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

Michael P. Lisanti, * Philipp E. Scherer, Jolanta Vidugiriene, Zhao Lan Tang, Anne Hermanowski-Vosatka, Ya-Huei Tu, Richard F. Cook, and Massimo Sargiacomo

The Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142-1479; The Rockefeller University, Laboratory of Molecular Parasitology, New York 10021-6399; Brigham and Women's Hospital, Harvard Medical School, Department of Pathology, Boston, Massachusetts 02115; and Biopolymers Laboratory, Howard Hughes Medical Institute, Center for Cancer Research, Department of Biology, MIT, Cambridge, Massachusetts 02139

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]) (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
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 CD36 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 phagocytosis (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 Ca2+ pump; IP3-sensitive Ca2+ 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 NH2-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-angi 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, anti-ERK/MAP kinase, GRB-2, Vav, PTP-1D, annexin II-heavy chain, Ras-GAP, ISGF, SHC, PLC-γ2, Rap 2 (Transduction Laboratories); CD 36 (Accurate); c-Src, Fyn (Oncogene Sciences, Manhasset, NY); c-Yes (gift of M. Stuefl, The Rockefeller University, NY); Lyn, Lck, c-Fgr, Rap1, Aro A, Bb, RB (Santa Cruz Biotechnology); JAK-2, Rap 1, Ras, casein kinase II (Upstate Biotechnology, Inc.); actin, justed to 40% sucrose by addition of 2 ml of 80% sucrose prepared in MBS.

Markers for caveolar plasma membrane (alkaline phosphatase, GPI-mannosidase II), lysosomal (β-hexosaminidase) and ER (c~-glucosidase II) were assayed as described previously (17, 101). Note that in order to preserve immuno-reactivity with anti-caveolin IgG (mAb 2234), it was necessary to omit fixation with glutaraldehyde.

For microsequecing, 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 NH2-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% hydroxylated Triton X-100/10% acetonitrile/100 mM Tris-HCI, pH 8.0, for 24 h at 37°C (34). Eluted peptides were isolated by HPLC and subjected to NH2-terminal sequencing, using a (model 447A/120A) pulsed-liquid protein sequencer (Appl. Biosystems, Foster City, CA). NH2-terminal and internal peptide sequences were used to scan existing databases, using the programs ATLAS or BLAST.

Detection of G-Actin

G-Actin (Calbiochem, Inc.) was radio-iodinated to a high specific activity using lodogen as suggested by the manufacturer (Pierce, Rockford, IL). Isolated caveolin-rich membrane domains (~200 μg in 200 μl MBS) were allowed to bind for the probe for 1 h at 4°C, and unbound material was removed by centrifugation before flotation on sucrose density gradients. Control experiments omitting caveoma 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)/(100/30000 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 22P-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, 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-
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 m² 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. 1A). The flotation and protein composition of this light-scattering Triton-insoluble complex was unaffected when 1% Triton

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 Coo massie 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.
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 polyepitope 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. As a yield of 0.05% was previously obtained from cultured MDCK cells (canine kidney origin; [79]), these complexes are ~40-fold more 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 microsequencing. 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. NH2-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, SPA, 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, immuno-blotting 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, gelosolin, and non-muscle myosin II (Fig. 6). The identity of Rab 5 was also confirmed by immuno-blotting (not shown). Pert-
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, \(\beta\)-subunit; (F) p45; (G) \(\alpha\)-mannosidase II; (H) \(\beta\)-hexosaminidase; (I) \(\alpha\)-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 \(\sim 85\%\) of caveolin and \(\sim 55\%\) of alkaline phosphatase, and exclude \(\geq 98\%\) 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, \(\sim 42.5\)-fold, and alkaline phosphatase—a GPI-linked protein, \(\sim 27.5\)-fold), while they exclude markers for plasma membrane (<1-fold), Golgi (\(\alpha\)-mannosidase II; \(\sim 0.1\)-fold), lysosomes (\(\beta\)-hexosaminidase; \(\sim 0.2\)-fold), and ER (\(\alpha\)-glucosidase II; \(\sim 0.035\)-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).

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 \(\alpha\), 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 (<10-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-\(\gamma\), 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-\(\gamma\) 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) |      |      |
|-------------------------------|---------------------------------|--------------|-----------------------|--|--|
| GPI-linked                    |                                 |              | Reported              | Apparent |
| 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   |
| RAGE (receptor for advanced  | KXYRVXVYQ.. (I)                 | D            | 35-50                 | 48/45 |
| glycosylation end products)   | IYGKPEIVDPDA                    |              |                       |      |      |
| plasma membrane porin        | KXRVTQSNAFAV.. (I)              | D            | 31                    | 33   |
| caveolin; VIP-21              | KEIDLVRDPPK (I)                 | D            | 21-29                 | 25   |
| Cytoplasmically oriented      |                                 |              |                       |      |      |
| signaling molecules           |                                 |              |                       |      |      |
| G-protein, α, i subunits      | KXXXGIVEXHF... (I)              | D            | 39-41                 | 40   |
| annexein II, heavy chain      | XFK                              |              |                       |      |      |
| Rap 1                         | KINVNEIFYD... (I)               | D            | 21                    | 22   |
| 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   |
| myosin, regulatory light      | KEAFNMIDQN... (I)               | D            | 20                    | 18   |
| chain 2                       | XDGFIKD (I)                     |              |                       |      |      |
| Endocytic ligands/fluid phase |                                 |              |                       |      |      |
| uptake?                       |                                 |              |                       |      |      |
| albumin                       | EAHKSEIAHRY.. (N)               | A            | 68                    | 68   |
|                               | NDLG                            |              |                       |      |      |
|                               | KEIIAXXYND... (I)               | D            | 68                    | 65   |

The Journal of Cell Biology, Volume 126, 1994 118
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 IP3 receptor in endothelial cells. Mol. Biol. Cell. 4:234a.) (16) that does not involve detergent. These vesicles are 50-100-nm in diameter and are enriched in the IP3-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 NH2-terminal domain of Rab 5 is extremely susceptible to proteolysis (91). The identity of Rab 5 was independently confirmed by immuno-blotting (not shown).](image-url)
sine kinases, PTP 1D, annexin II, hetero-trimeric G-proteins, Rap GTPases, cytoskeletal elements, protein kinase C \( \alpha \), and MAP kinase, 42 kD), while excluding others (RB, PLC-\( \gamma \), 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 nonspecific 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 \( \nu \)-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, 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-
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 integrin (αVβ3) (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 glycosphospholipid 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 phosphoprotein. 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 transcytosis via caveolae (31, 105). As RAGE is an endogenous ligand, 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 PI(3,4,5)P3, a precursor for IP3 and diacylglycerol, which act on the IP3-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. |
| hetero-trimeric 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-GM1 (3). In addition, SP-A has been immunolocalized in part to plasma membrane-associated vesicular structures (50–100 nm in diameter) in situ (104). |

Due to 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 NH2 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-

---

Lisanti et al. Caveolae, Atherosclerosis, and Cell Transformation 121
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-Fyn, JAK-2. (~85–95%). (B) Phospho-tyrosine related molecules, v-Src-pck substrate (annexin II-heavy chain, ~65%), protein tyrosine phosphatase (PTP-ID, ~55%), SH-2 adaptor molecules (Hiv, ~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.
References

1. Abumrad, N., R. El-Maghrabi, E.-Z. Amri, E. Lopez, and P. A. Grimaldi. 1993. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during pre-adipocyte differentiation: homology with CD 36. J. Biol. Chem. 268:17655-17668.

2. Akino, H., Y. Tokumitsu, M. Noda, and Y. Nomura. 1993. Decrease in coupling of Gs in v-src transformed NIH-3T3 fibroblasts: possible involvement of tyrosine phosphorylation of Gs by pp60 v-src. Arch. Biochem. Biophys. 306:235-241.

3. Akino, T. 1992. Biochemical and clinical aspects of pulmonary surfactant proteins. Nippon Kyyo Shikkan Gakkai Zasshi 30:5-14.

4. Allen, L. F., R. J. Lefkowitz, M. G. Caron, and S. Coteccia. 1991. G-protein-coupled receptor genes as proto-oncogenes: constitutively activating mutation of the alpha 1 beta-adrenergic receptor enhances mitogenesis and tumorigenicity. Proc. Natl. Acad. Sci. USA. 88: 11,354-11,358.

5. Alonso, T., S. Srivastava, and E. Santos. 1990. Alterations of G-protein coupling function in phosphoinositide signaling pathways of cells transformed by ras and other membrane-associated and cytoplasmic oncogenes. Mol. Cell. Biol. 10:2645-2652.

6. Anderson, R. G. W. 1993. Caveolae: where incoming and outgoing messengers meet. Proc. Natl. Acad. Sci. USA. 90:10909-10913

7. Anderson, R. G. W. 1993. Plasmaemmal caveolae and G-protein-anchored membrane proteins. Curr. Opin. Cell. Biol. 5:647-652.

8. Anderson, R. G. W., B. A. Kamen, K. G. Rothberg, and S. W. Lacey. 1992. Porecytosis: sequestration and transport of small molecules by caveolae. Science (Wash. DC). 255:410-411.

9. Baldini, G., T. Hohi, H. Lin, and H. F. Lodish. 1992. Cloning of a Rab3 isotype predominately expressed in adipocytes. Proc. Natl. Acad. Sci. USA. 89:5049-5052.

10. Bamezai, A., K. L. Rock. 1991. Effect of ras-activation on the expression of G-protein-anchored proteins on the plasma membrane. Oncogene. 6:1445-1451.

11. Beranger, F., B. Goud, A. Tartifian, and J. de Gunzburg. 1991. Association of the Ras-antagonistic Rap 1/Krev-1 proteins with the Golgi complex. Proc. Natl. Acad. Sci. USA. 88:1606-1610.

12. Blachly-Dyson, E., et al. 1993. Cloning and functional expression in yeast of two human isoforms of the outer mitochondrial membrane channel, the voltage-dependent anion channel. J. Biol. Chem. 268:1835-1841.

13. Bomsel, M., and K. Mostov. 1992. Role of heterotrimeric G proteins in membrane traffic. Mol. Biol. Cell. 3:1317-1328.

14. Bosilla, E., K. Fischbeck, and D. Schotland. 1981. Freeze-fracture studies of muscle caveole in human muscular dystrophy. Am. J. Path. 104:167-173.

15. Deleted in Proof.

16. Bourguignon, L., H. Jin, N. Iida, N. Brandt, and S. Zhang. 1993. The involvement of ankyrin in the regulation of the IP3 receptor mediated internal calcium release from calcium storage vesicles in mouse T-lymphoma cells. J. Biol. Chem. 268:7290-7297.

17. Brada, D., and U. C. Dubach. 1984. Isolation of a homogeneous glucosylceramide II from pig kidney microsomes. Eur. J. Biochem. 141:149-156.

18. Bretscher, M., and S. Whytock. 1977. Membrane-associated vesicles in macrophages. Science (Wash. DC). 197:192-194.

19. Brett, J., et al. 1993. Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. Am. J. Pathol. 143:1699-1712.

20. Brown, D., and J. K. Rose. 1992. Sorting of G-protein-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell. 68:533-544.

21. Brown, L. F., et al. 1992. Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. Mol. Cell. Biol. 3:1169-1180.

22. Bucci, C., et al. 1992. The small GTPase Rab5 functions as a regulatory component in the early endocytic pathway. Cell. 70:715-728.

23. Caldwell, R. B., and S. M. Slapnick. 1992. Freeze-fracture and lanthanum studies of the retinal microvasculature in diabetic rats. Invest. Ophthal. Mol. Sci. 33:1610-1619.

24. Cao, L.-G., D. J. Fishkind, and Y.-L. Wang. 1993. Localization and dynamics of non-filamentous actin in cultured cells. J. Cell Biol. 123:173-181.

25. Couter, S., and M. Rodbell. 1992. Heterotrimeric G proteins in synaptonemarose are crosslinked by phenylenedimaleide, yielding structures comparable in size to crosslinked tubulin and F-actin. Proc. Natl. Acad. Sci. USA. 87:5842-5846.

26. Creutz, C. E. 1992. The annexins and exocytosis. Science (Wash. DC). 258:924-930.

27. Drabrova, L., and P. Draher. 1993. Thy-1 glycoprotein and src-like protein tyrosine kinase p55/p65 are associated in large detergent-resistant complexes in rat basophilic leukemia cells. Proc. Natl. Acad. Sci. USA. 90:3611-3615.

28. Drivas, G., A. Shih, E. Coutavas, M. Rush, and P. D'Estachio. 1990. Characterization of four novel ras-like genes expressed in a human teratocarcinoma cell line. Mol. Cell. Biol. 10:1793-1798.

29. Dupree, P., R. G. Parton, G. Raposo, T. V. Kurzchalia, and K. Simons. 1993. Caveolae and sorting of the trans-Golgi network of epithelial cells. EMBO (Fur. Mol. Biol. Organ.) J. 12:1597-1605.

30. Endemann, G., et al. 1993. CD 36 is a receptor for oxidized low density lipoprotein. J. Biol. Chem. 268:11811-11816.

31. Esposito, C., H. Gerlach, J. Brett, D. Stern, and H. Vlassara. 1989. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface properties. J. Exp. Med. 170:1387-1407.

32. Pan, J. Y., et al. 1983. Morphological changes of the 3T3-L1 fibroblast plasma membrane upon differentiation to the adipocyte form. J. Cell Sci.
33. Ferguson, M. A. J. 1991. Lipid anchors on membrane proteins. Curri. Opin. Struct. Biol. 1:522–529.
34. Fernandez, J., M. DeMott, D. Aberton, and S. M. Mische. 1992. Internal protein sequence analysis: enzymatic digestion for less than 10 μg of protein bound to PVDF or nitrocellulose membranes. Anal. Biochem. 201:255–264.
35. Forbes, M. S., M. Rannels, and E. Nelson. 1979. Caveolar systems and sarcoplasmic reticulum in coronary smooth muscle cells. J. Ultrastruct. Res. 76:325–339.
36. Fujimoto, T. 1993. Calcium pump of the plasma membrane is localized to caveolae. J. Cell Biol. 119:1507–1513.
37. Ghitescu, L., A. Fixman, M. Simonescu, and N. Simonescu. 1986. Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capacity endothelium: receptor-mediated transcytosis. J. Cell Biol. 102:1304–1311.
38. Glimcher, M. J., C. J. Longdahl, C. Styles, and G. R. Pink. 1992. Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and Ras. Cell. 68:1077–1090.
39. Glennie, J. R. 1989. Tyrosine phosphorylation of a 22 kD protein is correlated with transformation with Rous sarcoma virus. J. Biol. Chem. 264:20136–20166.
40. Glennie, J. R. 1992. The sequence of human caveolin reveals identity with VIP 21, a component of transport vesicles. FEBS Lett. 314:45–48.
41. Goldman, J. R., and D. Zakas. 1999. Novel tyrosine kinase substrates from Rous sarcoma virus transformed cells are present in the membrane cytoskeleton. J. Cell Biol. 108:2401–2408.
42. Goldberg, R. L., R. M. Smith, and L. Jarett. 1987. Insulin and alpha-2-macroglobulin receptors: different mechanisms in rat adipocytes. J. Cell. Physiol. 133:203–212.
43. Gupta, S. K., C. Gallego, G. L. Johnson, and L. E. Heasley. 1992. MAP kinase is constitutive activated in Gip2 and src transformed rat fibroblasts. J. Cell Biol. 117:797–804.
44. Hageman, J. L., and P. H. Fishman. 1982. Detergent extraction of cholera toxin and gangliosides from cultured cells and isolated membranes. Biochim. Biophys. Acta. 720:181–187.
45. Hase, J. F., and A. Holocher. 1994. Solubility in non-ionic detergents distinguishes between slowly and rapidly degraded plasma membrane proteins. J. Biol. Chem. 269:5981–5988.
46. Hasford, W. P., et al. 1992. Tyrosine phosphorylation of G protein alpha subunits by src family protein kinases: differential regulation in normal and transformed cells. J. Cell Biol. 118:1003–1014.
47. Hooper, N. M., and A. J. Turner. 1988. Ectoenzymes of the kidney plasma membrane: implications for the biogenesis of caveolae. J. Cell Biol. 111:2391–2400.
48. Hore, J. F., and A. Holocher. 1994. Solubility in non-ionic detergents distinguishes between slowly and rapidly degraded plasma membrane protein: implications for the biogenesis of caveolae. J. Cell Biol. 111:2391–2400.
49. Huang, X.-Y., A. D. Morielli, and E. G. Per'aim. 1993. Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled receptor related protein (LRP). J. Cell Biol. 122:789–807.
50. Izumi, T., Y. Shibata, and T. Yamamoto. 1988. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
51. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
52. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
53. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
54. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
55. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
56. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
57. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
58. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
59. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
60. Izumi, T., Y. Shibata, and T. Yamamoto. 1989. The cytoplasmic surface of caveolin-1, a major protein component of caveolae, is involved in the transduction of a G protein-coupled receptor-related signal. Am. J. Cardiol. 72:268–270.
89. Snelting-Havinga, I., et al. 1989. Immunoelectron microscopic visualization of the transcytosis of low density lipoproteins in perfused rat arteries. Eur. J. Cell Biol. 48:27–36.
90. Sorokin, S. P. 1983. The respiratory system. In Histology: Cell and Tissue Biology. L. Weiss, editor. Elsevier Biomedical, New York. 833–836.
91. Steele-Mortimer, O., et al. 1994. The N-terminal domain of a rab protein is involved in membrane-membrane recognition and/or fusion. EMBO (Eur. Mol. Biol. Organ.) J. 13:34–41.
92. Stefanova, I., et al. 1993. LPS induces activation of CD-14 associated protein tyrosine kinase p53/56 lyn. J. Biol. Chem. 268:20772–20778.
93. Stefanova, I., V. Horajsi, I. 1. Annoteug, W. Knapp, and H. Stockinger. 1991. GPI-anchored cell surface molecules complexed to protein tyrosine kinases. Science (Wash. DC). 254:1016–1019.
94. Streuli, C. H., B. Patel, and D. R. Critchley. 1981. The cholera toxin receptor ganglioside GMI remains associated with the Triton X-100 cytoskeletons of BALB/c-3T3 cells. Exp. Cell Res. 136:247–254.
95. Strosberg, A. D. 1991. Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. Eur. J. Biochem. 196:1–10.
96. Thomas, P. M., and L. E. Samelson. 1992. The GPI-anchored Thy-1 molecule interacts with the p60 fyn protein tyrosine kinase in T cells. J. Biol. Chem. 267:12317–12322.
97. Torti, M., M. F. Crouch, and E. G. Lapetina. 1992. Epinephrine induces association of pp60src with Gi alpha in human platelets. Biochem. Biophys. Res. Commun. 186:155–161.
98. van Corven, E., P. Horidijk, R. Medema, J. Bos, and W. Moolenaar. 1993. Pertussis toxin-sensitive activation of p21ras by G-protein coupled receptor agonists in fibroblasts. Proc. Natl. Acad. Sci. USA. 90:1257–1261.
99. Vasile, E., and F. Antobe. 1991. An ultrastructural study of beta-very low density lipoprotein uptake and transport by valvular endothelium in hyperlipidemic rabbits. J. Submicrosc. Cytol. Pathol. 23:279–287.
100. Vasile, E., M. Simionescu, and N. Simionescu. 1983. Visualization of the binding, endocytosis, and transcytosis of low density lipoprotein in the arterial endothelium in situ. J. Cell Biol. 96:1677–1689.
101. Vidugiriene, J., and A. K. Menon. 1993. Early lipid intermediates in GPI-anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. J. Cell Biol. 121:987–996.
102. von Zastrow, M., and B. Kobilka. 1992. Ligand-regulated internalization and recycling of human beta-2 adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J. Biol. Chem. 267:3530–3538.
103. Wagner, R. C., P. Kreiner, R. J. Barnett, and M. W. Bitensky. 1972. Biochemical characterization and cytochemical localization of a catecholamine-sensitive adenylate cyclase in isolated capillary endothelium. Proc. Natl. Acad. Sci. USA. 69:3175–3179.
104. Walker, S. R., M. C. Williams, and B. Benson. 1986. Immunocytochemical localization of the major surfactant apoproteins. J. Histochem. Cytochem. 34:1137–1148.
105. Williams, S., J. Devenny, and M. W. Bittensky. 1981. Micropinocytotic ingestion of glycosylated albumin by isolated microvessels: possible role in pathogenesis of diabetic microangiopathy. Proc. Natl. Acad. Sci. USA. 78:2393–2397.
106. Ying, Y., R. G. W. Anderson, and K. G. Rothberg. 1992. Each caveola contains multiple GPI-anchored membrane proteins. Cold Spring Harbor Symp. 57:593–602.
107. Zurzolo, C., W. van't Hof, G. van Meer, and E. Rodriguez-Boulan. 1994. VIP21/caveolin, glycosphingolipid clusters and the sorting of GPI-anchored proteins in epithelial cells. EMBO (Eur. Mol. Biol. Organ.) J. 13:42–53.