Characterization of Caveolin-rich Membrane Domains Isolated from an Endothelial-rich Source: Implications for Human Disease

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Abstract. Caveolae are 50–100-nm membrane microdomains that represent a subcompartment of the plasma membrane. Previous morphological studies have implicated caveolae in (*a*) the transcytosis of macromolecules (including LDL and modified LDLs) across capillary endothelial cells, (*b*) the uptake of small molecules via a process termed potocytosis involving GPI-linked receptor molecules and an unknown anion transport protein, (*c*) interactions with the actin-based cytoskeleton, and (*d*) the compartmentalization of certain signaling molecules, including G-protein coupled receptors. Caveolin, a 22-kD integral membrane protein, is an important structural component of caveolae that was first identified as a major v-Src substrate in Rous sarcoma virus transformed cells. This finding initially suggested a relationship between caveolin, transmembrane signaling, and cellular transformation.

We have recently developed a procedure for isolating caveolin-rich membrane domains from cultured cells. To facilitate biochemical manipulations, we have applied this procedure to lung tissue—an endothelial and caveolin-rich source—allowing large scale preparation of these complexes. These membrane domains retain ~85% of caveolin and ~55% of a GPI-linked marker protein, while they exclude ≥98% of integral plasma membrane protein markers and ≥99.6% of other organelle-specific membrane markers tested. Characterization of these complexes by micro-sequencing and immuno-blotting reveals known receptors for modified forms of LDL (scavenger receptors: CD 36 and RAGE), multiple GPI-linked proteins, an anion transporter (plasma membrane porin), cytoskeletal elements, and cytoplasmic signaling molecules—including Src-like kinases, hetero-trimeric G-proteins, and three members of the Rap family of small GTPases (Rap 1—the Ras tumor suppressor protein, Rap 2, and TC21). At least a fraction of the actin in these complexes appeared monomeric (G-actin), suggesting that these domains could represent membrane bound sites for microfilament nucleation/assembly during signaling. Given that the majority of these proteins are known molecules, our current studies provide a systematic basis for evaluating these interactions in vivo.

CAVEOLAE (also known as plasmalemmal vesicles) are ~50–100-nm membrane domains that represent a sub-compartment of the plasma membrane. However, their specific morphology may vary widely, even within a given cell type; they may be flat or invaginated, expressed singly or in bunches like clusters of grapes (32, 76, 84). These structures are most abundant in simple squamous epithelia (e.g., capillary endothelial cells and type I pneumocytes), fibroblasts, smooth muscle cells, and adipocytes (18, 32, 35, 86). Although caveolae are thought to exist in most cell types, in vivo caveolar functioning has been most extensively studied in endothelial cells.

In capillary endothelial cells, numerous morphological studies indicate that caveolae function in the transcytosis of certain blood-borne macromolecules (including albumin, modified albumins, LDL, modified LDLs, and advanced glycosylation end products [AGE's][1]) (31, 38, 85, 89, 100, 105). As the endothelial caveolar transcytosis of lipoproteins is dramatically increased during hyperlipidemia, it has been proposed that this transport mechanism contributes to the sub-endothelial accumulation of LDL during atherosclerosis (85, 99). In light of these observations, it has been suggested

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1. *Abbreviations used in this paper*: AGE, advanced glycosylation end products; GPI, glycosyl-phosphatidylinositol; MBS, Mes-buffered saline; RAGE, receptor for advanced glycosylation end products; RSV, Rous sarcoma virus.

that endothelial caveolae may concentrate as yet "unidentified" scavenger receptors (defined as cell surface molecules that recognize modified LDL) (81), as the "classical" scavenger receptors are not expressed in endothelial cells (59). Recently, two novel scavenger receptors expressed by endothelial cells have been identified as CD 36 and receptor for advanced glycosylation endproducts (RAGE) (30, 68). Interestingly, both of these receptors can also act as mitogenic signaling molecules (30, 49, 68).

AGE formation (non-enzymatic glycosylation of serum proteins and lipids, including glycated LDL) and their cellular processing is thought to underlie the pathogenesis of diabetic vascular complications and normal aging (63). In diabetic states, elevated levels of serum glucose lead to accelerated AGE formation and their sub-endothelial accumulation (63). In accordance with these findings, AGEs undergo endothelial transcytosis via caveolae (31, 105), and, in diabetic animal models, both the size and number of endothelial caveolae increases at sites of diabetic microangiopathy (23). These results are consistent with the recent observation that RAGE—a lectin-like type I transmembrane protein—appears to be localized within endothelial cell caveolae (80).

Two additional functional roles for caveolae have been proposed from the study of other cell types. These include (*a*) the translocation of small molecules from the extracellular environment to the cell's cytoplasm via glycosylphosphatidylinositol (GPI) linked protein receptors coupled to an unknown anion transporter protein, in a process termed potocytosis (7, 8); and (*b*) as a sub-compartment of the plasma membrane involved in a subset of transmembrane signaling events (6, 73, 74, 79, 95).

Although the exact function of caveolae remains to be elucidated, several recent observations have led to a better understanding of their molecular components. Morphological studies indicate that caveolae concentrate certain classes of lipid-linked molecules (glyco-sphingolipids, cholesterol, and GPI-linked proteins), molecules that regulate calcium homeostasis (plasma membrane Ca^{2+} pump; IP_3-sensitive Ca^{2+} channel), and G-protein related signaling molecules (multiple G-protein-coupled receptors, G-protein modifying bacterial toxins and adenylate cyclase activity) (7, 36, 37, 43, 66, 71, 73, 74, 95, 102, 103).

In the case of the muscarinic acetylcholine receptor (a G-protein coupled receptor), redistribution to plasmalemmal caveolae was agonist-induced and reversible, but did not occur in response to receptor antagonists, consistent with the idea that these structures could participate in G-protein-coupled signaling (73). In addition, agonist-induced caveolar localization resulted in the local accumulation of cytoskeletal elements—tentatively identified as the actin-based cytoskeleton—in close contact with linear arrays of plasmalemmal caveolae (73). In further support of an association with actin, the cytoplasmic surface of caveolae is specifically labeled in situ with an actin-binding protein, gold-conjugated myosin subfragment I (51, 52) and dystrophin—an actin-binding protein implicated in the pathogenesis of Duchenne's muscular dystrophy—has been immunolocalized to caveolae in smooth muscle cells (69). In accordance with this localization, muscle cell caveolae undergo characteristic morphological changes in Duchenne's muscular dystrophy, but not in other forms of muscular dystrophy examined (14).

Caveolin, a 22-kD transmembrane phospho-protein (40, 42, 79), is an important structural component of caveolae and contributes to their characteristic granular cytoplasmic appearance (76). Immuno-gold localization of caveolin reveals that it is confined to plasmalemmal caveolae and to a small extent within discrete tubulo-vesicular structures in the vicinity of the *trans*-Golgi (29, 60). This may reflect the existence of caveolae at the level of the *trans*-Golgi network. Originally identified as the major v-Src substrate in RSV-transformed cells, the phosphorylation of caveolin on tyrosine residues is dependent on NH_2-terminal myristoylation of v-Src (40, 42). Similarly, myristoylation of v-Src is required to confer cellular transformation. Based on these studies, it has been implied that caveolin is the critical target for v-Src transformation. However, this observation has remained correlative. In light of the newly proposed view of caveolae as "signaling organelles," caveolin phosphorylation by v-Src could constitutively activate their signaling capabilities, leading to unregulated cell growth (6, 79).

As several caveolar membrane components (GPI-linked proteins, glyco-sphingolipids, and caveolin) are selectively resistant to solubilization by Triton X-100 at 4°C (20, 40, 42, 45, 48, 61), we recently investigated the origin and composition of low-density Triton-insoluble membrane domains isolated from tissue-cultured cells (79). The unusual Triton-insolubility of these domains has been attributed to their high glyco-sphingolipid content (20), as glycosphingolipids are intrinsically Triton-insoluble (45, 94). Our results indicated that these domains derive from the cell surface, are dramatically enriched in caveolin and a GPI-linked marker protein, but that they exclude ≥99% of total cell surface-labeled plasma membrane proteins and a battery of organelle-specific marker enzymes for non-caveolar plasma membrane, Golgi, lysosomes, and ER. Low-angle rotary shadowing of these domains revealed that they are ~50–100 nm in diameter and have a granular appearance. Similarly, by transmission EM, they are 50–100 nm in diameter or greater—depending on the cell type—and demonstrate a membrane bilayer. In accordance with the morphological observations that caveolae concentrate G-protein-coupled receptors and glycolipid binding bacterial toxins that modify G-proteins, these domains were specifically enriched in hetero-trimeric G-protein alpha subunits (79). In addition, in support of the observation that caveolin was first identified as a major v-Src substrate, these domains were also enriched in an endogenous Src-like kinase (c-Yes) and another v-Src substrate (annexin II) (79). As many similarities exist between these membrane domains and caveolae, we have suggested that these structures represent isolated caveolae (see [79] for discussion of this point). However, to distinguish these domains from caveolae seen in electron micrographs of intact cells, we have chosen the biochemically descriptive term, "caveolin-rich membrane domains," to refer to these complexes.

To systematically identify other components of these complexes, we have isolated caveolin-rich membrane domains from an endothelial-rich tissue source, i.e., lung, where caveolin demonstrates its highest known levels of expression. Our current characterization of these complexes reveals the identities of the major protein components of these membrane domains. It should be noted that although low-density Triton-insoluble membrane domains were initially isolated from cultured cells more than a decade ago (64), this is the first systematic characterization of their protein components. This first step should allow us and others to further evaluate

the relationship between caveolin-rich domains and caveolar functioning in situ.

Materials and Methods

Materials

Antibodies and their sources were as follows: caveolin, protein kinase C-α isoform, pan-ERK/MAP kinase, GRB-2, Vav, PI-3 kinase (p85), PTP-1D, annexin II- heavy chain, Ras-GAP, ISGF, SHC, PLC-γ, Rap 2 (Transduction Laboratories); CD 36 (Accurate); c-Src, Fyn (Oncogene Sciences, Manhasset, NY); c-Yes (gift of M. Sudol, The Rockefeller University, NY); Lyn, Lck, c-Fgr, Rapl, Rho A, Rho B, RB (Santa Cruz Biotechnology); JAK-2, Rap 1, Ras, casein kinase II (Upstate Biotechnology, Inc.); actin, non-muscle myosin II-heavy chain (Biomedical Technologies, Inc.); gelsolin (Sigma Chem. Co., St. Louis, MO); G protein-β common (Dupont/NEN, Wilmington, DE), Rab 3D (gift of Drs. G. Baldini and H. Lodish, Whitehead Institute), G protein-α subunits (gift of Dr. A. Spiegel, NIH, MD); Na^+K^+-ATPase, β-subunit (gift of Dr. M. Caplan, Yale University); p45, cell surface integral membrane protein (gift of Dr. J. F. Hare, Oregon Health Sciences University).

Isolation of Caveolin-rich Membrane Domains

Caveolin-rich membrane domains were prepared from mouse lung by a minor modification of an established protocol used for cultured cells (20, 79). Mouse lungs (one pair; 400 mg wet weight) were minced with a scissor and homogenized with 2 ml of Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl) containing 1% Triton X-100 and 1 mM PMSF. Homogenization was carried out initially with 10 strokes of a loose-fitting Dounce homogenizer, followed by a Polytron tissue grinder (three 10-s bursts; Brinkmann Instruments, Westbury, NY). The homogenate was adjusted to 40% sucrose by addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient (in MBS, lacking Triton X-100) was formed above the homogenate and centrifuged at 39,000 rpm for 16–20 h in a SW41 rotor (Beckman Instruments, Palo Alto, CA). A light-scattering band confined to fractions 4–7 is harvested, diluted threefold with MBS, and pelleted in the microfuge. Caveolin-rich membrane domains were snap frozen in liquid nitrogen and stored at −80°C. The majority of protein remained within the 40% sucrose region of the gradient (lower sucrose, ≥90% and insoluble pellet, ~8%). A similar procedure was also used to prepare caveolin-rich membrane domains from dog and human lung tissue.

Organelle-specific Membrane Marker Assays

Markers for caveolar plasma membrane (alkaline phosphatase, GPI-linked), non-caveolar plasma membrane (alkaline phosphodiesterase), Golgi (α-mannosidase II), lysosomal (β-hexosaminidase) and ER (α-glucosidase II) were assayed as described previously (17, 101). Note that these assay systems were not affected by the presence or absence of Triton X-100 in the initial homogenate.

Electron Microscopy

For routine transmission EM, samples were fixed with glutaraldehyde, postfixed with OsO_4, and stained with uranyl acetate and lead citrate, as described (79). For immuno-gold labeling of sections, samples were fixed in 2.5% paraformaldehyde in PBS (pH 7.2) for 2 h on ice. After washing with PBS, samples were dehydrated in graded dimethyl formamide and embedded in Lowicryl K4M resin. Thin sections were cut, incubated with anti-caveolin IgG (a 1:50 dilution) and bound antibodies visualized with 10-nm gold-conjugated secondary IgG (Zymed Labs., S. San Francisco, CA). Control experiments indicated that gold-labeling was specifically dependent on incubation with anti-caveolin IgG. Samples were examined under the Philips 410 TEM. Note that in order to preserve immuno-reactivity with anti-caveolin IgG (mAb 2234), it was necessary to omit fixation with glutaraldehyde and OsO_4, although these conditions are not optimal for structural preservation.

Microsequencing

For microsequencing, mouse lung caveolin-rich domains were fractionated using the detergent Triton X-114, that separates hydrophobic from hydrophilic proteins. Briefly, membrane domains were solubilized in Tris-buffered saline (10 mM Tris, pH 7.5, 0.15 M NaCl) containing 1% Triton X-114 plus protease inhibitors by incubation at 37°C for 20 min. This condition effectively solubilizes caveolin-rich membrane domains (79). After solubilization, extracts were partitioned into detergent-rich (D, detergent) and detergent-poor (A, aqueous) phases and proteins were collected by acetone and TCA precipitation, respectively. After separation by SDS-PAGE (10% acrylamide gels), proteins were transferred to Immobilon-P (Millipore, Bedford, MA) for NH_2-terminal sequencing and to nitrocellulose for internal sequencing. For internal microsequencing, bands were excised after Ponceau S staining and stored in Milli-Q water at −20°C. Excised bands were enzymatically digested with the endoproteinase Lys-C in the presence of 1% hydrogenated Triton X-100/10% acetonitrile/100 mM Tris-HCl, pH 8.0, for 24 h at 37°C (34). Eluted peptides were isolated by HPLC and subjected to NH_2-terminal sequencing, using a (model 447A/120A) pulsed-liquid protein sequencer (Appl. Biosystems, Foster City, CA). NH_2-terminal and internal peptide sequences were used to scan existing databases, using the programs ATLAS or BLAST.

Detection of G-Actin

Gc-Globulin (Calbiochem, Inc.) was radio-iodinated to a high specific activity using Iodogen as suggested by the manufacturer (Pierce, Rockford, IL). Isolated caveolin-rich membrane domains (~200 μg; in 200 μl MBS) were allowed to bind the probe for 1 h at 4°C, and unbound material was removed by centrifugation before flotation on sucrose density gradients. Control experiments omitting caveolae and competition experiments with excess cold Gc-Globulin demonstrated binding specificity.

Immunoblotting

Caveolin-rich membrane domains were solubilized with sample buffer and separated by a preparative SDS-PAGE mini-gel (10% acrylamide). After transfer, nitrocellulose sheets were cut into 3-mm strips and incubated with a variety of different primary antibodies in multi-well trays. Bound antibodies were visualized with the appropriate secondary antibody, as described by the manufacturer (Promega Corp., Madison, WI; Amersham, Buckinghamshire, England). Pertinent positives were used to estimate the relative enrichment of these markers in caveolin-rich membrane domains by immunoblotting of gradient fractions (1–8, 5–30% sucrose, 9–12, 40% sucrose, and 13, insoluble-pellet). Note that the insoluble pellet was solubilized using 10% SDS, repeated cycles of freeze-thawing, and, finally, dilution with sample buffer and boiling. Specific activities of caveolin-rich fractions were calculated (arbitrary units/mg protein) and compared with total cellular extracts, yielding fold-enrichments. Quantitation was performed with a Molecular Dynamics Inc. (Sunnyvale, CA) computing densitometer. To ensure that these estimates were made in the linear range, we (*a*) used multiple autoradiographic exposures and (*b*) monitored their linearity using the densitometer, essentially as described (62). As caveolin-rich domains are very abundant in lung tissue, a maximum fold-enrichment of only 50 can be obtained relative to total cell lysates. This maximum is calculated by assuming 100% of a molecule is confined to these domains, i.e., [(100/600 μg)/(100/30,000 μg)] = 50.

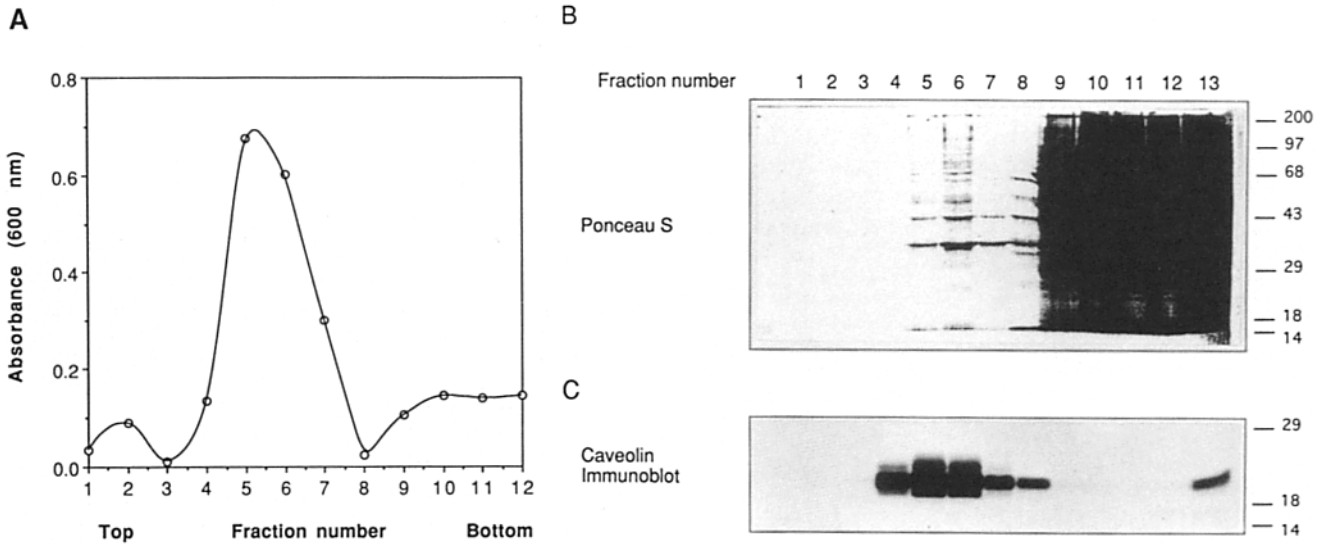
Northern Blot Analysis

poly $(A)^+$ RNA 1 (1 μg) prepared from a given murine tissue was separated on formaldehyde-agarose (1%) gels and blotted on Biotrans nylon membranes, UV-cross-linked, and probed with the ^{32}P-labeled caveolin or CD 36 cDNA. RNA preparation and hybridization conditions were as described previously (9, 79), except that blots were washed (2×; 30 min each) in 2× SSC/0.1% SDS at 50°C. cDNAs for canine caveolin and human CD 36 were cloned by PCR amplification of an MDCK cDNA library (79) or a human heart cDNA library (Stratagene, Inc., La Jolla, CA), using primers to the known sequences (60, 70). PCR products of the expected size were gel purified, cloned into pBluescript II KS^+, and their identity verified by double-stranded sequencing in both directions.

Results and Discussion

Isolation of Caveolin-rich Membrane Domains

We used an endothelial-rich source, lung tissue, in an attempt to isolate caveolin-rich membrane domains from whole tissue as caveolin—a caveolar marker protein—is most highly expressed in lung (41) (see also Fig. 7, this re-



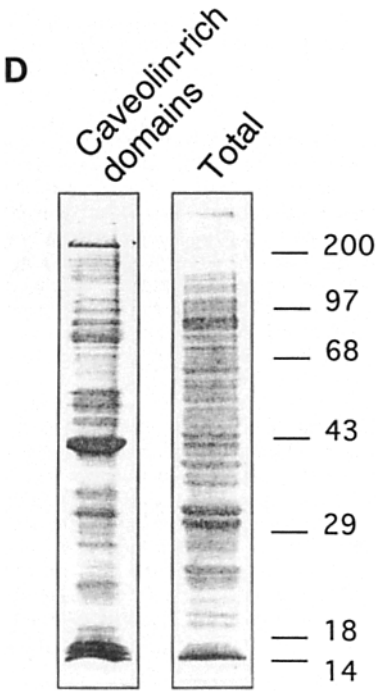


Figure 1. Isolation of caveolin-rich membrane domains. Sucrose gradient fractions were collected and analyzed by (*A*) light-scattering; (*B*) Ponceau S staining and (*C*) anti-caveolin immunoblotting. Fractions 1–8 represent the 5–30% sucrose layer, fractions 9–12 are the 40% sucrose cushion and fraction 13 is the insoluble pellet. (Fraction 13 was omitted from *A*, as it represents the insoluble pellet). Note that a single light-scattering band (fractions 4–7) contained ~85% of caveolin and excluded ≥98% of the cellular protein (based on independent protein determinations). (*D*) Comparison of caveolin-rich membrane domains with the total homogenate by Coomassie blue staining. Note that equivalent amounts of protein were loaded. Samples were delipidated by acetone precipitation before SDS-PAGE to allow better resolution of stained protein bands.

port). A possible explanation for the abundance of caveolin in lung may lie with the observation that caveolae are morphologically most abundant in simple squamous epithelia [defined as endothelial cells and type I pneumocytes (~5,000–10,000 caveolae per cell; (86)], smooth muscle cells (35), and fibrocytes (18)—the predominant cellular constituents of lung tissue. Quantitative morphometric estimates of the alveolar cell population indicate that >50% of the cells are simple squamous epithelia (~42% endothelial cells and ~11% type I pneumocytes) and ~35% interstitial cells that include primarily smooth muscle cells (pericytes), fibrocytes, and macrophages (90). Although the type I pneumocytes represent ~11% of the cell population, they are thought to cover 97–98% of the alveolar surface (~140 m^2 in humans), are twice the volume and four times the surface area of endothelial cells; endothelial cells cover roughly the same surface area. In contrast, type II pneumocytes occupy only 2–3% of the alveolar surface (90).

To this system, we applied our recently described procedure for isolating caveolin-rich membrane domains from cultured cells, which depends on the stringent criteria of (*a*) resistance to solubilization by Triton X-100 at 4°C and (*b*) buoyancy at a specific density in sucrose gradients. A single light-scattering band corresponding to a low-density, Triton-insoluble complex was observed mainly in fractions 5–6 of these gradients (the ~15–20% sucrose region; Fig. 1 *A*). The flotation and protein composition of this light-scattering Triton-insoluble complex was unaffected when 1% Triton

X-100 was included in the upper 5–30% sucrose layer, indicating that this Triton-insoluble complex does not "form" because of a lack of Triton in the upper portion of the gradient. These membrane domains excluded ≥98% of the total cellular protein which remained within the bottom-loaded 40% sucrose layer (fractions 9–13; Fig. 1, *B* and *C*), and their polypeptide composition was clearly distinct from the total homogenate (Fig. 1 *D*). A yield of ~600 μg was obtained from 30 mg of total protein—representing ~2% of the starting material.[2] As a yield of 0.05% was previously obtained from cultured MDCK cells (canine kidney origin; [79]), these complexes are ~40-fold mroe abundant in lung tissue. This is consistent with the observation that caveolin expression is highest in lung tissue and virtually undetectable in renal tissue (41) (see also Fig. 7, this report).

As compared with other gradient fractions, these complexes retained known caveolar marker proteins (~85% of caveolin and ~55% of alkaline phosphatase—a GPI-linked protein) and excluded ≥98% of three integral plasma membrane protein markers (alkaline phosphodiesterase, Na^+K^+-ATPase, and p45) and ≥99.6% of the other organelle-specific membrane markers tested (Fig. 2). Similarly, our previous findings with cultured cells indicated that MDCK-derived caveolin-rich domains exclude ≥99% of cell surface-labeled proteins and a battery of organelle-specific marker enzymes for non-caveolar plasma membrane, Golgi, lysosomes, and ER (79).

By transmission EM, caveolin-rich membrane domains appeared as ~50–100-nm vesicular structures and as membrane fragments that were often curved or U-shaped (Fig. 3 *A*). This variation in shape is in accordance with previous studies indicating that caveolae may be flat or invaginated and may exist singly or in bunches (76, 84); however, deeply invaginated caveolae are most conspicuous and appear as flasks attached perpendicularly to the plasma membrane. If these curved-domains or open-flasks were then dissociated from the plasma membrane and, therefore, no longer oriented perpendicularly to the plane of the membrane, sectioning of these structures would be expected to yield fields of 'sealed' vesicles and curved membrane fragments. This could also contribute to the apparent variation in shape we observe.

These structures were heavily labeled when thin sections were stained with anti-caveolin IgG, indicating that these complexes contain caveolin (Fig. 3 *B*). This is in accordance with our observation that ~85% of caveolin is recovered within these membrane domains. These results also demonstrate that the caveolin content of these domains is not contributed by a minor fraction of these complexes, as >90% of the discernable structures were immuno-labeled.

Characterization of Caveolin-rich Membrane Domains

To identify the major protein components of caveolin-rich membrane domains, we subjected these complexes to micro-sequencing. We first separated their components into hydrophobic and hydrophilic fractions by partitioning with the detergent Triton X-114 and subjected them to analysis by SDS-PAGE (Fig. 4). After Coomassie-blue staining, most proteins were found to partition with the detergent-rich phase. We identified a major 22-kD band as caveolin by immuno-blotting with mono-specific antibodies (not shown). As expected for an integral membrane protein, caveolin partitioned with the detergent phase. NH_2-terminal and internal sequencing of the major protein bands revealed multiple GPI-linked proteins, certain cell surface integral membrane proteins (CD 36, RAGE, plasma membrane porin and caveolin), a v-Src/pkc substrate (annexin II), GTPases (G α i subunits, Rap 1, TC 21, and Rab 5), putative luminal content molecules (albumin, SP-A, and osteopontin), and components of the actin-based cytoskeleton (Fig. 4; Table I). (A few novel protein sequences corresponding to 3 or more unknown proteins were also detected). These results support our enzyme-marker studies demonstrating that these domains exclude markers for non-caveolar plasma membrane, Golgi, lysosomes, and ER, but are enriched in a GPI-linked marker protein and caveolin (see above). As solubilized caveolin exists as a hetero-oligomeric complex (61) with other unknown integral cell surface proteins of 85, 50, 30–40, and 14 kD, CD 36 (85 kD), RAGE (45–48 kD), and plasma membrane porin (33 kD) could represent the other cell surface components of these immuno-isolated caveolin hetero-oligomers.

As monomeric actin has recently been visualized in intact cells as a plasma membrane–associated "beaded" structure by fluorescence microscopy with a specific probe (Gc-Globulin) that recognizes only G-actin (24), these structures might represent caveolae. This would explain our observation that actin is one of the most abundant protein components in caveolin-rich membrane domains. To test this hypothesis, we examined the binding of radio-iodinated Gc-Globulin to isolated caveolin-rich membrane domains. These structures specifically bound Gc-Globulin as determined by flotation in sucrose density gradients (Fig. 5), indicating that they contain G-actin. This is in accordance with the observation that monomeric actin beaded structures and caveolin are both concentrated at the leading edge of motile cells (24, 76). In addition, the cytoplasmic surface of caveolae can be specifically labeled in situ with an actin-binding protein, i.e., gold-conjugated myosin II-subfragment 1 (51).

In support of our results from micro-sequencing, immunoblotting of caveolin-rich membrane domains revealed a significant number of positives. These antibody studies confirmed the identities of a number of components we assigned based on micro-sequence analysis, i.e., CD 36, caveolin, G protein α i subunits, annexin II, Rap-1, actin, gelsolin, and non-muscle myosin II (Fig. 6). The identity of Rab 5 was also confirmed by immuno-blotting (not shown). Perti-

2. Empirically, we find that 400 mg of lung tissue results in ~30 mg of detectable protein, 7.5% of the wet weight. As protein represents ~15–22% of the wet weight of various organs, our yield caveolin-rich domains may be an overestimate due to an underestimate of the starting material. This difference may reflect the abundance of connective tissue components in lung that cannot be completely solubilized in SDS for protein determinations. If we recalculate our yield, using an estimated protein content of 15–22%, then these domains represent ~0.65–1%. Even with this "lower yield" caveolin-rich domains would still be 10–20-fold more abundant in lung than previously observed for renal epithelial cells, in accordance with tissue Northerns. In addition, this lower yield would also increase the fold-enrichments we observe by a factor of two-to-three, but would not change the amount of a given protein that we have expressed as a percentage of the total homogenate.

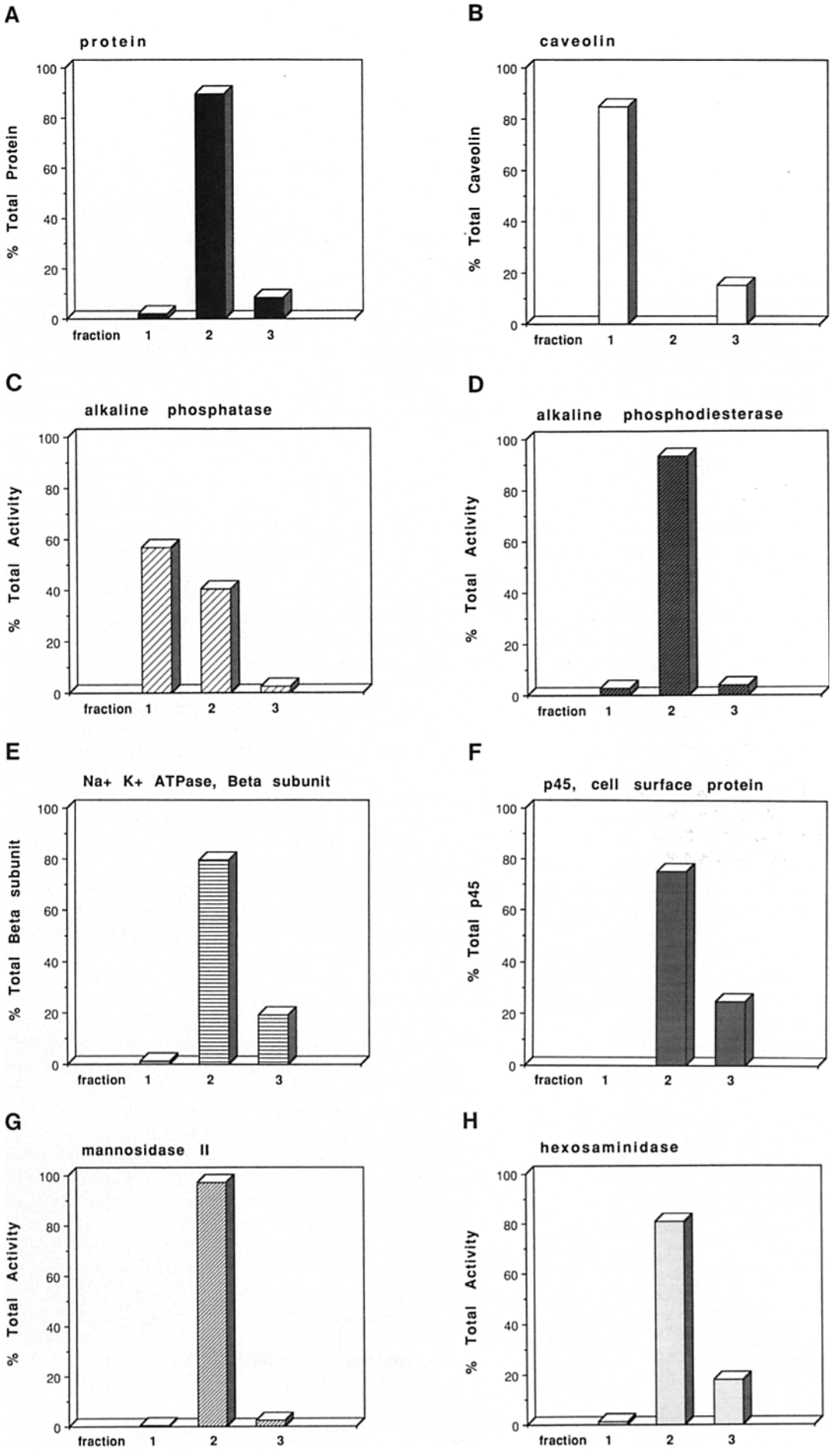


Figure 2. Continued next page.

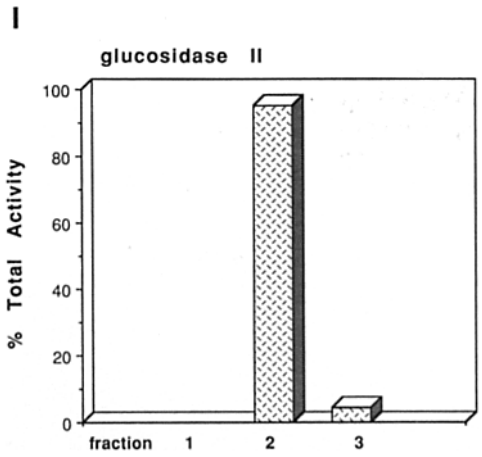


Figure 2. Profiles of organelle-specific membrane markers across the gradient. (*A*) Cellular protein; (*B*) caveolin; (*C*) alkaline phosphatase; (*D*) alkaline phosphodiesterase; (*E*) Na^+K^+-ATPase, β-subunit; (*F*) p45; (*G*) α-mannosidase II; (*H*) β-hexosaminidase; (*I*) α-glucosidase II. Fractions 1, 2, and 3 represent caveolin-rich membrane domains, the 40% lower sucrose layer, and the pellet, respectively. The sum of the protein/activity in these three fractions was equivalent to the amount present in total cell homogenates. Note that caveolin-rich domains (fraction 1) retain ~85% of caveolin and ~55% of alkaline phosphatase, and exclude ≥98% of integral plasma membrane markers (alkaline phosphodiesterase, Na^+K^+-ATPase, and p45) and ≥99.6% of the other organelle-specific membrane markers tested. When these results are expressed as fold-enrichments, caveolin-rich domains are enriched in caveolar marker proteins (caveolin, ~42.5-fold, and alkaline phosphatase—a GPI-linked protein, ~27.5-fold), while they exclude markers for plasma membrane (≤1-fold), Golgi (α-mannosidase II; ~0.1-fold), lysosomes (β-hexosaminidase; ~0.2-fold), and ER (α-glucosidase II; ~0.035-fold).

Note that p45 is a non-GPI-linked plasma membrane protein that can be labeled from the cell surface, partitions with the hydrophobic phase of Triton X-114 extracts, and is inextractable by washing with carbonate or NaOH, behaving as an integral membrane protein (46).

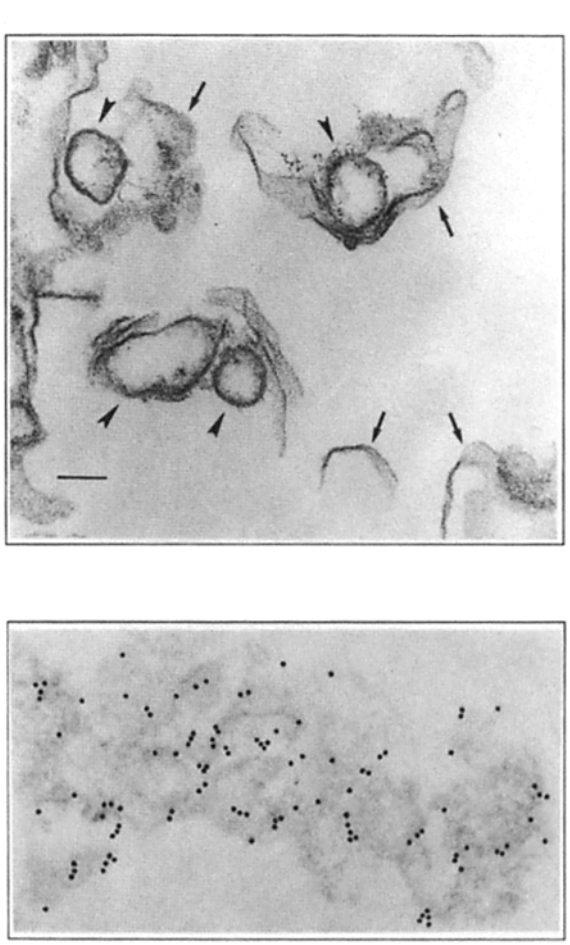


Figure 3. Visualization of caveolin-rich domains by transmission electron microscopy. (*A*) These domains appeared as ~100-nm vesicular structures (*arrowheads*) and as curved or U-shaped membrane fragments (*arrows*). Bar, 0.1 μm; (*B*) Caveolin-rich domains were fixed with paraformaldehyde and processed for immunolabeling with anti-caveolin IgG (see Materials and Methods). Bound IgG were visualized with a 10-nm gold-conjugated secondary antibody. Magnification is the same as in *A*. Note that in order to preserve immuno-reactivity with anti-caveolin IgG (mAb 2234), it was necessary to omit fixation with glutaraldehyde and OsO_4.

nent positives were used in studies of fractionated gradients to estimate whether these molecules were specifically enriched in caveolin-rich membrane domains, relative to total cellular extracts (Fig. 6, *A–G*). We find that CD 36, cytoskeletal elements, non-receptor tyrosine kinases, a v-Src/pkc substrate (annexin II), SH-2 adaptor proteins, a protein tyrosine phosphatase (PTP-1D), protein kinase C α, MAP kinase (42 kD, ERK-2 isoform), casein kinase II, PI-3 kinase (p85), hetero-trimeric G-protein subunits, and the Ras-related GTPases, Rap 1 (Krev-1; Ras tumor suppressor gene) and Rap 2—are all associated with caveolin-rich domains. Although the enrichment of a given protein varied from as high as 50-fold (representing 100% of a given molecule) to as low as threefold (representing 6% of the total cellular expression) in one case, these enrichments were greater than that observed for non-caveolar plasma membrane markers (≤1.0-fold) or other organelle-specific marker enzymes (0.035–0.2-fold; see legend of Fig. 2). In contrast to the enriched molecules, several signaling molecules are specifically excluded from these complexes (including Rho A and B, Rab 3D, Ras, Ras-GAP, PLC-γ, RB), remaining confined to the lower sucrose layer (40% sucrose loading zone) (Fig. 6, *D* and *H*). Other well known signaling molecules were also excluded (MAP kinase [44 kD, ERK-1 isoform] and MEK [MAP kinase kinase or ERK kinase]; not shown). Similarly, our previous results with caveolin-rich domains prepared from tissue-cultured cells indicated that Ras-GAP and PLC-γ were excluded from these domains (79).

Consistent with the hypothesis that CD 36 represents a transmembrane component of caveolin-rich domains in situ, Northern analysis reveals that CD 36 and caveolin share a similar tissue distribution and both are most highly expressed in lung and muscle tissues (Fig. 7), where caveolae are morphologically abundant. Similarly, the expression of

Table I. Micro-Sequencing of Murine Lung Caveolin-rich Membrane Domains

Identity	Sequence		TX-114 Phase	Molecular Weight (kD)	
				Reported	Apparent
GPI-linked					
membrane dipeptidase	KLAQTXXNIP	(I)	D	47	45
carbonic anhydrase IV	KDNVRPLQ	(I)	D	35–39	35
Thy-1	XEVXAGSH	(N)	D	25–30	28–31
Transmembrane					
CD36	GXDRNXGLIA	(N)	D	88	85
	KTGTTVYRQFX.. IFDVQNPDDVA	(I)			
	KELLWGY	(I)			
RAGE (receptor for advanced glycosylation end products)	KXNYRVXVYQ.. IXG	(I)	D	35–50	48/45
	KXNYXVXVYQ.. IPGKPEIVDPA	(I)			
plasma membrane porin	KXRVTQSNFAV.. GYK	(I)	D	31	33
	KXNTDNTL	(I)			
	KXD/LEFQL/VX.. XNXXD	(I)			
caveolin; VIP-21	KEIDLVNRDPK	(I)	D	21–29	25
	KIDFEDVIAEPE... GTXSFXXI	(I)			
Cytoplasmically oriented signaling molecules					
G-protein, α, i subunits	KXXGIVEXHF... XFK	(I)	D	39–41	40
annexin II, heavy chain	KSALSGHLETV.. ILGLLK	(I)	A	36	35
	KGDLENAFXNL.. XQXIQN	(I)			
	KLMVALAK	(I)			
Rap 1	KINVNEIFYDL... VXQINXK	(I)	D	21	22
	KXXQXFALVYS... IXA	(I)			
TC 21*	KXXYMXXXAK	(I)	D	21	22
Rab 5	RGAQXA	(N)	D	25	14–17
Cytoskeletal elements					
gelsolin	XVEHPEFLXA	(N)	D	90	90
actin	KEITALAPSTMK	(I)	D	42	42
	KAGFAGDDAP... RAVFPSIV	(I)			
	KDLYANTVLSG.. GTTMYPGIAD	(I)			
	KYPIE	(I)			
myosin, regulatory light chain 2	KEAFNMIDQN... XDGFIDK	(I)	D	20	18
	KLNGTDPEDVI... XNAFAXFD	(I)			
Endocytic ligands/fluid phase uptake?					
albumin	EAHKSEIAHRY.. NDLG	(N)	A	68	68
	KSEIAXXYND... LXE	(I)	D	68	65

Table I. (continued)

Identity	Sequence		TX-114 Phase	Molecular Weight (kD) Reported	Apparent
pulmonary surfactant protein A (SP-A)	KGEPGERGLP.. GFPAYLD	(I)	D	28–36	35/40
	KXQILQTMGVL.. SLQGSML	(I)			
	TEVXAGSPGIP.. GTPGNXGL	(N)	A	28–36	35
	KGEPGERGLP.. GFPAXXXEELQT.. ALYXIK	(I)			
osteopontin; SSP-I, secreted phosphoprotein I	DSGXXXEXQ	(N)	D	45–55	35–38

* TC 21, a member of the Rap sub-family of GTPases, is ~57% identical to Rap 1 (28). *N*, NH_2-terminal sequencing; *I*, internal sequencing; *D*, detergent phase; *A*, aqueous phase.

the RAGE protein is also highest in lung and muscle tissues (19).

To diminish the possibility that caveolin-rich membrane domains "form" because of the inclusion of detergent during the initial homogenization step, we attempted to isolate these complexes in the absence of detergent. For this purpose, we used an established protocol for the isolation of "light vesicles" from lymphoma or endothelial cells (Bourguignon, L., N. Iida, L. Sobrin, and G. J. Bourguignon. 1993. Identification of an IP_3 receptor in endothelial cells. *Mol. Biol. Cell.* 4:234*a*.) (16) that does not involve detergent. These vesicles are 50–100-nm in diameter and are enriched in the IP_3-receptor, a caveolar marker protein (37), but exclude other markers for plasma membrane, Golgi, lysosomes, and ER (16). When we isolated these light vesicles from lung—an endothelial-rich source—we found (*a*) that they contain caveolin; (*b*) that they have a similar buoyant density and protein composition as caveolin-rich membrane domains (Fig. 8); and (*c*) these light vesicles are Triton-resistant (~50–70% Triton-insoluble by protein determinations). In addition, our preliminary studies indicate that these light vesicles also contain a subset of signaling molecules (non-receptor tyro-

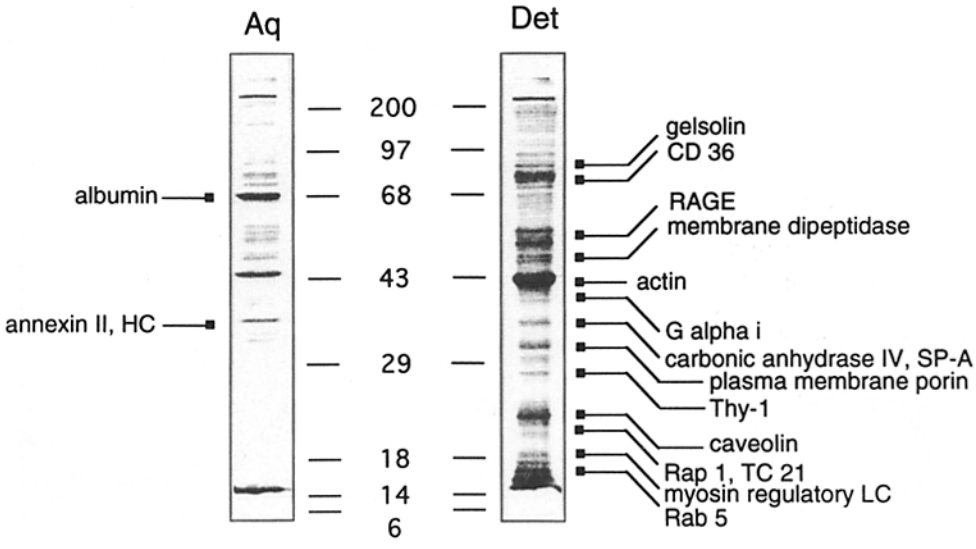


Figure 4. Summary of the protein components of caveolin-rich domains identified by microsequence analysis. Coomassie blue-stained profile of caveolin-rich domains fractionated with the detergent Triton X-114. Note that most proteins partition with the detergent-rich phase (*Det*), while few are associated with the detergent-poor phase (*Aq*). The major Coomassie blue-stained bands were subjected to microsequence analysis and their assigned identities are shown (see Table I for the specific sequences). Note that an unusually low molecular weight was obtained for Rab 5 (14–17 kD). This is consistent with the recent observation that the NH_2-terminal domain of Rab 5 is extremely susceptible to proteolysis (91). The identity of Rab 5 was independently confirmed by immuno-blotting (not shown).

sine kinases, PTP 1D, annexin II, hetero-trimeric G-proteins, Rap GTPases, cytoskeletal elements, protein kinase C α, and MAP kinase, 42 kD), while excluding others (RB, PLC-γ, and Ras-GAP)—as we have shown for caveolin-rich membrane domains (data not shown).

Prospects

In this paper, we report the isolation of caveolin-rich membrane domains from whole tissue. The known functions of the molecules we identified are summarized in Table II and are correlated with earlier morphological studies of caveolae (plasmalemmal vesicles) in situ. The components we identified may represent resident elements of caveolae at steady-state, whereas other depleted molecules may have a regulated affiliation (possibly ligand-induced) or may have been dissociated by the harsh treatments inherent in subcellular fractionation. As such, our current results provide a critical starting point and a systematic basis for future immunolocalization studies.

As many of the proteins we have identified as components of caveolin-rich membrane domains are lipid modified, the possibility has been raised that these components associate simply because of the inclusion of a detergent during the initial homogenization step. We feel that this is unlikely for the following reasons: (*a*) GPI is added at the level of the ER, however, GPI-linked proteins become Triton-insoluble and buoyant only after exiting the ER and entering the Golgi complex, indicating that their insolubility is specific to their immediate micro-environment (20); (*b*) we have identified a subset of small GTPases (Rap 1, Rap 2, and TC 21) that are lipid modified and are associated with caveolin-rich membrane domains. In contrast, other Ras-related GTPases that share over 50% sequence identity with the Rap family and undergo the same lipid-modifications were excluded from these complexes: (*c*) other investigators have demonstrated that multiple hetero-trimeric G proteins are concentrated in the same complex that exists in the absence of detergent, is resistant to dissociation by Triton-like detergents, and is dispersed by octyl-glucoside ([25, 53] and references therein), as we have observed for caveolin-rich membrane domains (this report and [79]); and (*d*) preliminary experiments isolating caveolin-rich domains in the absence of detergent suggests that these domains have a similar protein composition, that is independent of detergent extraction, and that they exist in cells that have not been exposed to Triton X-100 (see above). Taken together, these results argue against non-specific aggregation due to lipid modification. More likely, their Triton-insolubility derives from their immediate lipid micro-environment that is rich in glyco-sphingolipids as suggested previously (20), since glyco-sphingolipids are localized to cell surface caveolae (66) and glyco-sphingolipids are intrinsically Triton-insoluble (45, 94).

Tyrosine phosphorylation of a small subset of cellular proteins is thought to play a critical regulatory role in mitogenic-signaling events. One of these substrates for tyrosine phosphorylation, caveolin, was first identified as a major target of v-Src in RSV-transformed cells (40, 42). This initial finding suggested a relationship between caveolin, cellular transformation, and transmembrane signaling. Consistent with these observations, we find that known signaling molecules are associated with caveolin-rich membrane domains (this report and [79]). These findings could provide a compartmental framework for understanding certain transmembrane-signaling events. In support of this proposal, several independent reports demonstrate both physical and functional interactions, "cross-talk", between G-protein-coupled receptors, hetero-trimeric G-proteins, non-receptor tyrosine kinases, protein kinase C, MAP kinase, SH-2 adaptors, and Ras-related signaling molecules (2, 4, 5, 44, 47, 50, 67, 77, 87, 97, 98). Morphological studies independently suggest that multiple G-protein-coupled receptors, G-protein modifying bacterial toxins, and adenylate cyclase are localized to or internalized by caveolar domains (See Introduction). In addition, recent evidence indicates that activators of protein kinase C cause caveolae to undergo dramatic morphological changes which functionally regulates the caveolar uptake of small molecules, such as folate (88). In this regard, caveolin undergoes phosphorylation on both tyrosine and serine resi-

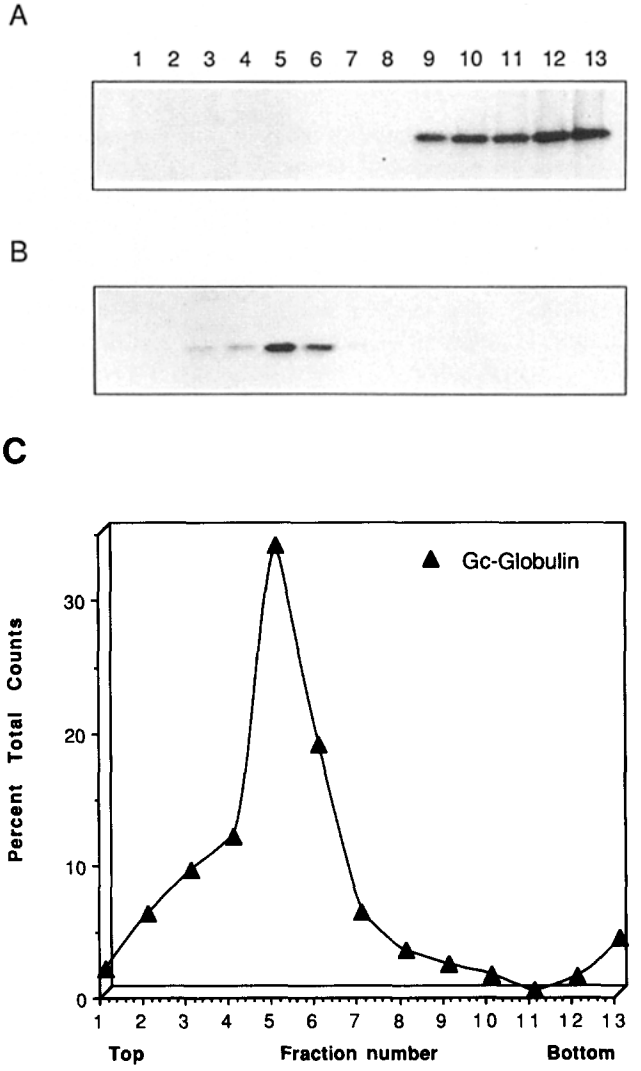


Figure 5. Detection of monomeric actin in caveolin-rich domains. The buoyancy of radio-iodinated Gc-Globulin was examined by density-gradient centrifugation as for the isolation of caveolin-rich domains. (*A*) Gc-Globulin alone; (*B*) Gc-Globulin bound to caveolin-rich domains; (*C*) graphical representation of *B*. Note that only bound Gc-Globulin attains buoyancy and cofractionates with caveolin-rich domains, suggesting that these structures contain G-actin.

Table II. Molecules Present in Caveolin-rich Membrane Domains and Their Known Functions

Molecule	Known functions and relationship to caveolae
albumin	Serum carrier for amino acids, fatty acids, sterols, hormones, bile acids, and bilirubin (81). Undergoes receptor-mediated transcytosis across endothelial cells via caveolae (38).
osteopontin	Widely expressed RGD-containing secreted glyco-phospho-protein with intrinsic Ca^{2+}-binding and phosphorylation activity. Associated with the luminal (apical) aspect of epithelial cells, including lung (21). Transduces cytoskeletal rearrangements via the intergrin ($\alpha V\beta_3$) (65) and the plasma membrane Ca^{2+}-ATPase, a known caveolar component (36).
GPI-linked proteins	Diverse group of proteins anchored to the cell surface via a glycophospholipid tail (33). Are found concentrated in plasmalemmal caveolae (106). Can act as signaling molecules that stimulate mitogenesis and cytoplasmic tyrosine phosphorylation (75, 93) and physically associate with multiple Src-like kinases (Lyn, Fyn, Lck, and c-Yes) (27, 79, 92, 93, 96). Also, GPI-linked proteins can function as receptors for the caveolar uptake of small molecules via a process termed potocytosis (8).
caveolin	22-kD transmembrane phospho-protein. Originally identified as a major v-Src substrate in RSV-transformed cells (40). Both cellular transformation and caveolin phosphorylation are dependent on the membrane attachment of v-Src (40). Recently identified as a caveolar protein component by immuno-gold labeling in situ (29, 60, 76).
CD 36	Widely distributed glycoprotein receptor for oxidized LDL, expressed in both endothelial cells and macrophages (30). Also binds long chain fatty acids and physically associates with multiple Src-like kinases (Lyn, Fyn, c-Yes, and c-Src) (1, 49). Contains a consensus sequence for interaction with Src-like kinases in its short cytoplasmic domain. Interestingly, endothelial cell caveolae bind, endocytose and transcytose LDL, and modified forms of LDL (89, 100).
RAGE	Receptor for advanced glycosylation end products (AGEs), including glycated LDL (68). Also functions as a signaling molecule (68). Previous localization studies reveal the presence of RAGE in caveolae (80) and AGEs undergo endothelial transcytois via caveolae (31, 105). As RAGE is an endogenous lectin, it could function to recognize GPI or other glycolipids concentrated in caveolae.
plasma membrane porin	Voltage-dependent anion channel; slight preference for anions (2:1), with a molecular cut off of ~6 kD. Localized to the cell surface, but homologous with mitochondrial porins (12, 56). May have implications for the uptake of small molecules through caveolae as potocytosis occurs via an unknown anion-transport protein (57). Interestingly, plasma membrane porin has recently been immunolocalized to plasmalemmal vesicles in situ (72).
Src-like kinases	Caveolin and annexin II (an actin-binding protein involved in membrane fusion events) were first identified as major v-Src substrates in RSV-transformed cells (26, 40).
protein kinase C	Activators of protein kinase C prevent the uptake of small molecules via caveolae (88). Both caveolin and annexin II contain consensus sites for protein kinase C phosphorylation.
gelsolin	Severs and sequesters actin in a monomeric state (54). Attaches to PIP_2, a precursor for IP_3 and diacylglycerol, which act on the IP_3-receptor and protein kinase C, respectively. The latter molecules have been shown to localize to either caveolae (37) or caveolin-rich domains (this report).
actin	The cytoplasmic coat of caveolae can be labeled in situ with an actin-binding protein, gold-conjugated myosin subfragment I (51, 52). Also, dystrophin (an actin-binding protein) has been immunolocalized to caveolae in smooth muscle cells (69). Here, we identify actin and three actin-binding proteins (gelsolin, annexin II, and myosin II) as components of caveolin-rich membrane domains by microsequencing.
heterotrimeric G-proteins	Dual role in signal transduction and cellular transport. Implicated in TGN vesicular budding and transcytosis (13). Substrates for glycolipid binding bacterial toxins, such as cholera toxin—that is internalized via caveolae (66). Certain G-protein coupled-receptors have been localized to caveolae in situ and this association is agonist-induced and reversible (73, 74).
rap 1	Ras-related GTPase (11) suppresses the tumorigenicity of ras and middle T antigen (55, 58). Middle T is thought to exert its effect through activation of Src-like kinases. Rap 1 is the mammalian homologue of RSR1, a gene product that controls yeast cell polarity (39, 78). Interestingly, caveolin and associated proteins are thought to participate in the generation of apical cell polarity in epithelial cells (29, 61, 79, 107). Given the antagonistic relationship between rap 1 and ras, it is interesting to note that expression of activated ras selectively disrupts apical cell polarity in epithelia (82) and leads to the selective intracellular retention of GPI-linked proteins in fibroblasts (10).
rab 5	Plasma membrane and early endosomal marker involved in endosome–endosome fusion events (22). This association is consistent with evidence that caveolae participate in endocytic and transcytotic transport processes.
SP-A	Surfactant protein A, a member of the family of C-type lectins, recognizes cell surface glyco-sphingolipids, including galactosyl-ceramide, and asialo-GM_2 (3). In addition, SP-A has been immunolocalized in part to plasma membrane-associated vesicular structures (50–100 nm in diameter) in situ (104).

dues in vivo and in vitro (40, 79) and contains conserved cytoplasmic consensus sites for phosphorylation by protein kinase C (Ser-37) and casein kinase II (Ser-88). Similarly, the unique NH_2 terminus of annexin II is a dual substrate for v-Src (Tyr-23) and protein kinase C (Ser-25) (26).

Finally, it should be noted that several proteins that we have identified here as components of caveolin-rich membrane domains by micro-sequencing of their major Coomassie-blue stained bands (caveolin, RAGE, GPI-linked proteins, plasma membrane porin, albumin, actin, and SP-

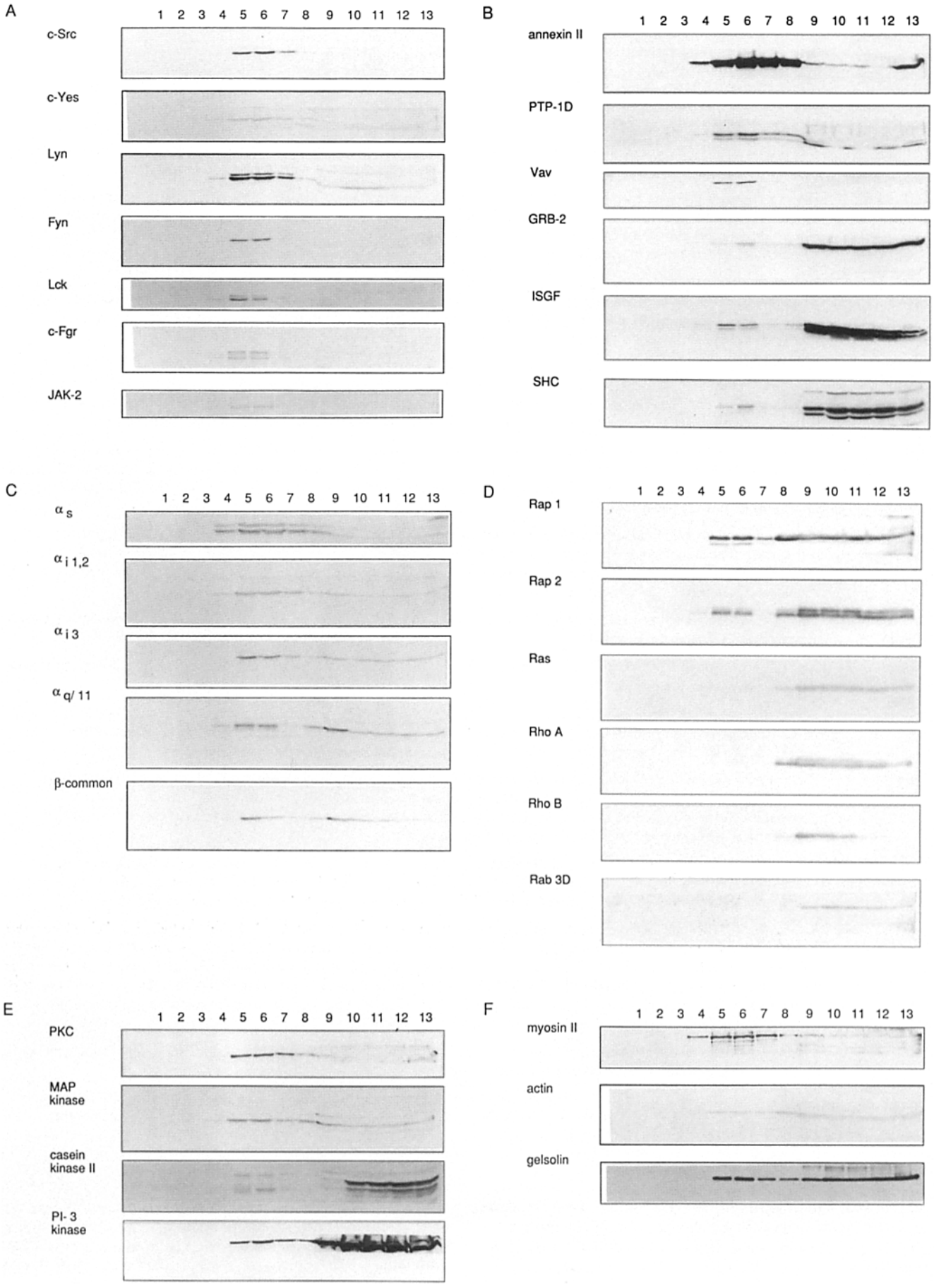

A
1 2 3 4 5 6 7 8 9 10 11 12 13
c-Src
c-Yes
Lyn
Fyn
Lck
c-Fgr
JAK-2
B
1 2 3 4 5 6 7 8 9 10 11 12 13
annexin II
PTP-1D
Vav
GRB-2
ISGF
SHC
C
1 2 3 4 5 6 7 8 9 10 11 12 13
αs
αi 1,2
αi 3
αq/ 11
β-common
D
1 2 3 4 5 6 7 8 9 10 11 12 13
Rap 1
Rap 2
Ras
Rho A
Rho B
Rab 3D
E
1 2 3 4 5 6 7 8 9 10 11 12 13
PKC
MAP kinase
casein kinase II
PI-3 kinase
F
1 2 3 4 5 6 7 8 9 10 11 12 13
myosin II
actin
gelsolin

Figure 6.

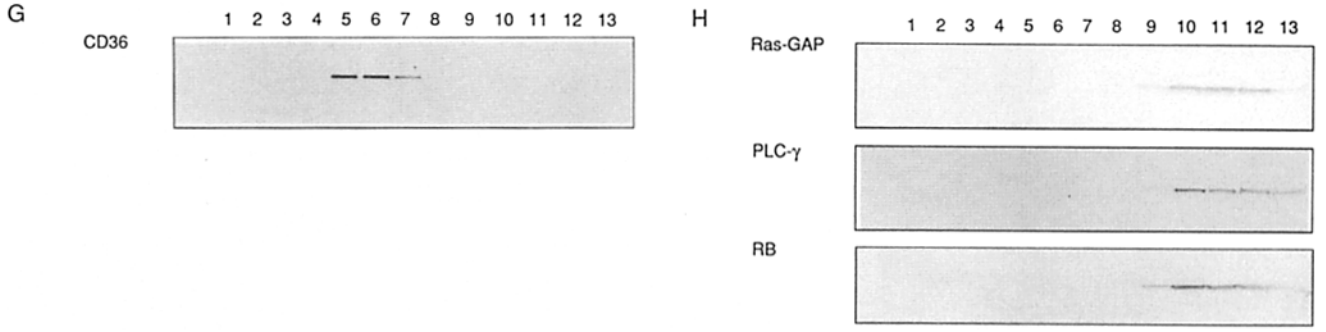


Figure 6. Immunoblotting of caveolin-rich domains. An aliquot of each 1-ml gradient fraction (1–8, 5–30% sucrose; 9–12; 40% sucrose; and 13, insoluble-pellet) was collected by acetone precipitation, separated by SDS-PAGE, and transferred to nitrocellulose. Fractions 1–13 were then subjected to immunoblotting with antibodies to signaling molecules or cytoskeletal elements. Fractions 4–7 represent the caveolin-rich membrane domains. Note that in the case of immunoblotting with anti-CD 36 IgG, human lung tissue was used as available antibodies do not react across species. The amount of given protein recovered in caveolin-rich domains is expressed as a percentage relative to the total homogenate. To calculate the fold-enrichment, divide this percentage by a factor of 2 (See Materials and Methods). (*A*) Non-receptor tyrosine kinases. *c-Src*, *c-Yes*, *Lyn*, *Fyn*, *Lck*, *c-Fgr*, *JAK-2*. (~85–95%). (*B*) Phospho-tyrosine related molecules. v-Src-pkc substrate (*annexin II-heavy chain*, ~65%), protein tyrosine phosphatase (*PTP-ID*; ~55%), SH-2 adaptor molecules (*Vav*, ~90–98%; *GRB-2*, ~12%; *ISGF*, ~8%; *SHC*, ~7%). Note that certain SH-2 adaptor molecules—GRB-2, ISGF, and SHC—were least associated. The steady-state distribution of some of these adaptor molecules could reflect a regulated affiliation with caveolin-rich domains. (*C*) Hetero-trimeric G proteins. α-subunits (*s*, ~80%; *i 1,2*, ~80%; *i 3*, ~55%; *q*,11, ~50%) and β-subunit (~40%). (*D*) Ras-related GTP-binding proteins. *Rap 1*, ~33%; *Rap 2*, ~30%. Note that *Ras*, *Rho A*, *Rho B*, and *Rab 3D* are excluded. (*E*) Other kinases. *PKC* α, ~80%; *MAP-kinase*, ~42%; *casein kinase II* (α and α′ subunits, ~16%); *PI-3 kinase* (p85, ~6%). Note that of the kinases detected, PI-3 kinase (p85) was least associated. (*F*) Cytoskeletal elements. *non-muscle myosin II-heavy chain*, ~75%; *actin*, ~30%; *gelsolin*, ~20%. (*G*) Transmembrane *CD-36*, ~90–95%. (*H*) Additional examples of excluded signaling molecules. *Ras-GAP*, *PLC-γ*, *RB*.

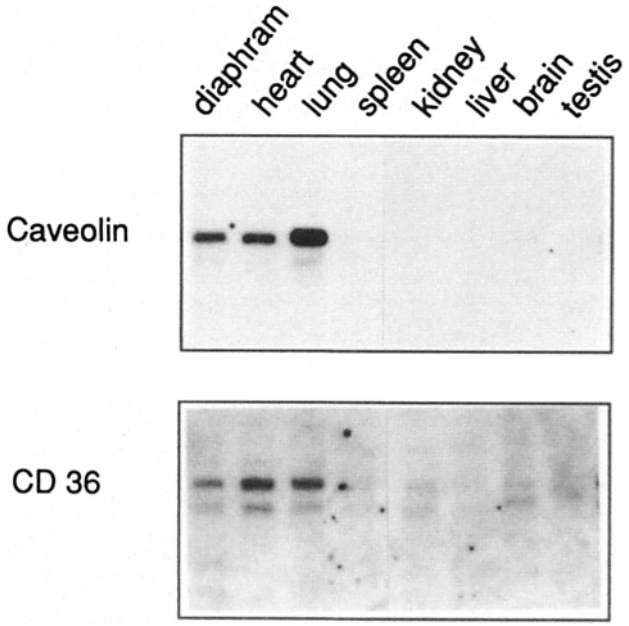


Figure 7. Caveolin and CD 36 are both highly expressed in lung and muscle tissues. Comparative Northern blot analysis of the tissue distribution of caveolin and CD 36. Each lane contains 1 μg of poly $(A)^+$ RNA prepared from a given murine tissue, as indicated. After the initial hybridization with caveolin or CD 36 cDNA probes, the blot was stripped and reprobed with an actin cDNA probe (as published previously, see Baldini et al., 1992 [Fig. 4 within]) or a murine cytosolic hsp 70 cDNA probe (not shown) as controls for equal loading.

A), have been previously morphologically localized to plasmalemmal vesicles or caveolae in situ (see Introduction and Table II for specific references). Similarly, others have demonstrated that specific lipids (such as glyco-sphingolipids) that are highly enriched in caveolin-rich complexes (20), are morphologically localized to caveolae in situ (66). These biochemical and morphological correlates provide further evidence that these domains represent isolated caveolae.

In conclusion, isolation of caveolin-rich membrane domains from lung tissue has allowed us to identify a significant number of their protein components. As almost all of these molecules are known proteins whose genes have been cloned and to which well-characterized antibodies have been generated, the availability of these molecular probes should facilitate the evaluation of these putative functional interactions in situ.

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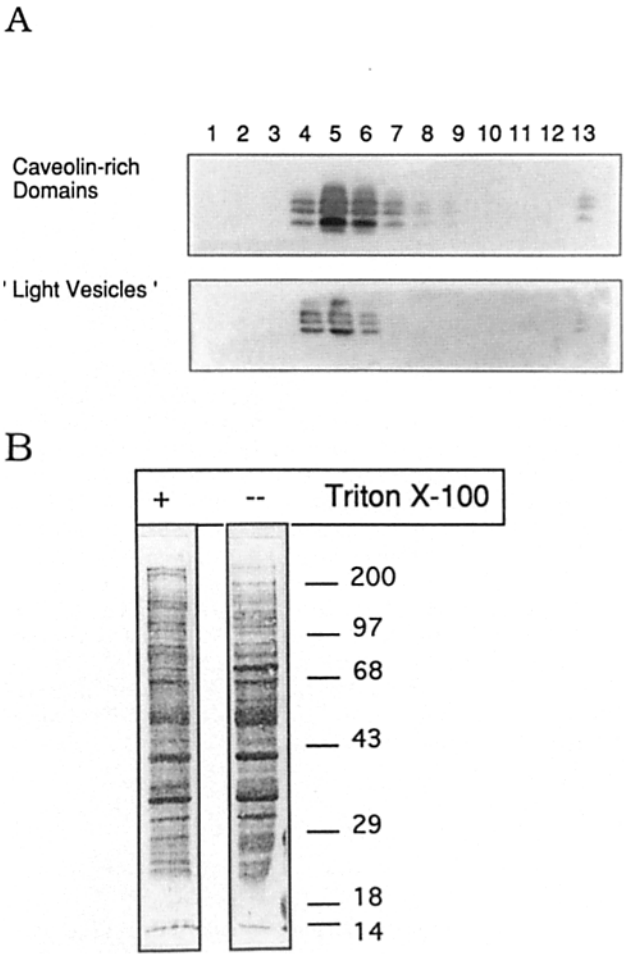

Figure 8. Caveolin-rich domains and "light vesicles." Comparison of these two membrane preparations reveals (*A*) that they contain caveolin and have a similar buoyant density in sucrose gradients, and (*B*) they contain some of the same major protein bands (most notably 68 kD, 50 kD, 42 kD, 35–36 kD, 32 kD, and 22–29 kD and 14 kD) by Coomassie blue-staining. In *A*, both preparations were refloated after isolation and yielded a single light-scattering band in the ~15–25% sucrose region of the gradient (fractions 4–7). Caveolin immunoblotting revealed that this light-scattering peak coincided with the distribution of caveolin. In (*B*) + Triton X-100 is caveolin-rich domains; –Triton is light vesicles.

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