Purification and Characterization of Smooth Muscle Cell Caveolae

Wen-Jinn Chang,* Yun-shu Ying,* Karen G. Rothberg,* Nigel M. Hooper,§ Anthony J. Turner,§ Hervé A. Gambliel,* Jean De Gunzburg, Ill Susanne M. Mumby,* Alfred G. Gilman,* and Richard G. W. Anderson*

*Departments of Cell Biology and Neuroscience, and †Pharmacology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235; §Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom; and ‡Institut National de la Santé et de La Recherche Médicale, Unité 248, Faculté de Médecine Lariboisière-Saint Louis, Paris, France

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 Gα and Ga 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 (15,19) 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 (43, 54). 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 pp60v-src is tightly coupled to the transformation of chick embryo fibroblasts by the Rous sarcoma virus (13). This suggests that abnormal cell-cell and cell-substratum interactions, which result in the transformed phenotype, may be the consequence of caveolin being phosphorylated on tyrosine. GPI-anchored membrane proteins themselves have been implicated as the source of inositol phosphoglycans (IPG) that act as second messengers for a variety of different hormones (37, 38, 42). In addition, 1,4,5-trisphosphate (IP3) sensitive calcium channels (12), as well

© The Rockefeller University Press, 0021-9525/94/07/127/12 $2.00
The Journal of Cell Biology, Volume 126, Number 1, July 1994 127-138 127
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 caveolae 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., Fairlawn, NJ. PMSF was from Boehringer Mannheim GmbH (Germany). DTT and paraformaldehyde were from Fluka Chemical Corp. (Ronkonkoma, NY). SDS was from Pierce (Rockford, IL). n-Octyl-β-D-glucopyranoside (octyl-glucoside) was from Calbiochem Co. (La Jolla, CA). Monoclonal anti-DNP IgG was from Oxford Biomedical Research (Oxford, MI). Goat anti-λβ rabbit IgG conjugated to gold (10 nm) was from Jackson ImmunoResearch Laboratories (Westbury, NY) for 2 x 30 s at 27,000 rpm. The crude homogeneate 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 1 h at 30,000 g. Pellet B was resuspended in Buffer B (50 mM Mops, pH 7.4). To these were added 2 mg/ml heat-denatured BSA and 30% sucrose. 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 D (sucrose concentration, 0.25 M) and centrifuged for 60 min at 82,000 g in a Beckman SW 28 rotor at 4°C. The 15-25% 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 a tight-fitting 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 1 h at 100,000 g. Membrane material accumulated above the interface between both sucrose concentrations 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) was 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 0.5% Triton X-100 in buffer D that consisted of 1.5 ml of 42% sucrose, 3 ml 25% sucrose, and 3.5 ml 15% sucrose. Two ml of Triton X-100 treated Membrane III was layered on the top of the sucrose gradient and centrifuged for 2 h at 100,000 g. The pellicle at the interface between both concentration 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 was collected, resuspended in buffer A, and stored at -80°C. This fraction was designated Caveolae.
Pies (20 μl) were mixed with 5 μl of 10% SDS/1 mM DTT, followed by heating in a water bath with 5 μl of 10% SDS/1 mM DTT for 15 min at 100°C and incubation for 30 min at 10°C using a solution containing 20% methanol, 0.025 M Tris, and 0.192 M glycine (pH 8.5) as the electrode buffer.

Before loading on gels, samples 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 15 min in buffer J, and then incubated with either 125I-conjugated goat anti-mouse IgG or goat anti-rabbit IgG at 1:2,000 dilution in buffer J for 1 h at room temperature. The blots were rinsed with several changes of buffer J over 1−2 h and air-dried. [α-32P]GTP 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]GTP Binding.** [α-32P]GTP binding was carried out according to the method of Lapetina and Reep (24). The nitrocellulose containing transferred proteins was rinsed briefly in buffer N. The nitrocellulose was then incubated for 90 min at room temperature with [α-32P]GTP in buffer N (1 μCi/ml, specific activity, 5000 Ci/mmol) in the absence or presence of 1 μM GTP. The blots were rinsed with several changes of buffer N over 1−2 h and air-dried. [α-32P]GTP binding was visualized by autoradiography using Kodak X-Omat AR film.

**PL-PLC Treatment.** PI-PLC treatment was carried out using a modification of a method of Lissanti 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 boiled for 3 min. The samples were subjected to SDS-PAGE and transferred to nitrocellulose paper.

**Immun-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 2001) 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).

**Immun-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 Q. 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).

**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. 2 A). 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. 2, B 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....
Figure 1. Diagrammatic representation of the protocol used to purify caveolae from chicken gizzard smooth muscle.

(data not shown). Fig. 3 shows that when Membrane II was loaded onto a sucrose step gradient and centrifuged at 100,000 g for 2 h, the membranes at the 15%/25% interface had the highest caveolin specific activity. The 25% fraction had nearly the same activity, but the other fractions were quite low. We pooled the two fractions with the highest activity, treated them with 0.5% Triton X-100 (2-3 mg of protein in each treatment) and loaded the material onto a second sucrose step gradient (Fig. 4). The 15%/25% interface in this gradient had the highest activity, with a 1.8-fold increase over the Membrane III. Very little material was recovered in the denser fractions (data not shown). We washed the 15%/25% gradient had the highest activity, with a 1.8-fold increase over the Membrane III. Very little material was recovered in the denser fractions (data not shown). We washed the 15%/25% interface with carbonate and designated it the caveolae fraction. From 100 g wet weight of tissue, we obtained 1.5 mg of protein in the caveolae fraction. This fraction contained 1-5% of the caveolae present in the starting material.

The above assay indicated that, compared to the plasma membrane fraction, there was ~5-fold enrichment in caveolin in the caveolae fraction. To further assess the purity of the caveolae preparation, we analyzed four different fractions 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 caveolae 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 caveolae 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 caveolae 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 caveolae fraction (compare plasma membrane lane with caveolae 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 caveolae fraction was not above background (1,609 cpm/min/mg of protein). Therefore, Golgi membranes did not contaminate the caveolae 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 caveolae-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 caveolae 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 caveolae 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 caveolae 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. 7A). These fractions typically contained numerous, cup-shaped pieces of membrane. At higher magnification (Fig. 7A, 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. 7B). 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.

decorated with anti-caveolin IgG. A high magnification view shows how the gold was often associated with the striated coat (Fig. 7 B, inset). We did not see any gold labeling when an irrelevant monoclonal IgG was substituted for monoclonal anti-caveolin IgG (Fig. 7 C).

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 G\textsubscript{a} (Fig. 8 A) and G\textsubscript{a15} (Fig. 8 B) are enriched in caveolae. Scanning densitometer reading of the blots indicated that the specific blotting activity of G\textsubscript{a} was increased eightfold compared to plasma membranes while G\textsubscript{a15} was increased sixfold. An antibody that recognizes both G\textsubscript{a1} and G\textsubscript{a12} 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-G\textsubscript{a1}/G\textsubscript{a12} IgG (data not shown).

In contrast to the G\textsubscript{a} subunits, the specific blotting activity of the G\textsubscript{b} 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 G\textsubscript{b} from the Membrane III fraction (data not shown). Therefore, we used immunogold electron microscopy to determine if G\textsubscript{b} 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-G\textsubscript{b} 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 G\textsubscript{b}.

Chang et al. Purification of Caveolae
(small gold, Fig. 10 B) to the same pieces of membrane. Therefore, caveolae appear to contain both $G_a$ and $G_\beta$ subunits of the heterotrimeric GTP-binding proteins but that only $G_a$ is detergent resistant.

We also found that several small GTP-binding proteins were enriched in the caveolae fraction (Fig. 11, A and B). [$\alpha$-32P]GTP binding to proteins transferred to nitrocellulose revealed two prominent bands that were increased $\sim$2-fold in density compared to plasma membranes. In an effort to identify other GTP-binding proteins, we used a peptide anti-

**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 arrowheads 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.

Chang et al. Purification of Caveolae
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.

Figure 7. Whole mount electron microscopic visualization of untreated, purified caveolae (A) or purified caveolae labeled by immunogold with either anti-caveolin IgG (B) or irrelevant IgG (C). Samples of Membrane IV were briefly treated with octyl-glucoside on ice, fixed, and air-dried onto grids. The grids were then either viewed directly (A) or processed to localize anti-caveolin IgG binding sites (B) or non-immune IgG binding sites (C). Bar, 0.3 μm (inset, 0.2 μm).
Immunoblot detection of either Gs (A) or Gi3 (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 Gs (A) or Gi3 (B) using 125I-goat anti-rabbit IgG to detect the rabbit IgG.

even though caveolin is enriched only ~5-fold relative to the plasma membrane.

In contrast to caveolin, GPI-anchored membrane proteins were enriched ~15-fold in the caveolae fraction. The anti-CRD IgG detected at least nine bands on immunoblots. Scanning densitometry of the four most prominent bands showed that they were enriched from 13–18-fold. The relative enrichment was independent of the band intensity. This suggests that the GPI-anchored proteins are purifying as a unit but that there are variable amounts of the individual proteins within the unit. These findings are consistent with electron microscopic studies showing that caveolae contain clusters of GPI-anchored proteins composed of multiple protein species (54).

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. 5 A). 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 ( starvation, Fig. 5 A) 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 Ga 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 Ga 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 Ga1 and Ga2 are associated with isolated caveolae. We also successfully used immunogold cytochemistry to localize the other two Ga subunits to caveolae (data not shown).

The caveolae-associated Ga most likely corresponds to a subset of all the Ga that is associated with the plasma membrane. This subgroup may participate in one or more signaling activities that originate in caveolae. Interestingly, Ga was the least enriched Ga 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 Ga 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 Ga must remain in caveolar membranes during the purification because of a tight interaction with a structural component of the caveolae. Ga appears not to hold the Ga 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 Ga (25) may control the association with caveolae.
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. Non-receptor, 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^ck and p59^ctk 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_s subunits that we detected in purified caveolae share with p59^ctk and p56^ck the Met-Gly-Cys motif at the NH_2 terminus. Furthermore, all are palmitoylated at position 3 (9, 30, 33), and, with the exception of G_m, 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^src 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^src and the association of the kinase with GPI anchor protein complexes. G_m 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_s 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. [32P]GTP blots showed that at least two proteins of 30 and 28 kD were enriched. Moreover, an anti-RaplA/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 Rap1 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 Gp, 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.

This study would not have been possible without the dedicated assistance of Grace Liao who prepared the caveolae from chicken gizzards. We would like to thank Dr. Douglas Lublin for sharing his results before publication.

This work was supported by grants: HL 20948, GM 34497, and GM 43169 from the National Institutes of Health; BE30-0 from the American Cancer Society; 91R-077 from the American Heart Association; the Perot Foundation; The Lucille P. Markey Charitable Trust; and The Raymond Willi Chair of Molecular Neuropharmacology.

Received for publication 9 December 1993 and in revised form 11 March 1994.

References

1. Anderson R. G. W. 1993. Caveolae: where incoming and outgoing messengers meet. Proc. Natl. Acad. Sci. USA. In press.
2. Anderson, R. G. W. 1993. Potocytosis of small molecules and ions by caveolae. TIC. 3:69-71.
3. Anderson, R. G. W., B. A. Kamen, K. G. Rothberg, and S. W. Lacey. 1992. Potocytosis: sequestration and transport of small molecules by caveolae. Science (Wash. DC). 255:410-411.
4. Beranger, L., B. Goud, A. Tavitian, and J. de Gunzburg. 1991. Association of the Ras-antagonistic Rap1/Krev-I proteins with the Golgi complex. Proc. Natl. Acad. Sci. USA. 88:1006-1010.
5. Cerneus, D. P., E. Ueffing, G. Posthuma, G. J. Strous, and A. van der Ende. 1993. Detergent insolubility of alkaline phosphatase during biosynthetic transport and endocytosis. Role of cholesterol. J. Biol. Chem. 268:3150-3155.
6. Chang, W.-J., K. G. Rothberg, B. A. Kamen, and R. G. W. Anderson. 1992. Lowering of the cholesterol content of MA104 cells inhibits receptor mediated transport of folate. J. Cell Biol. 118:63-69.
7. Cinek, T., and V. Horejsi. 1992. The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. J. Immunol. 149:2262-2270.
8. Corvazier, E., J. Enouf, B. Papp, J. D. Gunzburg, A. Tavitian, and S. Levy-Toledano. 1992. Evidence for a role of ras protein in the regulation of human platelet Ca2+ fluxes. Biochem. J. 281:325-331.

Figure 11. Radiolabeled GTP blot (A), immunoblot with anti-Rap1A/B IgG (B), and immunogold localization of Rap1A/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 Rap1A/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-Rap1A/B IgG as described. Bar, 0.3 μm.
28. Lucchesi, P. A., R. A. Cooney, C. Mangsen-Baker, T. W. Honeyman, and T. W. Honeyman, and S. M. Mumby, S. M., C. Kleuss, and A. G. Gilman. 1994. Palmitoylation of G protein α subunits: structural requirements and modulation by receptor agonists. Proc. Natl. Acad. Sci. USA. In press.

29. Mumby, S. M., C. Kleuss, and A. G. Gilman. 1994. Palmitoylation of G protein α subunits: structural requirements and modulation by receptor agonists. Proc. Natl. Acad. Sci. USA. In press.

30. Mumby, S. M., C. Kleuss, and A. G. Gilman. 1994. Palmitoylation of G protein α subunits: structural requirements and modulation by receptor agonists. Proc. Natl. Acad. Sci. USA. In press.

31. Delet in proof.

32. Palade, G. E. 1953. Fine structure of blood capillaries. J. Appl. Physiol.