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A detergent-free method for purifying caveolae membrane from tissue culture cells

(G proteins/epidermal growth factor receptor/folate receptor)

ERIC J. SMART, YUN-SHU YING, CHIEKO MINEO, AND RICHARD G. W. ANDERSON

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235

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ABSTRACT **Current methods for purifying caveolae from tissue culture cells take advantage of the Triton X-100 insolubility of this membrane domain. To circumvent the use of detergents, we have developed a method that depends upon the unique buoyant density of caveolae membrane. The caveolae fractions that we obtain are highly enriched in caveolin. As a consequence we are able to identify caveolae-associated proteins that had previously gone undetected. Moreover, resident caveolae proteins that are soluble in Triton X-100 are retained during the isolation.**

Caveolae were originally identified with the electron microscope as flask-shaped membrane invaginations (50–100 nm in diameter) on the surface of epithelial (1) and endothelial cells (2). Subsequent studies of endothelial cells suggested that they form plasmalemmal vesicles that move across the cell during transcytosis. More recently, caveolae have been identified as the vehicle for internalizing small molecules such as 5-methyltetrahydrofolate by a process called potocytosis (3). During potocytosis, caveolae undergo an internalization cycle that involves membrane invagination, the formation of plasmalemmal vesicles, and the return of these vesicles to the plasma membrane. The cycle is regulated (4) so that caveolae may be at different stages of internalization within the same cell. Rapid-freeze, deep-etch electron microscopy shows that in whole images of the plasma membrane many caveolae are not invaginated, yet are clearly distinguished from other membrane by the presence of a characteristic membrane coat (5).

Recently, two methods were introduced for purifying caveolae (6, 7). One method relies on the fact that portions of the caveolae membrane are insoluble in detergents such as Triton X-100 (6). The other is designed to purify caveolae from tissues using routine cell fractionation techniques followed by treatment with Triton X-100 (7). Analysis of caveolae fractions from these two procedures indicates that they are enriched in sphingomyelin (8, 9) and cholesterol (8, 10). In addition, they have a high concentration of the integral membrane protein caveolin (6, 7) as well as multiple glycosylphosphatidylinositol (GPI)-anchored membrane proteins (7). These fractions also contain several different signal transducing molecules such as protein kinase C α (PKC_α) (4, 11) and heterotrimeric GTP binding proteins (6, 7).

Caveolin (5), GPI-anchored membrane proteins (12), cholesterol (13), glycolipids (14), and PKC_α (4) have all been shown to be enriched in invaginated caveolae by electron microscopic cytochemistry. We have used these markers to develop a third purification scheme that does not rely on detergents. In addition to the molecules already found enriched in caveolae, this method preserves resident prenylated proteins such as heterotrimeric $G_{\beta\gamma}$.

MATERIALS AND METHODS

Materials. Medium 199 with Earl's salts minus folic acid was prepared in the laboratory by standard methods (15). Dulbecco's modified Eagle's medium (DMEM), glutamine, trypsin-EDTA, and penicillin/streptomycin were from GIBCO. Fetal calf serum was from Hazleton Research Products, Inc. (Lenexa, KS). The analytical silica gel thin-layer chromatography plates and solvents (heptane, petroleum ether, ethyl ether, acetic acid, and 2-propanol) were from J. T. Baker. [^{3}H]Acetate (4.13 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]uridine diphosphate galactose (50 Ci/mmol) were obtained from DuPont. The sulfuric acid/dichromate spray was from Supelco. The alkaline phosphatase substrate system, the silver stain kit, and the Bradford assay kit were from Bio-Rad. Percoll was from Pharmacia. OptiPrep was from GIBCO. The β subunit of cholera toxin was from Sigma.

Antibodies. Antibodies and suppliers are as follows: anti-caveolin IgG, John Glenney (Glentech, Lexington, KY); anti-PKC_α IgG, anti-epidermal growth factor receptor (EGFR) IgG, and anti-paxillin IgG, Transduction Laboratory (Lexington, KY); anti-annexin I IgG, Zymed; goat anti-cholera toxin β-subunit IgG, Calbiochem; anti-actin IgG, Helen Yin (University of Texas Southwestern Medical Center, Dallas); anti-G_β IgG and anti-$G_{\alpha i}$ IgG, Susanne Mumby (University of Texas Southwestern Medical Center); anti-β-adaptin IgG, M. S. Robinson (University of Cambridge, Cambridge, U.K.); anti-human transferrin receptor IgG, Ian Trowbridge (Salk Institute); anti-folate receptor IgG, Centacore (Philadelphia). Anti-low density lipoprotein (LDL) receptor IgG and anti-clathrin IgG were developed in the laboratory.

Buffers. The following buffers were used: buffer A (0.25 M sucrose/1 mM EDTA/20 mM Tricine, pH 7.8); buffer B (0.25 M sucrose/6 mM EDTA/120 mM Tricine, pH 7.8); buffer C (50% OptiPrep in buffer B); buffer D (20 mM Tris, pH 7.6/137 mM NaCl/0.5% Tween 20).

Cell Culture. Normal human fibroblasts were obtained by skin biopsy, cultured in a monolayer, and set up according to a standard format. On day zero, 2.5×10^5 cells were seeded into 100-mm dishes with 5 ml of DMEM supplemented with 100 units of penicillin per ml, 100 units of streptomycin per ml, and 10% (vol/vol) fetal calf serum. The medium was changed on day 3 and day 5. The day 5 medium contained either 10% (vol/vol) fetal calf serum or 10% (vol/vol) human lipoprotein-deficient serum. The cholesterol pool was radiolabeled by incubating day 6 cells for 24 hr in the presence of [^{3}H]acetate (75 μCi per dish). All experiments were carried out on day 7. Monolayers of MA104 cells were cultured in folate-free medium 199 supplemented with 5% (vol/vol) fetal calf serum and 100 units of penicillin/streptomycin per ml. Cells for each experiment were set up according to a standard format (16).

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Abbreviations: GPI, glycosylphosphatidylinositol; PKC_α, protein kinase C α; EGF, epidermal growth factor; EGFR, EGF receptor; PNS, postnuclear supernatant fraction; LDL, low density lipoprotein.

On day zero, 3.0×10^5 cells were seeded into a T-75 culture flask and cultured for 5 days.

Purification of Caveolae. All steps were carried out at 4°C. A plasma membrane fraction was prepared from ten 100-mm dishes or T-75 flasks of confluent tissue culture cells (7–8 mg of total protein). Each dish or flask was washed twice with 5 ml of buffer A and the cells were collected by scraping in 3 ml of buffer A. The cells were pelleted by centrifugation for 5 min, 1400 × *g* in an IEC Centra-4B centrifuge. The cells were resuspended in 1.0 ml of buffer A, placed in a 2-ml Wheaton tissue grinder (catalog no. 0841416A), and homogenized with 20 strokes of the Teflon homogenizer. We transferred the suspension to a 1.5-ml centrifuge tube and centrifuged at 1000 × *g* for 10 min in an Eppendorf 5415C centrifuge. The postnuclear supernatant fraction (PNS) was removed and stored on ice. The pellet from each tube was resuspended in 1.0 ml of buffer A, homogenized, and centrifuged at 1000 × *g* for 10 min again. The two PNSs were combined (≈4 mg of total protein), layered on the top of 23 ml of 30% Percoll in buffer A, and centrifuged at 84,000 × *g* for 30 min in a Beckman Ti 60 rotor. The plasma membrane fraction was a visible band ≈5.7 cm from the bottom of the centrifuge bottle. The membrane fraction, which contained ≈0.6 mg of protein, was collected with a Pasteur pipette, adjusted to 2.0 ml with buffer A, and placed in a Sorvall TH641 centrifuge tube on ice. We placed a 3-mm (diameter) sonication probe equidistant from the bottom of the tube and the top of the solution and sonicated the sample two successive times (total power, 50 J/W per sec each time) using the automatic settings of a Vibra Cell sonicator (model VC60S, Sonics & Materials, Danbury, CT). The sample was then incubated on ice 2 min to maintain the sample at 4°C before a second round of two sonications. The process was repeated once again, for a total of six sonication bursts. An aliquot of the sonicate was saved before mixing the remainder with 1.84 ml of buffer C and 0.16 ml of buffer A (final OptiPrep concentration, 23%) in the bottom of the same TH641 tube. A linear 20% to 10% OptiPrep gradient (prepared by diluting buffer C with buffer A) was poured on top of the sample and then centrifuged at 52,000 × *g* for 90 min in a Sovall TH641 swinging bucket rotor. The top 5 ml of the gradient (fractions 1–7) was collected, placed in a fresh TH641 centrifuge tube, and mixed with 4 ml of buffer C. The sample was overlaid with 2 ml of 5% OptiPrep (prepared by diluting buffer C with buffer A) and centrifuged at 52,000 × *g* for 90 min at 4°C. A distinct opaque band was present in the 5% OptiPrep overlay about 4–5 mm above the interface. This band was collected and designated caveolar membranes. Typically we obtained 10 μg of protein in this band.

Enzyme Assays. Alkaline phosphatase was measured by the method of Engstrom (17). Galactosyltransferase and NADPH cytochrome *c* reductase were assayed using methods adapted from Graham (18). Lactate dehydrogenase was measured with a standard kit from Sigma.

Protein Determination. Protein concentrations were determined by the Bio-Rad Bradford assay. A 100-μl sample of each gradient fraction was mixed with 5 ml of diluted (1:4) Bradford reagent and the absorbance was measured at 595 nm. OptiPrep interfered with other standard protein determination methods including the micro-Bradford assay.

Electrophoresis and Immunoblots. A 75-μg sample of the whole cell lysate or 5 μg of the PNS, plasma membrane fraction, and the caveolar membrane fraction was concentrated by trichloroacetic acid precipitation and washed in acetone. Pellets were suspended in Laemmli (19) sample buffer that contained 1.2% 2-mercaptoethanol and heated at 95°C for 3 min before being loaded onto gels. Samples that were immunoblotted with anti-folate receptor IgG did not contain 2-mercaptoethanol in the sample buffer. Proteins were separated in a 12.5% SDS/polyacrylamide gel using the method of Laemmli (19). The separated proteins were then transferred to nylon. The nylon was blocked in buffer D that contained 5% dry milk for 1 hr at room temperature. Primary antibodies were diluted in buffer D that contained 1% dry milk and incubated with the nylon samples for 1 hr at room temperature. The nylon was washed four times, 10 min each in buffer D plus 1% dry milk. The second antibody (goat anti-mouse IgG, goat anti-rabbit IgG, or rabbit anti-goat IgG, all conjugated to horseradish peroxidase) was diluted 1:30,000 in buffer D plus 1% dry milk and incubated with the nylon for 1 hr at room temperature. The nylon was then washed and the bands were visualized using enhanced chemiluminescence.

Quantification of Immunoblots. The relative fold enrichment of the indicated proteins in the caveolar membrane fraction was determined by comparative density of the reactive bands on immunoblots. Different protein concentrations of the caveolae and whole plasma membrane fractions were separated on 12.5% SDS/polyacrylamide gels and immunoblotted with the appropriate antibody using enhanced chemiluminescence. Multiple exposures were obtained and the one that yielded a linear increase in band density for both fractions was used. Scans were quantified with a Molecular Dynamics (model P.D.) densitometer to determine the relative band intensity in each fraction.

Other Assays. The amount of cholesterol in the caveolae fraction was determined as described (10). Whole mount Immunogold labeling of caveolae membranes was done as described (7) with the modification that unfixed samples (2 μl) were mounted on carbon-coated grids for 20 min before being fixed with 3% paraformaldehyde for 10 min.

RESULTS

The first step in the purification protocol is to isolate plasma membranes by Percoll gradient fractionation (20). We found that fraction 4 from this gradient contained all of the plasma membrane marker, alkaline phosphatase, but lacked galactosyltransferase, NADPH cytochrome *c* reductase, and lactate dehydrogenase (data not shown). Therefore, these fractions contain plasma membrane that is not contaminated with either cytoplasm or membranes from other compartments. The quality of the plasma membrane preparation was found to be very sensitive to the amount of protein loaded on the gradient. The plasma membrane fraction was then broken into small pieces by sonication. The indicated settings were required to properly disrupt the membrane for subsequent stages in the isolation procedure. Caveolae membrane was separated from the remainder of the plasma membrane using two OptiPrep density gradients. The first gradient (OptiPrep 1) separated the bulk of the membrane protein from lighter material that floated in the gradient. Fig. 1 shows the protein profile of the gradient (dashed line). Almost all of the protein was in fractions 9–15 (●, Fig. 1*A*). We then pooled the top 7 fractions and used a discontinuous flotation gradient (OptiPrep 2) to concentrate the lightest material. Fig. 1 shows the protein profile from this gradient (solid line). Essentially all of the protein on OptiPrep 2 was in fraction 3 (■, Fig. 1*A*).

The distribution of caveolin in the two gradients was determined by immunoblotting (Fig. 1*B*). All of the protein in each fraction was separated by gel electrophoresis and immunoblotted with anti-caveolin IgG. While fractions 3–11 in OptiPrep 1 (OptiPrep 1, Fig. 1*B*) had the most intense caveolin blotting activity, nearly every fraction contained some of the protein. By contrast, virtually all of the caveolin on the OptiPrep 2 gradient was in fraction 3 (OptiPrep 2, Fig. 1*B*). Typically this fraction contained ≈10 μg of the original 700 μg of protein in the plasma membrane.

We set up a standard immunoblot assay to measure the relative concentration of various proteins in fraction 3 (designated, caveolae fraction). This involved separating samples of whole cell lysate (75 μg per lane), PNS (5 μg per lane),

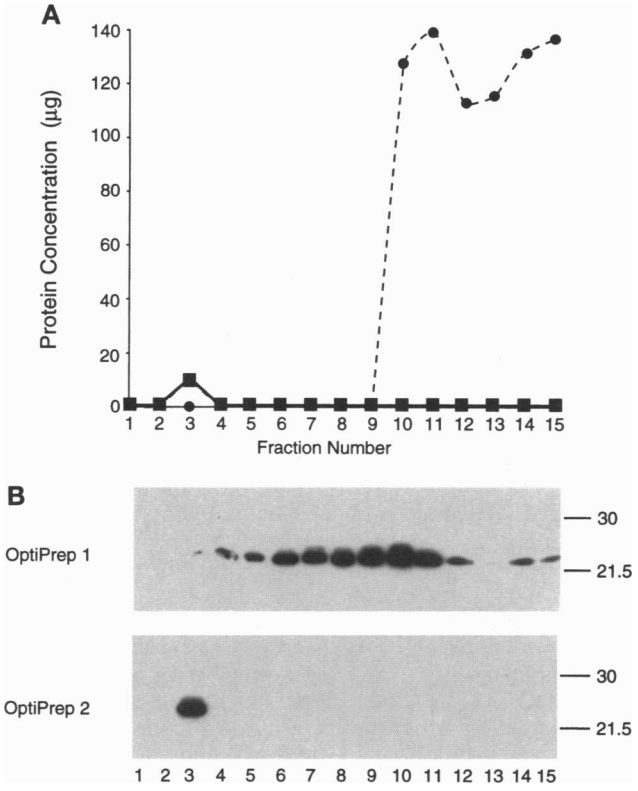

FIG. 1. Protein content (*A*) and anti-caveolin immunoblot (*B*) of fractions from the OptiPrep gradients. (*A*) Purified plasma membranes were sonicated and loaded on the bottom of the first OptiPrep gradient and spun for 90 min. Each fraction (0.65 ml) was analyzed for protein content (●). Fractions 1–7 from this gradient were pooled and spun on the second OptiPrep gradient for 90 min before the protein content was determined (■). (*B*) The total protein in each fraction of OptiPrep 1 and OptiPrep 2 was separated by electrophoresis and immunoblotted with anti-caveolin IgG as described.

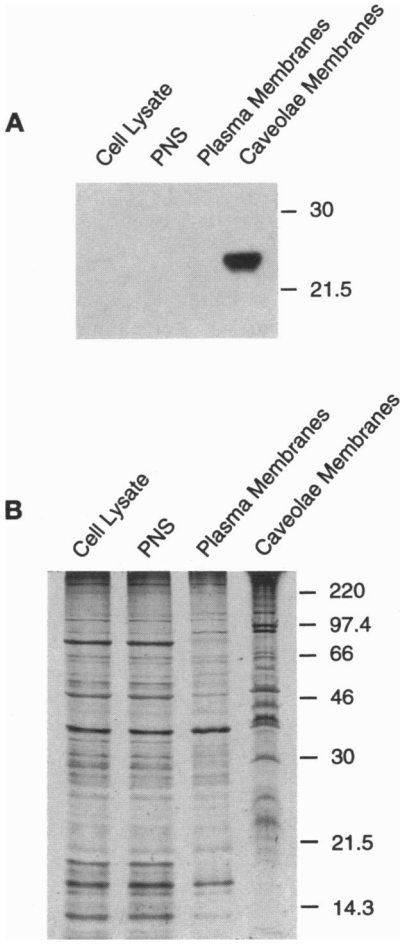

FIG. 2. Standard immunoblot assay (*A*) and protein profile (*B*) of the fractions used in this assay. (*A*) Four fractions were used in the standard immunoblot assay; 75 μg of whole cell lysate, 5 μg of the PNS, 5 μg of plasma membrane, and 5 μg of caveolae. Each of these samples was separated on 12.5% SDS/polyacrylamide gels and immunoblotted with anti-caveolin IgG. (*B*) The same fractions (10 μg per lane) were separated by electrophoresis and the protein bands were visualized by silver staining.

plasma membrane (5 μg per lane), or the caveolae fraction (5 μg per lane) by electrophoresis and immunoblotting with the antibody of interest. We loaded 15 times more whole cell lysate to have a positive control for the antibody. Fig. 2 shows that with anti-caveolin IgG only the caveolae fraction (caveolae, Fig. 2*A*) had an intense reactive band. Immunoblot quantification showed that the caveolae band was ≈2200 times more intense in the caveolae fraction than in the plasma membrane fraction. Silver staining of replicate gels (Fig. 2*B*) showed that the caveolae fraction had a unique protein profile. Very few of the bands in this fraction matched bands in the plasma membrane, indicating a high degree of enrichment for a specific set of proteins. Finally, we found that the caveolae fraction contained 4% of all the membrane cholesterol.

Electron microscopic images of the caveolae fraction are shown in Fig. 3. The dominant structure in fractions from human fibroblasts was a round membrane profile that averaged 70 nm in diameter. Immunogold labeling with anti-caveolin IgG showed that these membranes contained caveolin but that there were also small fragments of membrane that labeled with the antibody (Fig. 3*A*). Gold labeling was not seen when a nonimmune monoclonal antibody was substituted for the anti-caveolin IgG (Fig. 3*C*). Finally, we prepared caveolae from MA104 cells and used Immunogold to label the membrane with anti-folate receptor IgG (Fig. 3*B*). These caveolae had the same general morphology as the fibroblast caveolae. Many of these caveolae were decorated with gold particles.

On the basis of these analyses, the caveolae fraction appears to be a highly purified preparation of caveolae. We next carried out an immunoblot analysis to characterize the protein composition. Previous studies have shown that PKC$_\alpha$ (4), heterotrimeric G$_\alpha$ (6, 7), and actin (7, 11) are enriched in caveolae. We found that all three of these proteins were also enriched in detergent-free caveolae (G$_\alpha$, actin, PKC$_\alpha$, Fig. 4*A*). The intensity of the G$_{\alpha i}$ immunoreactive band in the caveolae fraction was 25-fold greater than in the plasma membrane fraction. By contrast, the clathrin-coated pit-associated proteins, clathrin (CL, Fig. 4*B*) and β-adaptin (AP$_\beta$, Fig. 4*B*), were not detected in the caveolae fraction. Two cytosolic proteins that can associate with plasma membrane, annexin I and paxillin, were present in the plasma membrane fraction but excluded from the caveolae fraction (An I, Pax, Fig. 4*B*).

We also used immunoblotting to assay for the presence of different membrane receptors. We found that the intensity of the GPI-anchored folate receptor band in the caveolae fraction was 42-fold greater than in the plasma membrane fraction (FR, Fig. 4*A*). The caveolae fraction did not contain the coated pit-targeted receptors for LDL (LDLR, Fig. 4*B*) and transferrin (TFR, Fig. 4*B*), even though they were easily detected in the plasma membrane. We were surprised to find, therefore, that the EGFR, another receptor that is internalized by coated pits (21), was highly enriched in the caveolae fraction (EGFR, Fig. 4*A*). Quantitative analysis showed that the receptor band was 100 times more intense in immunoblots of caveolae than of plasma membranes.

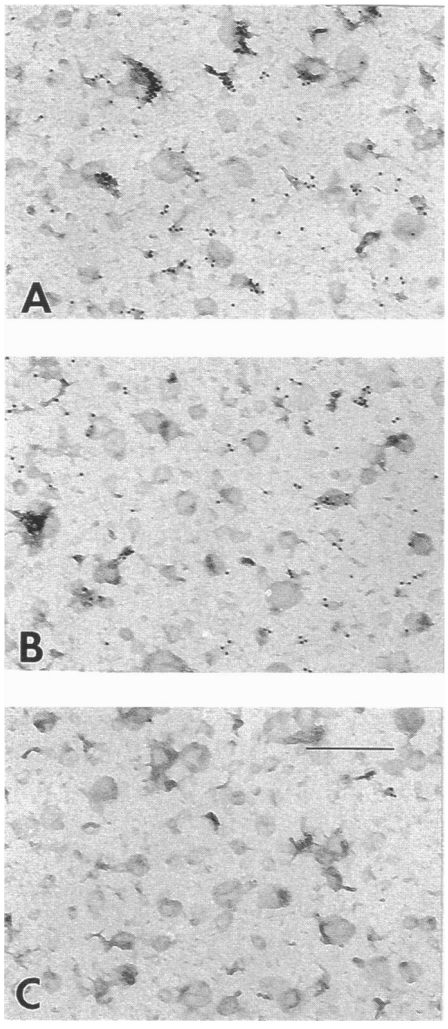

FIG. 3. Electron microscopic analysis of caveolae fraction. Samples from fraction 3 of OptiPrep 2 were applied to electron microscopy grids, fixed, and processed for Immunogold labeling using either monoclonal antibody (mAb) anti-caveolin IgG (*A*), mAb anti-folate receptor IgG (*B*), or mAb nonimmune IgG (*C*). (Bar = 0.3 μm.)

We noted previously that Triton X-100 removed heterotrimeric G_β from smooth muscle caveolae (7). Fig. 4 shows that caveolae isolated without detergents are highly enriched in G_β (G_β, Fig. 4*A*). By quantitative analysis, the intensity of the reactive band in caveolae fractions was 150-fold greater than in plasma membrane fractions. Since G_β is always tightly associated with G_γ (22), all three subunits of the heterotrimeric complex must be enriched in caveolae.

To determine if bound ligands would copurify with detergent-free caveolae, fibroblasts were incubated for 60 min at 4°C with the β subunit of cholera toxin before caveolae were isolated. Cholera toxin binds to GM1 ganglioside (23), a glycolipid that is enriched in caveolae. Fig. 5 shows that the dimeric and monomeric β subunits were enriched in the caveolae fraction relative to the plasma membrane.

DISCUSSION

We have developed a detergent-free method for purifying caveolae membrane that can be used to isolate caveolae from tissue culture cells as well as tissues. In the process, we have discovered that caveolae have a unique buoyant density that causes them to separate from other membrane domains. They may behave this way on gradients because of a high lipid-to-protein ratio. Judging from the gel electrophoresis pattern,

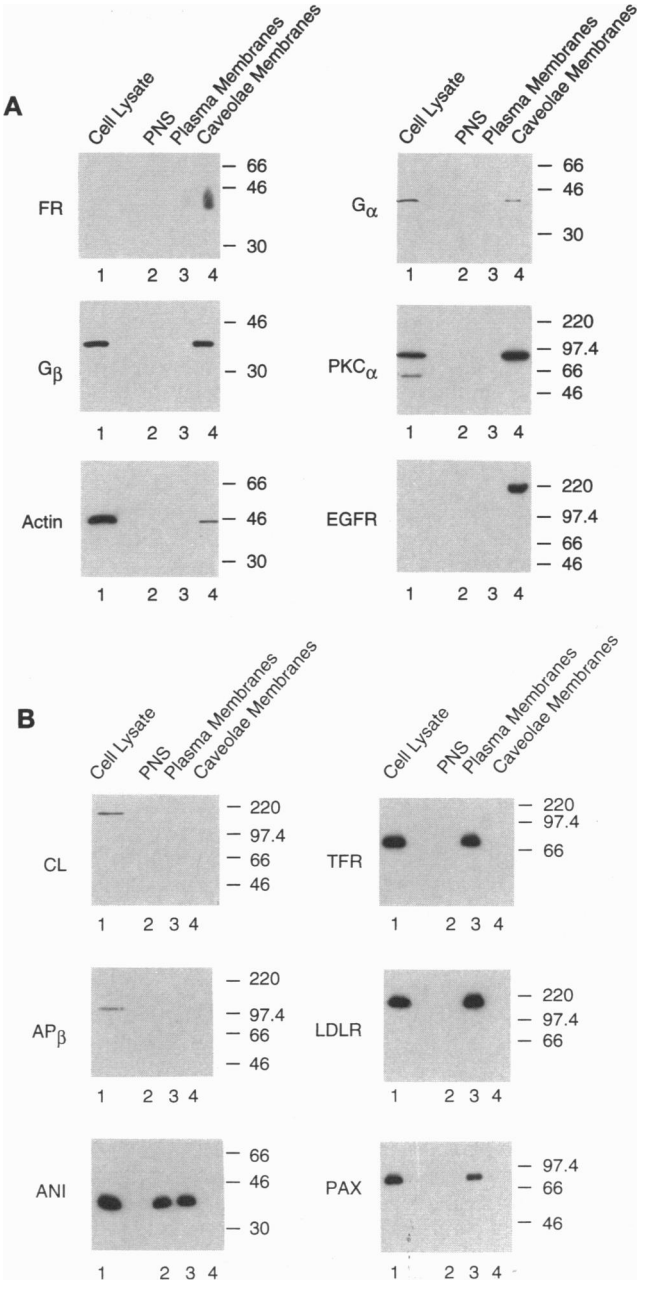

FIG. 4. Immunoblot detection of proteins that were enriched (*A*) or not enriched (*B*) in caveolae. Immunoblots were set up as described in the legend to Fig. 2 except that for the LDL and transferrin receptor the lanes that normally received 5 μg per lane received 20 μg of protein. The nylon membranes were blotted with the indicated antibody as described. FR, folate receptor; G_α, α subunit of heterotrimeric GTP binding protein; G_β, β subunit of heterotrimeric GTP binding protein; CL, clathrin; TFR, transferrin receptor; AP_β, β-adaptin; LDLR, LDL receptor; ANI, annexin I; PAX, paxillin.

these caveolae are purer than those obtained with other methods (6, 7). We also found that caveolin was enriched, although the increase in specific blotting activity appeared to be anomalously high. We do not know why the increase in specific activity was so high for this protein but not for other proteins we tested.

All of the proteins we tested that had previously been found to be enriched in Triton X-100-insoluble material were also enriched in detergent-free caveolae membranes. This indicates that detergent-insoluble complexes contain portions of caveolae membrane. They do not contain all of the resident caveolae

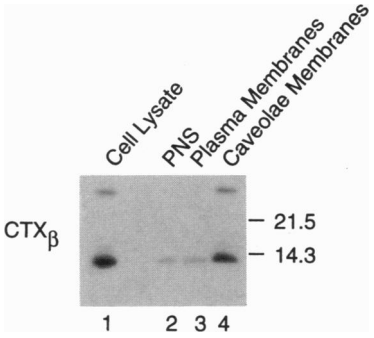

FIG. 5. Detection of bound cholera toxin in purified caveolae. Cells were incubated in the presence of the β subunit of cholera toxin (CTX_β) at 4°C for 1 hr before the cells were fractionated. Immunoblotting (see Fig. 2) was used to detect the β subunit with anti-β subunit IgG.

proteins. We found, for example, that heterotrimeric G_β was enriched in our caveolae fractions but not in detergent-treated caveolae membranes (7). This protein is tightly associated with heterotrimeric G_γ (24), a geranylgeranylated membrane protein (25). This raises the possibility that caveolae are rich in a variety of prenylated proteins that are removed when they are exposed to Triton X-100. Most likely, caveolae isolated without detergents reflect the composition of the membrane at the time it was removed from the cell. If so, it should be possible to replace the morphologic description of the membrane with a molecular definition of the organelle.

The original identification of caveolae as a site for clustered GPI-anchored membrane proteins was determined using immunocytochemical methods (12, 26). Subsequently it was found that GPI-anchored proteins were present in Triton X-100-insoluble aggregates that had about the same size as a caveola (27). The molecular basis for this insolubility has been traced to the unique lipid composition of this membrane domain (28). Now we have found that caveolae isolated without detergents are also highly enriched in the folate receptor. This indicates that GPI-anchored membrane proteins are indeed clustered in caveolae (12). Further research is needed to determine why antibodies against GPI-anchored proteins appear to affect the clustered organization of these antigens in living cells (29, 30).

Finding that the EGFR was enriched in human fibroblast caveolae was unexpected. This receptor is known to be rapidly internalized by clathrin-coated pits after ligand binding (21). The cells we used were grown continuously in serum prior to isolating the caveolae so that we do not know whether or not any EGF was bound to these receptors. These results strongly suggest that caveolae have an important role to play in EGF-mediated signal transduction.

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