Immunoisolation of Caveolae with High Affinity Antibody Binding to the Oligomeric Caveolin Cage

TOWARD UNDERSTANDING THE BASIS OF PURIFICATION*

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Defining the molecular composition of caveolae is essential in establishing their molecular architecture and functions. Here, we identify a high affinity monoclonal antibody that is specific for caveolin-1α and rapidly binds caveolin oligomerized around intact caveolae. We use this antibody (i) to develop a new simplified method for rapidly isolating caveolae from cell and tissue homogenates without using the silica-coating technology and (ii) to analyze various caveolae isolation techniques to understand how they work and why they yield different compositions. Caveolae are immunoisolated from rat lung plasma membrane fractions subjected to mechanical disruption. Sonication of plasma membranes, isolated with or without silica coating, releases caveolae along with other similarly buoyant microdomains and, therefore, requires immunoisolations to purify caveolae. Shearing of silica-coated plasma membranes provides a homogeneous population of caveolae whose constituents (i) remain unchanged after immunoisolation, (ii) all fractionate bound to the immunobeads, and (iii) appear equivalent to caveolae immunoisolated after sonication. The caveolae immunoisolated from different low density fractions are quite similar in molecular composition. They contain a subset of key signaling molecules (i.e. G protein and endothelial nitric oxide synthase) and are markedly depleted in glycosylphosphatidylinositol-anchored proteins, β-actin, and angiotensin-converting enzyme. All caveolae isolated from the cell surface of lung microvascular endothelium in vivo appear to be coated with caveolin-1α. Caveolin-1β and -2 can also exist in these same caveolae. The isolation and analytical procedures as well as the time-dependent dissociation of signaling molecules from caveolae contribute to key compositional differences reported in the literature for caveolae. This new, rapid, magnetic immunoisolation procedure provides a consistent preparation for use in the molecular analysis of caveolae.

In the last few years, there has been a burst of new research on defining the molecular architecture and function of one type of non-clathrin-coated plasmalemmal in various degrees on the surface of many mammalian cell types. In large measure, this surge of experimentation has resulted from the development of a number of techniques purporting to purify these invaginated microdomains from various cells and/or tissues. Cumulatively, the similarities between the lists of molecules identified in these preparations has been rather striking. It is interesting that this remarkable overall agreement has been minimally appreciated or acknowledged but rather only the few, yet significant differences have come to the forefront of discussion about this field. As with most fields growing rapidly in the modern era of research, this field has experienced its growing pains and controversies. Time resolves most scientific disputes, and here we wish to contribute new constructive information that may provide a firm basis for better understanding the basics of caveolae purification as well as the basis of both the similarities and differences found in the major methods currently devised for caveolae isolation.

Defining the molecular composition of caveolae is a critical step in dissecting and ultimately defining their function. Two groups (1, 2) reported purification of caveolae in 1994 using a methodology that evolved from the identification of caveolin (also called VIP21) as a marker protein of caveolae (3–5) and the development of a subfractionation procedure for following the synthesis and trafficking of glycosylphosphatidylinositol (GPI)-anchored proteins through the Golgi compartment (6). Cells or tissue are homogenized and exposed to Triton X-100 at 4 °C before using centrifugation to isolate the low buoyant density Triton-resistant membranes (TRM) enriched in caveolin, GPI-anchored proteins, cytoskeletal proteins, and signaling

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2 The abbreviations used are: GPI, glycosylphosphatidylinositol; TRM, Triton-resistant membranes; VAMP, vesicle-associated membrane protein (synaptobrevin); PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; P, the silica-coated luminal endothelial cell plasma membranes isolated from rat lungs; H, rat lung homogenate; V, caveolar fraction isolated by shearing of silica-coated endothelial cell plasma membranes (P); P-V, repelleted silica-coated membranes stripped of caveolae by sonication; PC, phosphate-buffered saline; SM, aliquot of starting material (usually V, PC, or AC) before immunoisolation; UB, unbound fraction containing material not binding to immunoaffinity beads; BD, bound fraction with material binding to immunoaffinity beads; PM, plasma membrane fraction derived from rat lung using Percoll density centrifugation; MBS, MFA-buffered saline; MfA, 2-(N-morpholinio)ethanesulfonic acid; CAV, caveolin monoclonal antibody (clone 2234); eNOS, endothelial nitric oxide synthase; uPAR, urokinase plasminogen activator receptor; ACE, angiotensin-converting enzyme; 5′ NT, 5′-nucleotidase; AC, low density caveolar fraction isolated after sonication of PM; HD, high density membranes left after sonication of PM; OC, caveolae immunoisolated using simplified procedure; pAb, polyclonal antibody; ELISA, enzyme-linked immunosorbent assay; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
From these methodologies, there has been general agreement from many laboratories that signaling molecules can be found in caveolae (1, 2, 7–10, 13, 15, 19, 21–24, 26–28). Moreover, various different assays show that caveolin can interact directly with various signaling molecules including G proteins and eNOS (29–33). Caveolin may act as a scaffolding protein that regulates signaling molecules by preferentially binding them in an inactive state (23, 29–33). Despite these data, one report in 1997 (34) concluded differently using a methodology that mixed different aspects of past procedures (13, 19, 24) in a new arrangement to immunosolivate caveolae. Instead of shearing, sonication was used in this study to dislodge caveolae from silica-coated rat lung endothelial cell plasma membranes before isolating the low density, caveolin-enriched vesicles by sucrose gradient centrifugation. This fraction was subjected to immunosoliation overnight using a rabbit polyclonal antiserum raised to a synthetic peptide similar to N-terminal region of caveolin. Although several signaling molecules were concentrated in low density, caveolin-rich vesicles released by sonication, little to no signal for many of these molecules such as eNOS and G proteins was reported in the immunosolivated caveolae. It was concluded from the data shown using volume equivalent analysis that caveolae are not signaling centers. In the end, these latter findings have extended uncertainty in the field from what had been a growing consensus about the lack of concentration of GPI-anchored proteins in caveolae under non-perturbed conditions to now the possible lack of many, if not all, lipid-anchored and possibly other signaling molecules in caveolae. This issue becomes of greater concern because many of the functions ascribed to caveolae, such as signaling, mechanotransduction (21, 35), and even vesicular transport (19, 21, 25), may rely on the presence of key molecules discovered to be in caveolae through the use of these subfractionation techniques.

In this work, we identify a high affinity, caveolin-specific monoclonal antibody that rapidly binds the oligomeric caveolin cage surrounding caveolae. By using this antibody, we have developed a method not only for rapidly purifying caveolae from cell and tissue homogenates but also for testing the purity of caveolae isolated using different methodologies. We identify factors that are important in purifying and analyzing caveolae and that explain the few but key compositional differences detected in various caveolar preparations.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies against caveolin were purchased from Transduction Laboratories (Lexington, KY) (rabbit polyclonal (pAb) and mouse monoclonal (clone 2297 and 2234)) and from Zymed Laboratories Inc. (South San Francisco, CA) (mouse monoclonal (Z034)). The M-450 Dynabeads were purchased from Dynal (New Hyde Park, NY). All other reagents/supplies were obtained as in our past work (13, 18, 19, 25).

**Western and Protein Analysis—**As described previously (13, 18, 19, 25), the proteins of various tissue fractions were solubilized and separated by SDS-PAGE for direct analysis by silver staining or for Western analysis by electrotransfer to nitrocellulose filters followed by immunoblotting using enhanced chemiluminescence and densitometric quantification using ImageQuant. Briefly, nitrocellulose filters from each gel were probed using primary antibody (diluted from 1:500 to 1:5000 in Blotto (5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20)) followed by the appropriate hors eradish peroxidase-labeled reporter antibodies (diluted 1:1000). Protein concentrations were measured using the micro-bicinchoninic acid protein assay kit with bovine serum albumin (BSA) as a standard.

**Subfractionation of Rat Lung to Isolate Silica-coated Endothelial Cell Plasma Membranes and Caveolae—**The luminal endothelial cell plasma membranes and caveolae were isolated directly from rat lung tissue using an *in situ* silica-coating procedure as described in detail (36). Briefly, the rat lungs were perfused via the pulmonary artery with a colloidal silica solution to coat the luminal surface of the endothelium and allow selective isolation of the silica-coated endothelial cell plasma membranes (P) from the lung homogenate (H) by centrifugation. The caveolae are separated from P by shearing and then isolated by sucrose...
density centrifugation in a low buoyant density fraction (V) well separated from the silica-coated membrane pellet stripped of caveolae (P-V). Alternatively, as described in Ref. 34, caveolae are separated from P by sonication and then isolated by sucrose density gradient centrifugation in a low buoyant density fraction (PC) away from the repelled and sonicated silica-coated membranes (P-C).

ELISA—The reactivity of caveolin antibodies with the silica-coated endothelial cell plasma membranes (P) was assessed by ELISA. Briefly, equal aliquots of P (5 μg in 100 μl) were placed in each well of a 96-well tray for drying overnight. After washing, the wells were blocked for 1 h with wash buffer (2% w/v albumin and 2 mM CaCl₂ in phosphate-buffered saline (PBS)), incubated with wash buffer alone or containing antibody, washed, incubated with reporter antibody conjugated to horseradish peroxidase (1:500), and washed again. A substrate solution (50 mM Na₂HPO₄, 25 mM citric acid, 1.2 mg/ml o-phenylenediamine dihydrochloride, and 0.03% H₂O₂) was added. The reaction was stopped with 4 mM H₂SO₄ before reading the signal at 450 nm using a Molecular Device Thermomax microplate reader.

Immunoaffinity Isolation of Caveolae—Magnetic immunoisolations were performed similar to our past work (19, 20). The new M450 Dynal beads conjugated with anti-mouse or -rabbit IgG were washed 3 times with PBS by resuspension and magnetic separation and then incubated for 4–8 h with the desired antibody (2 × 10⁶ M450 beads and 25 μg of IgG). After washing (3 times, PBS), the beads were resuspended and incubated for 1 h at 4°C with various starting membrane fractions (SM) before washing and magnetic separation to isolate two fractions: material bound to the beads (BD) versus material not bound to the beads (UB). SM, BD, and UB were subjected to SDS-PAGE and Western analysis. It should be noted that because of concerns about nonspecific binding, the initial procedure was done with the addition of 1 mg/ml BSA as a blocker. But when we left out BSA, we did not see any evidence for nonspecific binding and got the same results except that the nonspecific bands from the BSA detected in the silver-stained gels were largely avoided (although not completely because the beads from the manufacturer are in a BSA solution). Thus, all experiments shown here lacked additional BSA.

Simplified Purification of Caveolae from Plasma Membranes (PM)—Homogenates of cultured cells or tissues were subjected to Percoll gradient centrifugation to isolate a plasma membrane fraction as described (36). Briefly, the lung tissue was homogenized in buffer (0.25 M sucrose, 1 mM EDTA/20 mM Tricine, pH 7.8), and the lung homogenate was filtered sequentially through 53- and 30-μm Nytex filters. Then the cell or filtered tissue homogenates were subjected to centrifugation (1000 × g for 10 min) at 4°C, and the supernatant was saved. The resulting pellet was resuspended in 3 ml of buffer and subjected again to homogenization and centrifugation as above. The two supernatants were combined and then mixed with 30 ml of 30% Percoll in buffer. After centrifugation using an SW28 rotor at 84,000 × g for 45 min at 4°C (no brakes), we collected a single membranous band readily visible about 2/3 from the bottom of the tube. To reduce volumes and concentrate the membranes, we sometimes pelleted the membranes by first diluting the suspension 2–3-fold with MBS before centrifugation at 4°C at either 15,000 × g for 2 h or 100,000 × g for 0.5 h. To isolate the caveolae, this PM fraction was resuspended in 1 ml of MBS and sonicated on “high” (2 × 10 s; Branson sonicator, maximum for microprobe) followed by immediate cooling on ice. This sonication procedure was repeated 3 times. The sonicated membranes are mixed with 50% Optiprep to yield a final concentration of 23% in a 3-ml total volume. After adding a continuous layer of 20 to 10% Optiprep (~2.5 ml) to the SW55 tube, the suspension was spun at 22,500 × g for 90 min at 4°C in an SW55 rotor. Depending on the amount of PM loaded, one usually could see 5–7 distinct membranous bands. We collected the first 2 bands or the top quarter of the tube (when bands were not readily visible). To reduce volumes, we sometimes concentrated the membranes to a pellet by centrifugation. To immunoslate the caveolae, the low density material or its membrane pellet resuspended in 100 μl of MBS was processed as described above.

RESULTS

Rat Lung Subcellular Fractionation—We developed a method for subfractionating rat lung tissue to isolate first the luminal endothelial cell plasma membranes and then to discard and isolate their caveolae (13, 18, 19, 36). Briefly, the rat lung microvasculature was perfused in situ at 10–13°C via the pulmonary artery with a solution of positively charged colloidal silica particles that coated the intimal endothelial cell surface membrane directly in contact with the circulating blood and created a stable silica pellicle that specifically marked this membrane and facilitated its purification from tissue homogenates by centrifugation. Electron microscopy showed that the sedimented pellets (P) contain silica-coated endothelial cell plasma membranes and little contamination from other tissue components. P displayed ample enrichment for various endothelial cell surface markers relative to the starting whole lung homogenate (H) while being markedly depleted in markers of intracellular organelles or other cell surfaces. The caveolae attached on the cytoplasmic side of the membranes opposite to the silica coating were stripped from these membranes by shearing during homogenization at 4°C. Then they were subjected to sucrose density centrifugation to yield a low buoyant density fraction of intact caveolar vesicles (V) well separated from the pellet containing resedimented silica-coated membranes stripped of the caveolae (P-V). V was enriched in caveolar markers and markedly depleted of molecules found elsewhere in P (21).

Characterization of an Antibody Reactive with Caveolin Cage of Caveolae—As part of the development of a process for isolating and analyzing caveolae, we previously utilized various antibodies with magnetic beads to immunosolate from V those caveolae containing specific antigens mapped to caveolae by Western analysis of V (19, 20, 22). For instance, we used VAMP antibodies to pull down the caveolar vesicles in V and then examined the immunosolated vesicles for caveolin by Western analysis (19). In doing so, we were able to confirm that a specific protein such as VAMP did indeed exist in the same caveolar cage containing caveolin. The problem was that we could not do the complementary experiment (caveolin-based immunoisolations followed by Western analysis for the other protein) because the available antibodies to caveolin did not react effectively with oligomerized caveolin on intact caveolae. Over the last 4 years, we tested many antibodies to caveolin without success. This disappointing trend was broken early in 1997 when we tested a new monoclonal antibody just released commercially by Transduction Laboratories.
As shown in Fig. 1, unlike various other antibodies, monoclonal antibody clone 2234 that we call CAV not only is specific for caveolin but also binds caveolin in its native state as an oligomeric structural cage surrounding intact caveolae. We found many antibodies for caveolin including CAV that were quite specific by Western analysis (see Fig. 1A for some of the antibodies tested). Fig. 1B shows that CAV only recognized the upper band (α-isoform) on the immunoblot of V, whereas two bands are readily apparent with the polyclonal antibody. Previous work (37) showed that CAV specifically binds the α-isoform of caveolin-1 via a specific epitope found in the N-terminal segment that is not present in the β-isoform.

Next we examined the ability of CAV to react with caveolae stripped and isolated from the silica-coated endothelial cell plasma membranes. CAV bound to intact caveolae in V to allow immunoisolation with the magnetic beads. Fig. 1C shows several immunoisolations. By using CAV, we found caveolin only bound to the immunobeads and not in the unbound fraction. The nitrocellulose filters stained with Ponceau S showed that all of the proteins detected in V fractionated in the bound rather than unbound fraction (data not shown; confirmed using more sensitive staining (see Fig. 7)). In contrast, the other caveolin antibodies were ineffective with nearly all of the caveolin and protein signal detected in unbound fraction. Consistent with our ELISA results described below, the polyclonal antibody was poor but partially effective for immunoisolation.

As shown in Fig. 2, kinetic and binding analysis indicated rapid and high affinity binding of CAV to caveolin in the silica-coated endothelial cell plasma membranes (P) that have numerous attached intact caveolae. Titration of antibody concentration showed saturable binding reaching a maximum at about 10 nM (Fig. 2A). Time course studies (Fig. 2B) revealed rapid attainment of binding equilibrium in just 1 h. Scatchard analysis (Fig. 2C) indicated a binding constant of 2.5 nM. Other antibodies recognizing caveolin specifically by Western analysis (Fig. 1) showed little to no binding to P (Fig. 2A). The polyclonal antibody showed minor reactivity (probably low association rate/affinity) but only at much higher concentrations and at levels insufficient for immunoisolation (Fig. 1C). These other antibodies bound denatured caveolin but apparently not native caveolin in P.

These results cumulatively indicated that CAV is a high affinity antibody reacting quite rapidly and specifically with caveolin not only in its monomeric state after denaturation during Western analysis but also in its native oligomeric state surrounding intact caveolae found in P and V. Thus, CAV might be useful in immunoisolating caveolae from tissue and cell subfractions.

Comparison of Sonication Versus Shearing for the Isolation of Low Density Vesicles from the Silica-coated Plasma Membranes—Because caveolin can also be found intracellularly, for instance in trans-Golgi transport vesicles (4), starting with isolated plasma membranes is a logical first step in purifying caveolae from cells or tissues. The two approaches used for isolating plasma membranes before the caveolae have been the silica-coating technology and Percoll gradient centrifugation. The caveolae are dislodged from the plasma membranes by either sonication or shearing and then isolated by density centrifugation. Here, we first compared the effects of these two distinct types of mechanical disruption on fractionating silica-coated endothelial cell plasma membranes purified directly from rat lung tissue (see Fig. 3 for summary of membrane processing). In the next section, we examined the caveolar fraction derived from plasma membranes isolated using Percoll gradients.

It first should be noted that the silica-coated endothelial cell plasma membranes (P), as reported previously (see review Refs. 21 and 36), were quite enriched in caveolin and known endothelial cell surface proteins such as endothelial nitric oxide synthase (eNOS), urokinase-plasminogen activator receptor (uPAR), and angiotensin-converting enzyme (ACE) (>10-fold) but were depleted by >10-fold in proteins distinctive for other parts of the cell or tissue including e-COP, an endosomal and Golgi marker. These findings were consistent with a reasonable purification of the desired plasma membrane in P, thereby minimizing contamination from intracellular compartments.
such as the Golgi, which might complicate the analysis by contributing to the low density, caveolin-rich vesicular fraction that is isolated.

We found that both sonication and shearing were quite effective in not only removing the caveolae from P (>80% loss in caveolin signal in the resedimented silica-coated membranes [data not shown]) but also yielding a caveolin-enriched, low density vesicular fraction after continuous sucrose density centrifugation. Fig. 4A shows the Western analysis under equal protein loads. Relative to P, the low density fraction released by shearing (V) and by sonication (PC) were both quite enriched in caveolin by 10-fold. Although PC and V had many proteins in common (eNOS, G proteins, annexin II, and protein kinase C α), many other proteins enriched in P were readily detected in PC but not V, including the cytoskeletal protein β-actin, ACE, and the GPI-anchored protein uPAR. Neither e-COP nor the GPI-anchored protein 5′ nucleotidase (5′ NT) was apparent in V nor PC. Thus, V was quite distinct in molecular composition from P and PC.

Silver-stained SDS-PAGE gels further confirmed the differences between the fractions. Fig. 4B shows that each fraction (H, P, V, and PC) had its own distinct protein profile. V had the simplest profile, with the fewest detectable proteins, whereas PC had more protein bands than V but less than P. Although PC contained many, if not all, of the proteins apparent in V, it also contained several proteins found in P but not in V. Thus, both SDS-PAGE and Western analysis showed that sonication and shearing of P yielded a collectible vesicular fraction with the same low buoyant density but with quite different molecular composition. It appeared that sonication of P released additional low density vesicles not found in the shear-released vesicles V.

**Immuinoisolation and Characterization of Caveolae**

- **Fig. 3. Flow diagram of the subfractionation process to isolate PC and V fractions.**

- **Fig. 4. Protein analysis of low buoyant density membrane vesicles dislodged from the silica-coated plasma membranes by shearing versus sonication.** Protein analysis was performed on the rat lung homogenates (H) and silica-coated endothelial cell plasma membranes (P) as well as the low density fractions isolated after either shearing (V) or sonication (PC) of P (see Fig. 3 and “Experimental Procedures”). Different protein composition of PC and V is readily apparent by both Western analysis with antibodies to the indicated proteins (A) and SDS-PAGE followed by silver staining of the gels (B). The indicated fractions were analyzed equivalently under conditions of equal protein loading of the gels (5 μg per lane). Results shown in A and B are representative of three or more experiments (n ≥ 3).

- **Immuinoisolation of Shear- and Sonication-released Vesicles with Caveolin Antibodies—**We subjected PC and V to identical immunoaffinity isolation for 1 h using CAV bound to the magnetic beads. The starting material and the bound and unbound fractions were examined by Western analysis first to assess the relative distribution of the molecule in these fractions under equivalent volume conditions (the final volumes were equal in each fraction, and the same volume of each fraction was added to the gel lanes to maintain the same proportions).

Fig. 5 shows that although ample signals for each of the assessed molecules were seen in the starting material from PC, only a subset of these molecules were found in the vesicles bound to the caveolin antibody beads. Caveolin gave the strongest signal in bound fraction relative to both unbound fraction and starting material. Protein kinase C α as well as to a lesser extent eNOS and Gαs, were detected bound to the CAV beads, yet significant levels of each remained behind in the unbound fraction. Little to no signal for annexin II, β-actin, and uPAR was detected bound to the beads; each protein remained nearly completely in the unbound fraction. Thus, the sonication-released PC fraction contained a heterogeneous population of vesicles with the same buoyant density.
The results with the shear-released fraction V differed significantly as shown in Fig. 5. In each case tested where a signal was present in the starting material of V, very little to no signal was found in the unbound fraction, even when the film was overexposed and the signal in the starting material and bound fraction were much past saturation. Quantifying by scanning densitometry (using linear range exposures without signal saturation on the film and averaged for 3 or more experiments) confirmed that more than 95% of the signal in V for caveolin, G proteins, eNOS, and protein kinase Cα was recovered bound to the CAV beads. uPAR and β-actin were not detected in any of these fractions. Thus, nearly all of the caveolin in V was accessible and able to interact with the CAV immunobeads, resulting in quantitative isolation of nearly all of the starting material. This is in contrast with the results when PC was used as the starting material, where a significant amount of caveolin appeared inaccessible to immunoisolation.

To be certain that proteins in PC, for which a mild to nil signal was detected bound to the CAV beads, were not simply diluted out by maintaining equivalent volume conditions during the analysis, we loaded equal amounts of protein from each fraction onto the gels before Western analysis. Now a signal for eNOS, β-actin, and the GPI-anchored proteins eNOS, uPAR and 5'NT and uPAR, again consistent with past reports (24). Although not enriched, molecules such as annexin II as well as e-COP were easily detected in AC. As shown in Fig. 6B, the proteins detected in silver-stained gels for AC and PM were similar but clearly not identical.

We also subjected AC to CAV immunoisolation. Western analysis under equal protein conditions revealed that many proteins originally detected in AC did exist in the immunoisolated caveolin-coated vesicles (i.e. the caveolae), whereas many other proteins in the starting material were not constituents of these vesicles. Caveolin was found enriched in the bound fraction relative to unbound fraction. eNOS was clearly detected bound to the CAV beads, with little signal remaining behind in the unbound fraction. Annexin II partitioned rather equally between the three fractions. Conversely, the GPI-anchored proteins 5'NT and uPAR, as well as the Golgi/endoosomal marker e-COP and the cytoskeletal protein β-actin were not readily detected in the bound caveolae but rather remained behind in the unbound fraction. To be certain that the GPI-anchored proteins did not dissociate from their membranes into solution, we subjected the unbound fraction to centrifugation and found the GPI-anchored proteins in the membrane pellets (data not shown). Thus, CAV immunoisolation was useful in isolating the caveolin-coated caveolae in AC from other similarly buoyant membrane microdomains including those rich in GPI-anchored proteins and/or cytoskeletal proteins. From these and earlier results, we concluded that the caveolae, as isolated, can indeed be rich in signaling molecules while being markedly depleted in GPI-anchored proteins.

CAV Immunoisolates Nearly All Proteins and Caveolae in V but Not PC or AC—The low density, caveolin-rich vesicles isolated from each fractionation procedure (V, PC, and AC) were subjected identically to CAV immunoisolation for 1 h before performing SDS-PAGE as well as Western analysis for caveolin on the immunoseparated fractions. Fig. 7 shows that levels of e-COP, indicating the presence of contaminating Golgi and/or endosomal membranes. We then subfractionated PM as in Ref. 24 by sonication to produce small vesicles that are isolated by centrifugation, first as a broad band on a continuous Opti-Prep gradient hopefully to achieve selective separation and then on a step gradient to concentrate the low density buoyant vesicles. The resulting AC fraction was quite enriched in caveolin and eNOS (>5-fold) which is consistent with past reports (12, 24). It also was mildly enriched in β-actin and the GPI-anchored proteins 5'NT and uPAR, again consistent with past reports (24). Although not enriched, molecules such as annexin II as well as e-COP were easily detected in AC. As shown in Fig. 6B, the proteins detected in silver-stained gels for AC and PM were similar but clearly not identical.
nearly all of the membranes in V bound to the CAV beads. Nearly all the caveolin as well as other proteins detected originally in V fractionated bound to the CAV beads with little to none remaining behind in unbound fraction. Except for some bands coming from the IgG heavy and light chains and BSA (included by the manufacturer with the beads), the caveolin signal and protein profile of the starting material from V was identical to the bound fraction. Conversely, when we replaced CAV with a clathrin antibody as a control, little to none of the proteins and caveolin was detected in the bound fraction but remained nearly completely in the unbound fraction.

For the sonicated subfractions PC and AC, the results were strikingly different. Proteins were readily detected in both the bound and unbound fractions, consistent with the presence of significant populations of low density vesicles that were not reactive with the caveolin antibody. To be certain that these results were not caused by limited antibody, we increased the amount of antibody and immunobeads severalfold, only to get the same results (data not shown). The protein profiles of the bound and unbound fractions could be quite different; yet, the proteins detected bound to the CAV beads from PC and AC were very similar to each other, as well as to those seen in V (before and after immunoisolation). Thus, CAV was able to isolate a biochemically equivalent and consistent caveolar fraction from various different low density fractions including those with a heterogeneous population of distinct vesicles rather than just a homogeneous population of caveolin-coated vesicles.

This analysis agreed with the Western analysis (Figs. 5 and 6 as discussed above). Many molecules found in the sonication-released vesicles (either PC or AC) were split between the bound and unbound fractions, whereas all molecules detected in the shear-released vesicles (V) bound to the CAV beads, with little to none staying in the unbound fraction. The same proteins detected in V (before immunoisolation as well as after) were found in the caveolae immunoisolated from PC and AC. These results indicated that V, but not AC nor PC, contained a heterogeneous population of isolated caveolin-coated caveolae, not requiring immunoisolation to achieve further purity. For PC and AC, CAV immunoisolation was a necessary additional step to isolate caveolae to a higher level of purity.

The cytosol to the membrane, we tested for dissociation from the caveolae with time. First, we compared overnight versus 1-h incubations, both at 4 °C. As shown by Western analysis in Fig. 8, caveolin was almost completely detected in the bound fraction from both incubations, yet eNOS and Gαi were in the bound fraction after 1 h but largely absent from it after overnight incubation. Although eNOS and Gαi were initially a part of the floating low density membranes in V, the overnight incubation caused a substantial release of these signaling molecules from the caveolae apparently into solution. Hence, it was fortunate that the affinity of CAV was sufficient and the binding rapid enough that it was completely unnecessary to extend the incubation time beyond 1 h (see Figs. 1, 2, and 7).

This apparent dissociation was confirmed in experiments performed in the absence of antibody where we placed equal aliquots of P in solution for minutes to 16 h at 4 °C before sedimenting the membranes at 100,000 × g and processing the nonparticulate supernatants and membrane pellets for Western analysis. As early as 2–4 h, we detected a loss in eNOS and Gαi but not caveolin from the pelleted membranes, with a concomitant gain in the supernatant (data not shown). At 1 h or less, all of the signal detected for these three proteins was in the membrane pellet. Thus, with time, these signaling molecules dissociated from the membrane into solution. These findings provide at least a partial explanation for why various signaling molecules such as eNOS and G proteins were not readily detected in caveolae immunoisolated using overnight incubations as reported previously (34).

Finally, in testing the sodium carbonate procedure for isolating caveolae (23), we found even more dissociation of molecules from the membranes into solution upon sonication in the presence of sodium carbonate at high pH (less at pH 7 without sodium carbonate). We expected various peripheral membrane proteins, such as cytoskeletal proteins, to be removed from the membranes but were surprised at the significant membrane dissociation of lipid-anchored proteins but not caveolin (data not shown). Thus, to avoid this complication, we decided not to pursue this methodology further as a source for starting material for CAV immunoisolation.

Simplified Immunopurification of Caveolae from Plasma Membranes Not Coated with Silica—Based on the above results, we developed a simplified approach to isolating caveolae from cells and tissues not requiring the use of the silica-coating procedure (see “Experimental Procedures”). First, a plasma membrane-enriched fraction equivalent to PM was isolated from tissue and cell homogenates by standard centrifugation techniques using Percoll gradients (36). This PM was subjected to vigorous sonication and then loaded onto a continuous Optiprep gradient for centrifugation. We found that it was not advisable to collect the rather large number of fractions over a wide range of densities used to get AC (about half of the gradient volume) but rather collect only the top 20–25% of the gradient. This caveolin-rich fraction, which contained much less e-COP and GPI-anchored proteins than vesicles floating at higher densities (data not shown), was then subjected to CAV
immunoisolation. This procedure yielded purified caveolin-1-coated caveolae (OC) appearing equivalent in molecular composition to those derived by CAV immunoisolation of PC, V, and AC (see Table I). This procedure avoided an additional Opti-Prep centrifugation step in the AC isolation that essentially concentrates a wide density range of vesicles at the step gradient interface, and as shown in Fig. 6 yields an isolate rich in e-COP.

**DISCUSSION**

The monoclonal antibody CAV recognizes caveolin-1 with high affinity as a monomer as well as an oligomer surrounding intact caveolae. CAV immunoisolation provides in some cases a critical additional step to better isolate caveolae from low density, caveolin-enriched fractions. It also provides a useful test for assessing the purity of caveolar isolates. Shearing of the silica-coated endothelial cell plasma membranes (P) produces a low density caveolar isolate (V) that is different in molecular composition to the low density vesicles released by sonication (both PC from the silica-coated plasma membranes and AC from the Percoll-isolated plasma membranes). They (V, PC, and AC) are all quite enriched in caveolin but differ considerably in protein profile. Yet, when the caveolin-coated caveolae are immunoisolated from these fractions by using magnetic beads coated with CAV, the same molecules are found in V and in the immunoisolated fractions from PC, V, and AC (see Table I and Fig. 7). After sonication of plasma membranes, whether silica-coated or not, CAV immunoisolation becomes a necessary step to separate the caveolae away from other membranous components in the low density vesicular isolates. CAV immunoisolates caveolae that lack many proteins prevalent in the original sonication-released low density vesicles (PC and AC) such as the cytoskeletal protein β-actin, GPI-anchored proteins (SNT or uPAR), and in the case of AC, the Golgi/endosomal marker e-COP (Fig. 5–7). In contrast, CAV appears to isolate all of the membranes, proteins, and caveolae detected in V (Figs. 5, 7, and 8). Subjecting V to CAV immunoisolation provides no apparent change in molecular composition and thus no further enhancement in purification. V appears to be biochemically identical to the caveolae immunoisolated from AC, PC, and V as well as from the new simplified procedure (OC) (see Table I). Hence, V appears to contain a homogeneous population of caveolin-1-coated caveolae.

Sonication effectively dislodges, even from the silica-coated membranes, multiple microdomains of similar buoyant density and not just caveolae. Consistent with past reports (13, 18, 21, 36, 38), shearing is more selective in stripping away caveolae without appreciable disruption of the plasmalemma proper that is stabilized by the rather uniform coat of adherent silica particles. In retrospect, these findings may be expected because of the different nature of the two forces used on the membranes. Sonication creates alternating waves of expansive forces within the solution that evidently separates more of the plasma membrane from the silica coating, thereby allowing released noncaveolar regions to float into the low density fraction. Homogenization produces fluid shearing that cuts off the parts of the membrane that extend out away from the membrane as balloon-like invaginations directly exposed to the brunt of the shear forces and not supported by direct attachment to the silica coating. Interestingly, after sonication, some caveolin cannot be immunoisolated using CAV, probably because of the association of a subpopulation of caveolae with released membrane regions that vesiculate and thereby prevent access of the antibody. For instance, we have found in preparations also containing both GPI-anchored proteins and caveolin that caveolae can be found inside other larger vesicles (13).

Caveolin-1 oligomerizes to form a structural cage around the bulb of caveolae and may be necessary for the formation of caveolae (3, 39–41). Our experiments here provide direct evidence that most, if not all, caveolae found on the rat lung microvascular endothelium in vivo are coated with caveolin-1. Shearing of P can release nearly all of the caveolar invaginations detected by electron microscopy as well as reduce the caveolin signal by >90% (13, 18). All of the caveolin-1 in V is readily available for quantitative and nearly complete immunoisolation using CAV but not control antibodies. It appears that the homogeneous population of caveolin-1-coated caveolae in V (as discussed above) is representative of caveolae existing on the rat lung endothelial cell surface in vivo. Moreover, because CAV is specific for the α-isofrom of caveolin-1 and both α- and β-isofroms are found in CAV immunoisolated caveolae (see Fig. 1, B and C), it is evident that most, if not all, rat lung endothelial caveolae contain the α-isofrom and that both isofroms can exist in the same caveola. Because V also contains caveolin-2 but not caveolin-3 and caveolin-2 is easily detected in the CAV isolated caveolae, it appears that caveolin-1 and -2 can indeed exist in the same caveola in vivo.

Just like CAV, antibodies to dynamin, which may be another structural protein of caveolae, can immunoisolate nearly all of the proteins, caveolin and caveolae in V (20). Dynamin oligomerizes to form a collar around the neck of caveolae that upon activation hydrolyzes GTP to mediate the fission of caveolae from the plasma membrane and thereby release discrete transport vesicles (20). Antibodies to other proteins found in caveolae such as VAMP and albondin only isolate about 30–50% of the caveolin in V (19, 21, 38). Phosphotyrosine antibodies normally can immunoloaize 10–20% of the caveolae in V. This percentage increases to 50% after ligand-specific signaling in situ with platelet-derived growth factors (22) and even more to >90% with in situ mechanical stimulation by increasing vascular flow and pressure (35). Thus, subpopulations of vaeolae may exist within a cell type and molecules other than the caveolins may distinguish these subsets (see above). Key structural molecules such as caveolin and dynamin are probably present in all mature, fully formed caveolae, whereas other proteins, which are involved in more specialized caveolar func-

**Table I**

**Summary of proteins found in various plasma membrane and caveolae-containing low density fractions before and after immunoisolation with caveolin antibodies.**

| Molecule | AC | PC | V | V | PC | AC | OC |
|----------|----|----|---|---|----|----|----|
| Caveolin | +  | +  | + | + | +  | +  | +  |
| G proteins | +  | +  | + | + | +  | +  | +  |
| eNOS | +  | +  | + | + | +  | +  | +  |
| PKCa | +  | +  | + | + | +  | +  | +  |
| Src-like kinases | +  | +  | + | + | +  | +  | +  |
| Annexin II | +  | +  | + | + | +  | +  | +  |
| ACE | +  | +  | + | + | +  | +  | +  |
| Urokinase-PAR | +  | +  | + | + | +  | +  | +  |
| 5′-Nucleotidase | +  | +  | + | + | +  | +  | +  |
| β-Actin | +  | +  | + | + | +  | +  | +  |
| e-COP | +  | +  | + | + | +  | +  | +  |
| Clathrin | +  | +  | + | + | +  | +  | +  |

*Equal protein loads are used.*
tions such as signaling, mechanotransduction, or trafficking, may be relegated to a subset of caveolae.

Although GPI-anchored proteins are abundant in the low density, caveolin-enriched vesicular fractions isolated after sonication of the plasma membranes (as reported previously (24), CAV immunoisolation with these fractions yields a bound fraction consisting of caveolin-coated caveolae that are markedly depleted in GPI-anchored proteins and an unbound fraction rich in GPI-anchored proteins. Unfortunately, our attempts so far to use antibodies to various GPI-anchored proteins to immunoisolate GPI-rich vesicles from these fractions have failed, possibly because of poor accessibility. Nevertheless, it is clear that the tested GPI-anchored proteins are not normally concentrated in the isolated rat lung endothelial caveolae but rather exist in different microdomains. It is noteworthy that these distinct GPI-anchored protein microdomains actually do not require detergents for their formation and isolation. These results agree with our past findings (13, 25) but have been derived under different conditions, in this case from both silica-coated and naked plasma membranes. By performing the experiments in the absence of both detergents and silica coating, we thereby avoid concerns about artificial molecular redistribution induced by either the detergent or the silica particles. GPI-anchored proteins are also lacking in caveolae induced by GTP to bud under physiological conditions from the plasma membranes (either silica-coated or not) (20, 25). With high resolution immunofluorescence confocal microscopy, we find GPI-anchored proteins on the surface of cultured endothelial cells and fibroblasts in very small microdomains that are distinct from caveolin-coated caveolae (42). Antibody cross-linking of GPI-anchored proteins appears to cause their sequestration to caveolae and colocalization with caveolin (42, 43).

Because CAV immunoisolates caveolae coated specifically with caveolin-1, one must consider whether other caveolae may exist in our preparations that are not coated with caveolin-1 and contain GPI-anchored proteins. This possibility seems incongruous with the available data. As discussed earlier, all caveolae on the rat lung endothelial cell surface appear to be coated with caveolin-1. Moreover, electron microscopy shows that very few caveolar invaginations remain attached to the silica-coated plasma membranes after shearing (13, 18), and Western analysis using different caveolar markers (not just caveolin) provides further confirmation (13, 18, 20, 21, 36). Yet, the GPI-anchored protein content remains nearly constant in these sheared plasma membranes now lacking discernible caveolar invaginations (13, 36). These findings agree well with studies (14, 15) showing the presence of detergent-resistant GPI-anchored protein domains in cells not apparently expressing caveolin nor having morphologically distinguishable caveolae. Also keep in mind that other microdomains containing supramolecular complexes such as adhesion sites or cytoskeleton tethering sites at the plasma membrane can float with the detergent-resistant membranes and therefore are also likely to be present in the floating, low density vesicles released by sonication.

Of course, one can broaden the definition of caveolae from its intended, morphologically based derivation (literary meaning “cave-like structures”) to include possible flat domains which in the silica-coated membranes may be resistant to shearing and thus cannot be in V. One can envision that some of these flat domains may be coated with caveolin whereas others are not; both or either flat domain may be rich in GPI-anchored proteins. Past work (13, 18, 36) shows the following: (i) the silica coating is rather uniform over the plasma membrane, interacting with all flat noninvaginated regions of the membrane; and (ii) the GPI-anchored proteins reside in domains resistant to shearing and interact with the silica. The shear-independent caveolin signal remaining on the membrane after shearing may reflect caveolin-coated flat domains, possibly newly forming caveolae. Relative to the mature, shear-sensitive invaginations, the prevalence of this structure on the rat lung endothelial cell surface in vivo is low, as suggested by the much weaker caveolin signal detected after shearing. Like caveolar invaginations, such caveolin-coated flat domains are probably not the primary sites for concentrating GPI-anchored proteins because low density vesicles, which can be isolated from the sheared plasma membranes (P-V), are enriched in GPI-anchored proteins but lack caveolin (13). Thus, we prefer at this time to maintain what appears to be a very natural distinction by continuing to label the caves caveolae while using G domains as a name for the GPI-anchored protein domains that do share some characteristics with caveolae but ultimately differ in molecular composition, morphology, and probably derivation.

It has become evident over the last few years that starting with isolated plasma membranes is helpful in purifying caveolae and minimizing potential contamination from other sources (13). For instance, low density vesicles from the Golgi may contain caveolin and GPI-anchored proteins (4). Here as well as in earlier work (for review see Ref. 21), we find that relative to the starting homogenates, the silica-coating methodology isolates membranes consistently enriched by more than 10-fold in key endothelial cell surface proteins such as 5’NT, ACE, and caveolin while being more than 10-fold depleted in nonplasmalemmal markers such as the Golgi/endosomal marker e-COP. This level of purification contrasts with one report (34) where the silica-coated endothelial cell plasma membranes used for caveola isolation exhibited little caveolin and eNOS enrichment and yielded a heterogeneous low density caveolar fraction after centrifugation. The presence of non-plasma membranes in the starting material before further subfractionation may complicate the analysis and increase the impurity of the low density vesicles isolated before and possibly even after the immunoisolation step. This issue is especially relevant for caveola derived from plasma membranes isolated by Percoll gradient centrifugation (called the PM fraction here). Golgi elements as well as other intracellular membranes are present significantly in such preparations as shown here and previously (25, 36) by the significant signal for e-COP in PM (Fig. 6) as well as forewarned in textbooks on membranes and subcellular fractionation (44, 45).

The significant presence of caveolin in the Golgi of many cells, at least in culture, is likely to complicate further the analysis and immunoisolation of caveolae from such cells. This potential problem does not appear to be significant in the rat lung tissue used in our studies because we find by immunogold electron microscopy that nearly all the caveolin is associated with the caveolae of the endothelium and epithelium with little to none detected in intracellular organelles such as the Golgi.3 This more restrictive subcellular distribution for caveolin may have greatly assisted us in effectively immunoisolating caveolae, especially from the PM fractions. Although the new simplified immunoisolation procedure described here appears to reduce Golgi contamination, even from the low density vesicles derived from a sonicated, Percoll-isolated plasma membrane fraction, it still may be difficult to purify caveolae from cells, which express large amounts of caveolin in the Golgi, without first starting with highly purified plasma membranes markedly depleted of Golgi elements. This potential complication needs to be checked for each specific cell type or tissue used.

It is important to be cognizant of the effects of any isolation

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3 D. McIntosh and J. E. Schnitzer, unpublished observations.
procedure on the final isolate. We have discovered here, as shown in Fig. 8, that the processing time can be very important because some signaling molecules dissociate from the caveolae into solution. An overnight incubation contributes to an artifactual loss of signaling molecules such as eNOS and G proteins in the caveolar immunosolubilates that is not seen in the 1-h incubations. This dissociation may be anticipated because many signaling molecules are not integral to the membrane and can translocate back and forth from the cytosol to membranes where they are attached by lipid-anchoring and/or binding to lipids or other membrane proteins such as caveolin (30, 31). Cautious concern with how this dissociation may contribute to lower signals in general must be entertained. This dissociation may be occurring throughout the fractionation process as follows: the dilution during homogenization, the centrifugation to isolate the plasma membrane, and the dislodging and isolation of caveolae. Molecules originally concentrated in caveolae may not in the end be resolved as enriched in the final isolate. Because of these concerns, a mass balance of specific molecules is helpful to estimate the degree of such losses through the processing. We have reported previously in our mass balances of signaling molecules that up to 50% of the signaling molecules in P are lost during the V preparation (22).

Although it is clear from the results presented here that caveolae isolated by either sonication or shearing do indeed contain many signaling molecules, the way in which one analyzes for the presence of these molecules can give very different results. The determination of the relative concentration of a molecule between separated fractions requires normalization to content which for membranes can be readily defined by equivalent protein loads. Conversely, analysis by volume equivalence allows an assessment of the relative distribution of a molecule between the fractions. Because maintaining volume equivalence may require significant dilution, especially of small volume isolates, it is not surprising as shown in Fig. 5 that signaling molecules barely detected under volume equivalent conditions are readily apparent when analyzed under equal protein loads.

To best characterize caveolae, one must use proper analytical and preparative conditions in order to avoid changes in signal that may come from inadequately purified plasma membranes, time-dependent molecular dissociation, and dilution from volume equivalent analysis. Such factors elucidate in part the basis of the lack of detection of key signaling molecules in caveolae immuno-isolated in one recent study (34). In retrospect, it is not really surprising that signaling molecules including ENOS and G proteins appeared absent from caveolae that had been immunosolated using an overnight incubation and analyzed by volume equivalent immunoblotting (34). It appears that the polyclonal rabbit antiserum, which was raised in this study to a peptide resembling, but apparently not identical to, the N-terminal region of caveolin, lacks sufficient affinity and binding rate to allow rapid immunosolubilization. Binding inhibition assays show that the peptide used as immunogen is only about 10-fold more effective than an irrelevant control (not scrambled) peptide in reducing antibody binding to caveolin (34).

Many of the molecules reported to be enriched in the shear-purified caveolae of V (13, 18, 19, 21, 22) have also been immunolocalized to caveolae in endothelium and other cells by electron microscopy including caveolin (3, 5), the cholera toxin-binding sialoglycolipid GM1 (46), plasmalemmal calcium ATPase (47), inositol 1,4,5-trisphosphate receptor-like protein (48), and dynamin (20). Recently, we have performed electron microscopy on immunogold labeled rat lung tissue and found ENOS to reside preferentially in the caveoleae of vascular endothelium but apparently not epithelium (33). Finally, a recent study (26) using immunogold electron microscopy to investigate the organization of G proteins at the plasma membrane reveals that Goi is associated rather equivalently with caveolae as well as unidentified flat microdomains. This finding agrees with subfractionation analysis here and in a past report (19) that Goi is present in caveolae but not exclusively or significantly enriched. Although not apparently the case for the G proteins tested so far (members of Goa, Gob, and Goc classes), it remains to be seen whether any other heterotrimeric G proteins reside concentrated in caveolae.

By using the techniques described here, we have verified the presence or even enrichment of other key signaling molecules in caveolae including Ras, Raf, nonreceptor Src-like tyrosine kinases, phosphatidylinositol 3-kinase, platelet-derived growth factor receptors, phosphoinositides, ceramide, and neutral sphingomyelinase activity (but not acidic as reported using the AC preparation (49)) (22). In addition, we find little to no p42/p44 mitogen-activated protein kinase either at the plasma membrane or in caveolae. A recent study (50) using the AC preparation reports the concentration of the complete Ras/Raf/mitogen-activated protein kinase pathway in caveolae including both unstimulated and activated Erk1/2. Although we can confirm their presence in the AC fraction, we find little to no Erk1/2 in the bound fraction after CAV immunosolubilization. These results appear consistent with other reports (51, 52) showing a cytosolic distribution of mitogen-activated protein kinase with nuclear translocation upon activation.

The CAV immunosolubilization procedure developed here appears quite useful in purifying caveolae from low density vesicular fractions rich in caveolin. A simplified procedure is provided for use on cells and tissue to isolate caveolae away from other potential contaminating membranes. Although this immunoaffinity approach is inherently rather expensive in nature, it does provide an objective and necessary testing criterion for determining those molecules that reside in caveolae versus others that may be present in possible contaminating vesicles. At a minimum, it provides a way to test and confirm biochemically the existence of specific molecules in caveolin-coated vesicles. These vesicles must represent caveolae whenever either caveolin is expressed only in caveolae and not significantly elsewhere in other membranes (e.g. Golgi) or such intracellular membranes are not significantly present in the starting plasma membrane fraction. Keeping in mind these limitations, one procedure is now available for all use. It should allow comparison of caveolae under more equivalent conditions. Hopefully, other antibodies will be generated in the future that allow caveolae coated with other caveolin family members to be similarly purified and comparatively analyzed.

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