrane fraction, was mixed with an equal volume of 2.5 M sucrose to a final concentration of 42%. 4-ml aliquots of the sample (7-10 mg proteins) were added to centrifuge tubes, and then overlaid with 8 ml of 25% sucrose followed by 5 ml of 15% sucrose. The samples were then centrifuged for 2 h at 100,000 g. The material accumulated between these two sucrose layers, and the 25-42% sucrose layers. The 15-25% sucrose interface plus the 25% sucrose fraction were pooled, diluted in two times volume of buffer D and concentrated by centrifuging onto 42% sucrose cushion for 1 h at 100,000 g. The pellicle on the cushion (Membrane III), which was collected with a blunt-tipped Pasteur pipette and resuspended by 10 strokes of a tight fitting dounce homogenizer in buffer D.

Membrane III was adjusted to a protein concentration of 4.5 mg/ml with buffer D, with equal volume of 1% Triton X-100 (final Triton concentration 0.5% in buffer D) and incubated for 30 min with constant rocking. A sucrose step gradient was prepared using 0.1% BSA and 0.1% BSA.

**Radioimmunonassay.** After protein measurement, samples were first dissolved in 60 mM octyl-glucoside in water, and then diluted to desired concentrations in water. Aliquots of the sample (10 ng) were placed in individual wells of an Immuno 2, 96-well plate (Dynatech Lab., Inc., Chantilly, VA) and air-dried overnight. The wells were washed three times with buffer E, and then incubated in the presence of buffer F for 1 h at 37°C. The wells were washed an additional three times with buffer G before adding 50 μl of the anti-caveolin IgG (2 μg/ml in buffer H) to the wells and incubating for 1 h at 37°C. Each well was washed seven times with buffer G before adding 50 μl of [125I]streptavidin (2 μCi/ml in buffer H), preadsorbed with Dowex resin to remove free [125I]. Sp. Act. = 20-40 μCi/μg) was added to the wells.
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Electrophoresis and Immunoblot. Most of the samples were dissolved in sample buffer (23) and heated for 3 min at 100°C before being loaded onto gels. Samples for heterotrimeric G protein analysis were prepared by a modification of the method of Sternweis and Robinshaw (49). Protein samples were mixed with 5 μl of 10% SDS/1 mM DTT, followed by heating for 5 min at 100°C. The samples were then cooled to room temperature. A 5-μl aliquot of 0.3 M NEM (N-ethylmaleimide) was added to each sample and incubated for 10 min at room temperature. A 10-μl aliquot of 4× SDS sample buffer was then added to the samples and heated for 3 min at 100°C before loading on gels.

Proteins were separated by SDS-PAGE using the method of Laemmli (23). Proteins separated on the indicated percent gels were electrophoretically transferred to nitrocellulose membrane (pore size, 0.45 μm) by the method of Towbin (51). Transfer was performed at a 100 V for 60 min at 4°C using a solution containing 20% methanol, 0.025 M Tris, and 0.192 M glycine (pH 8.3) as the electrode buffer.

**[P]I IgG Blots.** Nitrocellulose membranes were rinsed briefly with buffer K and incubated with buffer L for 1 h at room temperature. The blots were then incubated with the indicated concentration of primary antibody (diluted in buffer J) for 1 h at room temperature or overnight at 4°C. The blots were washed three times for 5 min in buffer M and then incubated with alkaline phosphatase-conjugated to either goat anti-mouse IgG or goat anti-rabbit IgG at 5 × 10⁴ cpm/ml (sp. act., 20 μCi/μg of protein) in buffer J for 1 h at room temperature. The blots were washed three times for 5 min in buffer J, rinsed twice with buffer I, and then washed two times for 10 min in buffer I. Antibody binding was visualized by autoradiography using Kodak X-OMAT AR film.

**Alkaline Phosphatase BCIP/NBT Blots.** Nitrocellulose membranes were rinsed briefly with buffer K and incubated with buffer L for 1 h at room temperature. The blots were then incubated with the indicated concentration of primary antibody (diluted in buffer M) for 1 h at room temperature or overnight at 4°C. The blots were washed three times for 5 min in buffer M, and then incubated with alkaline phosphatase-conjugated to either goat anti-mouse IgG (1:2,000 dilution in buffer M) or goat anti-rabbit IgG (1:2,000 dilution in buffer M) for 2 h at room temperature. The blots were washed three times for 5 min in buffer K, and then processed to visualize the bands using the BCIP/NBT alkaline phosphatase substrate system.

**[α-32P]PGTP Binding.** [α-32P]PGTP binding was carried out according to the method of Lapetina and Reep (24). The nitrocellulose containing transfected proteins was rinsed briefly in buffer N. The nitrocellulose was then incubated for 90 min at room temperature with [α-32P]PGTP in buffer N (1 μCi/ml, specific activity, 3,000 Ci/mM) in the absence or presence of 1 μM unlabeled PGTP. The blots were rinsed with several changes of buffer N over 1–2 h and air-dried. [α-32P]PGTP binding was visualized by autoradiography using Kodak X-OMAT AR film.

**PI-PLC Treatment.** PI-PLC treatment was carried out using a modification of a method of Lisanti et al. (26). Membrane samples were dissolved in 60 mM octyl-glucoside. The samples were then incubated in the presence or absence of PI-PLC (4 U/ml) for 1 h at 37°C. The reactions were stopped by addition of Laemmli buffer and incubated for 3 min. The samples were subjected to SDS-PAGE and transferred to nitrocellulose paper.

**Imuno-Gold Labeling of Thin Sections.** Fresh chicken gizzard was fixed overnight in 3% (wt/vol) paraformaldehyde in buffer O containing 3 mM trinitrophenol, 4 mM KCl, and 2 mM MgCl₂. Vibratome sections (60–80 μm thick) of the fixed chicken gizzard were prepared and washed in buffer O containing 100 mM NH₄Cl for 30 min. The sections were then rinsed twice with buffer O and incubated in buffer P for 1–3 h at room temperature.

Primary antibodies, mouse monoclonal anti-caveolin IgG (designated 20B), and irrelevant mouse monoclonal IgG (designated 200i) were diluted in buffer P to a final concentration of 30 μg/ml. Groups of 6–8 sections were incubated overnight with each of the primary antibodies. This was followed by an 8-h incubation in the presence of 25 μg/ml of goat anti-mouse IgG conjugated to DNP in buffer P. Sections were washed after each incubation three times for 10 min each in buffer P. After a final wash, sections were rinsed twice in buffer O, fixed with 1% glutaraldehyde for 2 h in buffer O, washed with buffer O containing 100 mM NH₄Cl for 30 min, and rinsed twice in buffer O. Tissue sections were post fixed with 1% osmium tetroxide in buffer O for 2 h, dehydrated, embedded in Epon, sectioned, and processed to localize DNP groups by immunogold labeling as previously described (34).

**Imuno-Gold Labeling of Fractions.** Samples from purified fractions were mixed with an equal volume of 120 mM octyl-glucoside for 10 min on ice without vortexing to dissociate aggregates. This was followed by fixation with 3% paraformaldehyde in buffer O for 10 min. The fixed samples (2–3 μl) were air-dried onto carbon-coated grids. The grids were washed in buffer O followed by incubation with 100 mM NH₄Cl (in buffer O) for 30 min and buffer Q for 30 min. All the antibodies were diluted in buffer O. The grids were incubated 30 min each with primary antibodies (20 μg/ml) followed by 50 μg/ml of either goat anti-mouse IgG or goat anti-rabbit IgG and finally a 1:30 dilution of gold-conjugated rabbit anti-goat IgG. The grids were washed after each incubation three times for 30 min in buffer Q. After a final wash, grids were rinsed twice in buffer O, post fixed with 1% osmium tetroxide in buffer O for 10 min and finally stained sequentially for 10 min each with 1% tannic acid, 4% uranyl acetate, and 2% lead citrate.

**Other Methods.** SDS-PAGE was carried out according to the method of Laemmli (23). Protein was determined according to Lowry et al. (27) using BSA as a standard.

To measure galactosyltransferase, 10 μg of the indicated membrane fraction was incubated in the presence of 25 mM MnCl₂, 2.5 mM mercaptoethanol, 0.08 mg/ml [3H]UDP galactose (sp. act. = 172 Ci/mM), 10 mM Hepes-NaOH, pH 7.0 in the absence or presence of 66 mM N-acetylgalactosamine at 37°C for 1 h. The reaction was terminated with the addition of 0.3 M EDTA. The product (N-acetyl-lactosamine) was separated on a 1-cm column of Dowex-2-C₂.

The percent of the cell surface occupied by caveolae was determined by: measuring the average diameter of a caveola (75 nm) and using this value to calculate the membrane circumference of a caveola; counting the number of caveolae per linear length of membrane and multiplying this by the caveolar membrane circumference; and dividing total caveolar membrane circumference by the total linear surface counted plus the total caveolar membrane circumference.

**Results**

**Characterization of Purified Caveolae**

Caveolin is the only known protein marker for caveolae (39). Immunogold labeling has shown that at the cell surface this protein is found exclusively in caveolae (39). Since caveolin is also found randomly distributed in Golgi membranes (10, 21, 39), our strategy was to prepare plasma membranes from a tissue source that was rich in caveolae and track the purification of the organelle with anti-caveolin IgG. We chose chicken gizzard smooth muscle cells because they have abundant caveolae (Fig. 2A). Immunogold labeling of these cells with the same anti-caveolin IgG that was subsequently used to monitor purification showed that most of the gold labeling was confined to caveolae (Fig. 2B and C). We used the typical flask shaped morphology of the caveolar membrane to determine that caveolae represent ~18% of the total surface membrane in these cells. Most likely this is an underestimate because cells contain many uninvaginated caveolae that cannot be detected by thin section electron microscopy (39).

We developed a purification protocol that requires three steps (Fig. 1): preparation of plasma membranes stripped of peripheral membrane proteins (Membrane II); enrichment for light plasma membranes on a sucrose step gradient (Membrane III); and treatment of light plasma membranes with Triton X-100 followed by separation of soluble from insoluble material on a sucrose gradient (Membrane IV). We measured the specific activity of caveolin in each fraction using a radioimmune assay for bound anti-caveolin IgG. The specific activity (A) and the protein profile (B) of the various fractions obtained in a typical purification run is shown in Figs. 3 and 4. The starting material had significant activity, but when we separated the plasma membrane from the cytosol, most of this activity fractionated with the membrane.
had the highest caveolin specific activity. The 25% fraction loaded onto a sucrose Step gradient and centrifuged at (data not shown). Fig. 3 shows that when Membrane II was nearly the same activity, but the other fractions were quite low. We pooled the two fractions with the highest activities by both polyacrylamide gel electrophoresis (Fig. 5 A) and immunoblotting using anti-caveolin IgG (Fig. 5 B). We could identify at least 30 different bands that copurified with caveolae. The most prominent proteins are indicated by the arrowheads and caveolin by the arrow (Fig. 5 A). By immunoblotting (Fig. 5 B), three caveolin-specific bands (apparent MW, 22,000, 24,000, and ~300,000) were most intense in the caveola fraction. The high molecular weight band corresponds to a polymer of caveolin (14) while the two low molecular weight bands are isoforms of monomeric caveolin. Scanning densitometry of the caveolin-specific bands in the immunoblots indicated that caveolin was enriched ~5-fold in the caveola fraction relative to the plasma membrane and ~200-fold relative to the whole tissue starting material (Fig. 1).

Another test of the purification scheme is to show that marker proteins for other compartments are excluded from the caveola fraction. Fig. 5 A documents that multiple, Coomassie blue positive bands were excluded as caveolae were purified. The pattern also indicates that many of the proteins in the plasma membrane were not present in the caveola fraction (compare plasma membrane lane with caveola lane). We also measured the activity of galactosyltransferase, a marker for Golgi membranes. The activity in pellet B was 22,747 cpm/min/mg of protein, which was comparable to the activity of a control Golgi fraction isolated from rat liver (17,993 cpm/min/mg). By contrast, the activity in the caveola fraction was not above background (1,609 cpm/min/mg of protein). Therefore, Golgi membranes did not contaminate the caveola fraction. Finally, we used immunoblotting to measure the concentration of the cytoplasmic protein, annexin VI. This protein was excluded from caveolae (data not shown).

GPI-anchored membrane proteins are another marker for caveolae. Immunogold electron microscopy has shown that nearly every caveola is associated with a cluster of GPI-anchored proteins. On the other hand, the caveola-associated clusters only represent ~18% of the total number of GPI clusters on the cell surface (54). We used a polyclonal antibody that recognizes the cross-reacting determinant (CRD) epitope exposed when GPI-anchored proteins are released from membranes by PI-PLC (15) to determine if GPI-anchored proteins were enriched in the caveola fraction (Fig. 6, A and B). Immunoblots with this antibody detected at least nine different GPI-anchored membrane proteins that were highly enriched (Fig. 6, A and B). When we quantified the more intense bands by scanning densitometry we found that these GPI proteins were enriched 13-18-fold in the caveola fraction relative to the plasma membrane.

We next used whole mount electron microscopy and immunogold cytochemistry to characterize the morphology of the membranes in the caveola fraction (Fig. 7). Samples of the fraction were fixed, dried down onto the surface of a formvar-coated grid, and positively stained with heavy metals (Fig. 7 A). These fractions typically contained numerous, cup-shaped pieces of membrane. At higher magnification (Fig. 7 A, inset), these cups appeared to be partially decorated by a striated coat. We also saw smaller pieces of membrane interspersed between the cup profiles. These appeared to be fragments of caveolae because they contained remnants of the coat material and were positive for anti-caveolin IgG binding by immunogold cytochemistry (Fig. 7 B). Nearly all of the membrane fragments, plus the cup-shaped segments,
Figure 2. Electron microscopic visualization of chicken smooth muscle cells that were either unprocessed (A) or processed (B and C) to localize caveolin by immunogold cytochemistry. Fresh chicken gizzard was either fixed directly and embedded for electron microscopy (A) or fixed and processed to localize caveolin using either a monoclonal anti-caveolin IgG (B) or an irrelevant monoclonal IgG (C) as described. Arrowheads indicate caveolae. Bar, 0.5 μm.

GTP-binding Proteins Are Enriched in Caveolae

Sargiacomo et al. (43) recently reported that both small and heterotrimeric GTP-binding proteins coenrich with caveolin in Triton X-100 insoluble, membrane fractions prepared from tissue culture cells. We used specific antibodies to see if any of these regulatory proteins were also associated with smooth muscle caveolae. We used mono-specific peptide antibodies against various GTP-binding proteins to immunoblot the indicated fractions. Fig. 8 shows that both GS (Fig. 8 A) and Gi3 (Fig. 8 B) are enriched in caveolae. Scanning densitometer reading of the blots indicated that the specific blotting activity of Gs was increased eightfold compared to plasma membranes while Gi3 was increased sixfold. An antibody that recognizes both Ga1 and Ga2 showed that these subunits were also enriched (~3-fold) in the caveolae fraction (Fig. 9 A). When we used this antibody to immunogold label the caveolae fraction (Fig. 9 B), many of the caveolae profiles were heavily decorated with gold. Immunogold labeling was not seen when a preimmune IgG was substituted for the anti-Ga1/Ga2 IgG (data not shown).

In contrast to the Gs subunits, the specific blotting activity of the Ga subunit indicated that this subunit was no more concentrated in caveolae than in the starting material (compare starting material with caveolae, Fig. 10 A) even though there was some enrichment in the plasma membrane fraction. Immunoblot analysis of each fraction obtained during the purification showed that Triton X-100 removed Ga from the Membrane III fraction (data not shown). Therefore, we used immunogold electron microscopy to determine if Ga was present in caveolae before detergent treatment (Fig. 10, B, C, and D). The light membrane fraction obtained before detergent treatment (Membrane III, Fig. 10 C) had many cup-shaped membrane profiles that decorated with the anti-Ga IgG gold probe (arrows, Fig. 10 C). By contrast, we found substantially less gold associated with similar appearing pieces of membrane in the detergent-treated fraction (Membrane IV, Fig. 10 D). We verified that the labeled segments of membrane in Membrane III were caveolae by colocalizing both caveolin (large gold, Fig. 10 B) and Ga.
Figure 3. The specific activity of caveolin (A) and the protein distribution (B) in fractions obtained from the first sucrose step gradient.

Membrane II was mixed with 2.5 M sucrose to a final concentration of 42%, overlaid with a 15%-25% sucrose step gradient, and centrifuged at 100,000 g for 2 h at 4°C. The gradient was divided into five fractions: 15%; the 15/25% interface; 25%; the 25/42% interface; and 42% sucrose. Each fraction was assayed for the presence of caveolin using a radioimmune assay as described. The 15/25% interface and the 25% fraction were pooled and designated Membrane HI.

Figure 4. The specific activity of caveolin (A) and the protein distribution (B) in fractions obtained from the second sucrose gradient.

Membrane III was adjusted to a protein concentration of 4-5 mg/ml with buffer F, adjusted to 0.5% Triton X-100, and incubated for 30 min, all at 4°C. The sample was then loaded on the top of a sucrose step gradient and centrifuged at 100,000 g for 2 h at 4°C. The gradient was divided into four fractions: 7%; the 7/15% interface; 15%; and the 15/25% interface. Each fraction was assayed for the presence of caveolin using a radioimmune assay as described. The 15/25% interface was designated Membrane IV.

Discussion

Caveolae have remained a relatively obscure membrane spe-
Figure 5. Polyacrylamide gel electrophoresis (A) and anti-caveolin IgG immunoblotting (B) of different fractions obtained during the purification of caveolae. (A) Samples of the indicated fractions (75 μg per lane) were loaded on 6–15% gradient gels, separated by electrophoresis and stained with Coomassie blue as described. The arrow heads indicate proteins that are enriched relative to the plasma membrane. The arrow marks the position of caveolin. The * marks a protein band that appears to be more abundant in caveolae than caveolin. (B) Samples of the indicated fractions (5 μg per lane) were loaded on gels, separated by electrophoresis (11% gel) and transferred to nitrocellulose for blotting with anti-caveolin IgG (15 μg/ml). Anti-caveolin was detected using an 125I-labeled goat anti-mouse IgG. The arrow indicates the caveolin band.

Figure 6. Purified caveolae are enriched in multiple GPI anchored membrane proteins. Samples (10 μg) of the indicated fractions were solubilized in 60 mM octyl-glucoside and incubated in the presence (+) or absence (−) of PI-PLC for 1 h at 37°C. Each sample was then separated on an 11% gel by electrophoresis and immunoblotted with rabbit anti-CRD IgG (serum) using 125I-goat anti-rabbit IgG as described. An overnight (A) and a 3-d (B) exposure are shown.

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teins (41). We have used the relative enrichment of these markers as a guide for purification.

In any purification scheme a question always arises about the purity of the final preparation. A linear measure of the plasma membrane indicates that caveolae make up ~18% of the surface of chicken gizzard smooth muscle cells. If all of the non-caveolar membrane was removed from the plasma membrane, then a 5.5-fold enrichment for the caveolae segment is all that is needed for a complete purification. We used both a radioimmune assay for caveolin and whole mount electron microscopy to monitor purification. Anti-caveolin IgG was useful for monitoring caveolae behavior during the fractionation, but we could not use this marker to determine purity because we do not know the specific concentration of caveolin in each caveolae or whether all caveolae have caveolin. Therefore, we used electron microscopy to examine the fractions with the highest caveolin specific activity and found that the principal membrane component had the morphological characteristics of caveolae. Moreover, nearly all of the membrane profiles in these preparations decorated with anti-caveolin IgG gold particles (Fig. 7). Therefore, these fractions contain relatively pure caveolae.
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Figure 8. Immunoblot detection of either G\(_s\) (A) or G\(_{\alpha i3}\) (B) in different fractions obtained during the purification of caveolae. Samples (5 µg) of the indicated fraction were separated by electrophoresis on 11% polyacrylamide gels and immunoblotted with polyclonal peptide antisera against either G\(_s\) (A) or G\(_{\alpha i3}\) (B) using \(^{125}\)I-goat anti-rabbit IgG to detect the rabbit IgG. Bar, 0.3 µm.

Figure 9. Immunoblot (A) and immunogold (B) localization of G\(_{\alpha i1}/G_{\alpha i2}\) in fractions obtained during the purification of caveolae. (A) Samples (5 µg) of the indicated fraction were separated by electrophoresis on 11% polyacrylamide gels and immunoblotted with polyclonal peptide antisera (1:2,000 dilution) against G\(_{\alpha i1}/G_{\alpha i2}\) using \(^{125}\)I-goat anti-rabbit IgG to detect the rabbit IgG. (B) The caveolae fraction was processed for whole mount immunogold labeling with anti-G\(_{\alpha i1}/G_{\alpha i2}\) antisera (B) using an antibody sandwich that consisted of, goat anti-rabbit IgG followed by gold-conjugated rabbit anti-goat IgG. Bar, 0.3 µm.

An analysis of the polyacrylamide gel staining pattern for purified caveolae shows that they contain a complex mixture of proteins. Relative to the plasma membrane, many proteins are enriched, some are excluded, and still others are present at the same concentration (compare bands present in plasma membrane lane with those in the caveolae lane, Fig. 5). We determined by immunoblot and immunogold labeling (data not shown) that one of the proteins in the last category is actin (at the 45-kD marker, Fig. 5A). Despite the harsh salt and detergent treatments, actin remained bound to caveolae throughout the purification. This association may be due to the presence of a high affinity, actin-binding protein in caveolae or simply be the consequence of a non-specific interaction. Nevertheless, these results point out that many of the proteins that are not enriched may still be legitimate components of the organelle. By contrast, the enriched bands probably correspond to proteins that carry out a specific cellular function in caveolae. At least one of these proteins (\(\star\), Fig. 5A) appeared to be more abundant than caveolin. This raises the possibility that caveolae contain other structural proteins besides caveolin. Future sequence analysis may help to determine a function for these proteins.

Sargiacomo et al. (43) recently reported that heterotrimeric GTP-binding proteins are associated with caveolin-rich domains isolated from tissue culture cells. Furthermore, immunocytochemical studies have found that G\(_s\) colocalizes with caveolin in MA 104 cells (Mumby, S. M., Q. Yang, H. K. Hagler, A. G. Gilman, and K. H. Muntz, unpublished observations). We found that smooth muscle cell caveolae are also enriched in these proteins. Peptide antibodies that recognize different G\(_s\) subunits were used to monitor the specific blotting activity of each subunit during caveolae purification. Depending on the subunit, activity was found to increase from 3 to 8-fold relative to the plasma membrane. Immunogold cytochemistry confirmed that both G\(_{\alpha i1}\) and G\(_{\alpha i2}\) are associated with isolated caveolae. We also successfully used immunogold cytochemistry to localize the other two G\(_s\) subunits to caveolae (data not shown).

The caveolae-associated G\(_s\) most likely corresponds to a subset of all the G\(_s\) that is associated with the plasma membrane. This subgroup may participate in one or more signaling activities that originate in caveolae. Interestingly, G\(_{\alpha i2}\) was the least enriched G\(_s\) found in caveolin-rich domains from MDCK cells (43) but the most enriched subunit in smooth muscle cell caveolae fractions. This suggests that the caveolae found in each type of cell have a unique constellation of G\(_s\) subtypes that are there to carry out cell-specific signaling functions.

G proteins are largely solubilized from membranes by mild detergents such as Triton X-100. Therefore, the caveolae-associated G\(_s\) must remain in caveolae membranes during the purification because of a tight interaction with a structural component of the caveolae. G\(_{\alpha i3}\) appears not to hold the G\(_s\) in place because much of this complex was extracted from partially purified caveolae by Triton X-100. We favor the idea that amino-terminal acylation of G\(_s\) (25) may control the association with caveolae.
Figure 10. Immunoblot (A) and immunogold (B, C, and D) localization of Gα in fractions obtained during the purification of caveolae. (A) Samples (5 μg) of the indicated fraction were separated by electrophoresis on 11% polyacrylamide gels and immunoblotted with anti-Gα antisera (diluted 1:1,000) as described. Membrane II (C) or purified caveolae (D) were processed for whole mount immunogold labeling with anti-Gα antisera (diluted 1:1,000) as described in Fig. 9. Double labeling of Membrane II (B) was carried out by sequential addition of antibodies, using 5-nm gold for the anti-Gα antisera and 15-nm gold for the anti-caveolin IgG. Bar, 0.3 μm (inset bar, 0.1 μm).

There is now strong biochemical (5), morphological (40), and functional (6) evidence that it is the lipid environment of the caveolar membrane that attracts the fatty groups on the GPI-anchored proteins. Certain acylated cytoplasmic proteins may be attracted to caveolae for the same reason. Nonreceptor, tyrosine kinases have been found associated with caveolin-rich membranes (43) as well as clusters of GPI-anchored membrane proteins (7). Recently, Shenoy-Scaria et al. (45, Lublin, D. M., personal communication) showed that the interaction of p56<sup>lc</sup> and p59<sup>cm</sup> with GPI-anchored protein complexes in Triton X-100 extracts depends on a thioester link to palmitate at position 3 in the amino-terminal sequence, Met-Gly-Cys. Myristoylation of the glycine is also required. All of the G<sub>α</sub> subunits that we detected in purified caveolae share with p59<sup>cm</sup> and p56<sup>lc</sup> the Met-Gly-Cys motif at the NH<sub>2</sub> terminus. Furthermore, all are palmitoylated at position 3 (9, 30, 33), and, with the exception of G<sub>αs</sub>, all have been found to be myristoylated at the Gly. These data suggest that the presence of two acylated amino acids in tandem may direct proteins to specialized membrane domains such as caveolae.

This model makes the prediction that the introduction of tandem acyl groups into a protein would direct it to caveolae/GPI anchor complexes. We found that pp60<sup>src</sup> was not enriched in our purified caveolae preparations. However, pp60<sup>src</sup> has the sequence Met-Gly-Ser and, therefore, cannot be tandemly acylated. Lublin and co-workers (45, Lublin, D. M., personal communication) have now found that changing the Ser to a Cys causes both the tandem acylation of pp60<sup>src</sup> and the association of the kinase with GPI anchor protein complexes. G<sub>αs</sub> also appears to be enriched in caveolar preparations (43) but lacks the amino terminal myristoylation site (Gly). Instead, this protein has tandem cysteine residues at positions 9 and 10 that are both acylated (52). Therefore, tandem palmitoylation may work just as well to promote association with caveolae.

Acylation and deacylation of G<sub>α</sub> appears to be a dynamic process (30). Acylation of one or more of the tandem sites may be the method the cell uses to control the location of the subunit on surface and internal membranes. This added level of cellular organization in the signaling cascade could ensure that G proteins are spatially positioned to optimize their interaction with the appropriate receptor/effector.

We also found evidence for the presence of small GTP-binding proteins in caveolae. [γ<sup>32</sup>P]GTP blots showed that at least two proteins of 30 and 28 kD were enriched. Moreover, an anti-Rap1A/B IgG was positive by both immunoblot and immunocytochemistry. Rap1A/B shares extensive homology with ras protooncogenes (36) and can reverse the K-ras transformation of 3T3 cells (20). They also have been impli-
cated in the negative regulation of ATP dependent Ca++ transport activity (8). Recent immunocytochemical studies have shown that Ca++ ATPase is present in caveolae (11). Therefore, the ras related Rapl GTP-binding protein may be involved in regulating cytosolic calcium fluxes through caveolae.

There are now two methods available for obtaining caveolae: the biochemical method we have developed and an analytical method described by Sargiacomo et al. (43). Our method has two main advantages. First, the availability of large quantities of caveolae will facilitate the identification of many of the protein and lipid components of the organelle. Second, Membrane III is quite enriched in caveolae so that it is possible to obtain quantities of the organelle without a detergent treatment step. This is important because of the finding that resident proteins like Go, are removed by Triton X-100. Undoubtedly there are many important caveolae-associated molecules that will be removed using a purification scheme that depends on detergent. Nevertheless, the two types of preparations are in agreement that caveolae contain molecules that are known to participate in diverse signaling pathways. These isolation procedures set the stage for determining how caveolae function in the uptake of essential nutrients and in the delivery of signals to the cell.

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References


Figure 11. Radiolabeled GTP blot (A), immunoblot with anti-RaplA/B IgG (B), and immunogold localization of RaplA/B (C) in fractions obtained during the purification of caveolae. Samples (5 μg) of the indicated fraction were separated by electrophoresis on either 12.5% (A) or 11% (B) polyacrylamide gels and blotted with either 32P-labeled GTP (A) or immunoblotted (B) with affinity purified polyclonal anti-peptide IgG against RaplA/B using alkaline phosphatase--labeled goat anti--rabbit IgG to detect the primary antibody. (C) The caveolae fraction was processed for whole mount immunogold labeling with anti-RaplA/B IgG as described. Bar, 0.3 μm.


