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THE CAVEOLAE MEMBRANE SYSTEM

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ABSTRACT

The cell biology of caveolae is a rapidly growing area of biomedical research. Caveolae are known primarily for their ability to transport molecules across endothelial cells, but modern cellular techniques have dramatically extended our view of caveolae. They form a unique endocytic and exocytic compartment at the surface of most cells and are capable of importing molecules and delivering them to specific locations within the cell, exporting molecules to extracellular space, and compartmentalizing a variety of signaling activities. They are not simply an endocytic device with a peculiar membrane shape but constitute an entire membrane system with multiple functions essential for the cell. Specific diseases attack this system: Pathogens have been identified that use it as a means of gaining entrance to the cell. Trying to understand the full range of functions of caveolae challenges our basic instincts about the cell.

CONTENTS

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INTRODUCTION

Yamada proposed the name caveolae intracellularis (little caves) to define "a small pocket, vesicle, cave or recess communicating with the outside of the cell" in gallbladder epithelial cells (1). Although he did not attribute any special shape to these invaginations, nor did he distinguish coated from non-coated varieties, the name later became synonymous with "flask-shaped" or "omega-shaped" membrane owing to the prominence of such membrane profiles in endothelial and smooth-muscle cells. In fact, two years earlier Palade had described morphologically similar invaginations in endothelial cells (2). He later named them plasmalemmal vesicles (3) because they appeared to shuttle molecules across these cell.

Over the ensuing years, many studies supported Palade's hypothesis that caveolae were endocytic structures involved in the transcellular movement of molecules across endothelial cells (4). Unfortunately, little was learned during this time about how they might function in other cell types. The modern era of caveolae research was ushered in by two discoveries: (*a*) receptor-mediated uptake of folate by caveolae (5) and (*b*) caveolin, the first marker protein for caveolae (6). The former provided a general model for how caveolae might function in diverse cell types, whereas the latter was the critical tool needed to purify this membrane domain for analysis. Purified caveolae not only yielded information about their chemical composition but also resulted in the unexpected finding that caveolae are rich in a variety of cell-signaling molecules. Current research is focused on caveolae as a membrane system responsible for compartmentalizing signal transduction, thereby facilitating the integration of nutritional, mechanical, and humoral information at the cell surface.

DEFINING A CAVEOLA

The shift in research from the use of morphological tools to the use of biochemical tools has brought a changing perspective of caveolae. The original intent of the word caveolae was to describe membrane invaginations at the cell surface (Figure 1*A*), but membranes with the classic morphologic features of

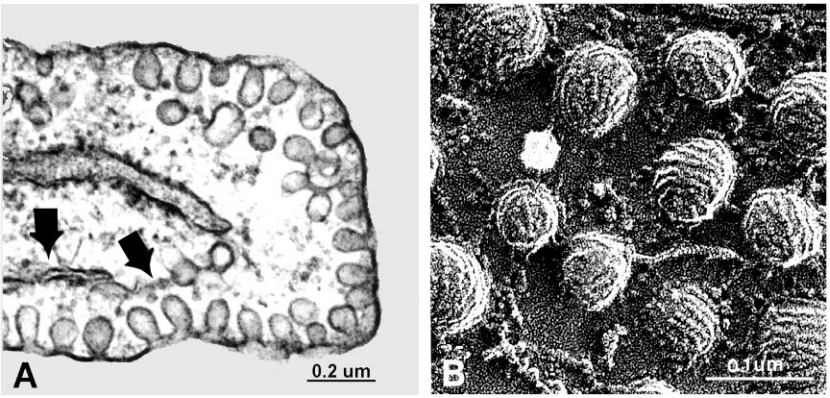

Figure 1 (*A*) Thin-section electron microscopy (EM) image and (*B*) rapid-freeze deep-etch image of fibroblast caveolae. *Arrows* point to endoplasmic reticulum near invaginated caveolae.

caveolae are not found in all cells. Purification methods using the caveolae marker protein caveolin established new criteria for identifying this membrane. These included (*a*) resistance to solubilization by Triton X-100 at 4°C (7); (*b*) a light buoyant density (8); and (*c*) richness in glycosphingolipids (GSLs), cholesterol, and lipid-anchored membrane proteins (Table 1). Membrane fractions with these properties, however, can be obtained from virtually all cells, even those not expressing detectable caveolin. The evidence presented in this review emphasizes that all cells have plasma membrane domains with the biochemical features of caveolae but only a subset of these membranes display the flask-shaped morphology. Caveolae assume a variety of shapes, including flat, vesicular, and tubular. They can be either open at the cell surface or closed off to form a unique endocytic/exocytic compartment. The use of the word caveolae in this review, therefore, is not restricted to membranes with a particular shape. Rather it is meant to encompass a membrane system with specific functions essential for normal cell behavior.

MOLECULAR COMPOSITION OF CAVEOLAE

The structure, function, and molecular composition of caveolae are dependent on the phase properties of a unique set of membrane lipids. Resident molecules freely move in and out of caveolae many times during their lifetime.

Caveolae Coat

Caveolae in endothelial cells (9) and fibroblasts (6) have a striated coat. Rapid-freeze deep-etch images show that the coat decorates membranes with different

Table 1 Partial list of molecules enriched in caveolae

Class of molecules	Name of molecule	Biochemical localization	Morphological localization	References
Lipid	Ganglioside	√	√	8, 143, 159, 160
	Sphingomyelin	√	—	43, 82, 191
	Ceramide	√	—	43, 82, 191
	Diacylglycerol (DAG)	√	—	191
	Cholesterol	√	√	19, 43, 216
Acylated protein	Heterotrimeric G_α and G_β	√	√	7, 8, 31, 35
	Src, Fyn, Hck, Lck	√	—	7, 53, 168, 169
	E-NOS	√	√	90, 91
	CD-36	√	—	32
	Caveolin	√	√	6, 7
Glycosylphospha-tidylinositol (GPI)-anchored protein	Folate receptor	√	√	58, 63
	Thy 1	√	√	32, 76
	Alkaline phosphatase	√	√	70, 72, 191
	Prion	√	√	76, 206, 207, 210
	Urokinase Rec	√	√	65, 217
	Multiple GPI proteins	√	—	31
	5′-Nucleotidase	√	√	203, 218–220
	CD14	√	—	221
Prenylated protein	Rap1A	√	√	31, 222
	Ras	√	—	36, 179
Membrane receptor	Platelet-derived growth factor (PDGF)	√	√	79, 223
	Insulin	—	√	224, 225
	Epidermal growth factor (EGF)	√	—	8, 179
	Receptor for advanced glycation end product (RAGE)	√	—	32
	Cholecystokinin (CCK) receptor	√	√	150
	m2 acetylcholine	√		181
	Tissue factor	√	—	226, 227
	β Adrenergic	—	√	100
	Bradykinin	√	—	83
	Endothelin	√	—	84
	SR-B1	√	√	121
Signal transducer	PKC_α	√	√	32, 99, 144
	SHC	√	—	79
	SOS	√	—	179
	GRB_2	√	—	179

(*Continued*)

Table 2 (*Continued*)

Class of molecules	Name of molecule	Biochemical localization	Morphological localization	References
	Mitogen-activated protein (MAP) kinase	√	√	32, 35, 79, 172
	Adenylyl cyclase	√	√	182–185
	SYP	√	—	79
	PI3 kinase	√	—	79
	Raf1	√	—	94, 179
	Calmodulin	√	—	90
	Phosphoinositides	√		192, 193
	Polyphosphoinositide phosphatase	√	—	193
	Engrailed	√	—	228
Membrane transporter	Porin	√	—	32
	IP_3 receptor	√	√	33, 190
	Ca^{+2} ATPase	√	√	33, 189, 229
	Aquaporin-1	√	—	135
	H^+ ATPase	√	—	230
Structural molecules	Annexin II	√	—	32, 231
	Ezerin	√	—	32
	Myosin	√	—	32
	VAMP	√	—	158
	NSF	√	—	158
	MAL	√	—	119, 232
	Actin	√	√	8, 31, 32
Miscellaneous	Atrial natriuretic Peptide	—	√	233
	Flotillin	√	—	234

amounts of curvature (Figure 1*B*), suggesting that it may control the shape of the membrane. The coat is composed of integral membrane proteins, one of which is caveolin (6). At least four caveolin gene products are present in mammals—caveolin-1α and -1β, -2, and -3 (10–14)—and possibly two in *Caenorhabditis elegans* (15). Each contains a 33-amino-acid hydrophobic domain that is thought to anchor the protein in the membrane, leaving the amino and carboxyl portions free in the cytoplasm (16). Caveolin-1 and -3 have cysteine residues at positions 134, 144, and 157 that in caveolin-1 are acylated (17). The expression of caveolin-1 in cells is correlated with the appearance of invaginated caveolae (18, 19) as well as the presence of the striated coat material (20). Although caveolin-1 is able to form homotypic oligomers both

in vitro (21–23) and in vivo (24), it probably does not have a mechanical function in shaping the membrane because invaginated caveolae sometimes lack the molecule (25, 26). Sequestration of membrane cholesterol with drugs such as filipin (6) or depletion of intracellular cholesterol (27) causes the coat to disassemble. At the same time, invaginated caveolae disappear. Because caveolin-1 appears to be a cholesterol-binding protein (28, 29) and cholesterol stabilizes caveolin-1 oligomers (23), sterol and caveolin-1 must work together to form the coat.

Purification

Caveolin-1 is the marker protein used to isolate caveolae by cell fractionation. Six methods have been reported for purifying caveolae from either tissues (30–35) or tissue culture cells (7, 8, 36). The methods fall into four categories: (*a*) flotation of detergent-insoluble membrane on sucrose gradients (7), (*b*) flotation of sonicated plasma membranes on OptiPrep gradients (8), (*c*) differential centrifugation of tissue homogenates (31), and (*d*) recovery, either by centrifugation (33) or immunoadsorption (34), from endothelial cell plasma membranes purified by adsorption to cationized silica. The caveolae obtained by these procedures are not strictly comparable, largely because no morphologic standard exists by which to judge purity. A coatlike material is visible in some preparations (Figure 2), but it generally is hard to recognize. Caveolin-1 is not the ideal molecule for assessing purity because the concentration in caveolae is variable (see below). Finally, the physical aids used during purification (e.g. cationized silica, Triton X-100, sonication, high pH carbonate, and immunoadsorption)

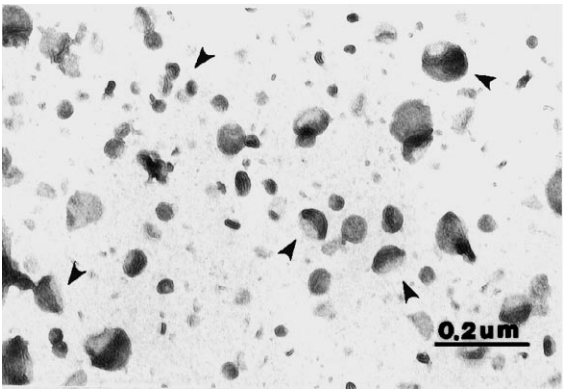

Figure 2 Whole-mount electron microscopy (EM) image of isolated caveolae prepared by the method of Chang et al (31). *Arrows* point to cup-shaped caveolae having a visible coat covering a portion of the membrane.

can alter the molecular composition of caveolae, making the comparison of results from different laboratories difficult. Detergents, for example, solubilize resident proteins (31), yielding extracted preparations of caveolae. The lack of a standard purification method, combined with the potential for contamination and extraction artifacts, obligates researchers to verify whenever possible all fractionation results by independent methods.

The resistance of core lipids to detergents at 4°C is a consistent property of caveolae (7). Simons & van Meer (37, 38) proposed that hydrogen bonding between GSLs caused the formation of GSL-rich membrane domains in the Golgi apparatus. Earlier reports had indicated that GSL-rich membranes (39–42) were insoluble in Triton X-100 at 4°C. Sucrose density centrifugation subsequently showed that GSL, cholesterol, and sphingomyelin (SPH), along with glycosylphosphatidylinositol-anchored membrane proteins (GPI protein), form a detergent-insoluble membrane domain with a light buoyant density (43). These light fractions are rich in caveolin-1 (7). Chang et al (31) directly demonstrated that caveolae are detergent insoluble by showing that partially purified caveolae, obtained without detergents, are resistant to Triton X-100. Caveolae in situ also are not extracted by Triton X-100 (44).

Because not all resident caveolae proteins are detergent insoluble, another useful identifier for caveolae is their light buoyant density relative to the bulk plasma membrane. They float on both velocity (8) and equilibrium (7) gradients independently of whether they have been treated with Triton X-100. Light membrane fractions with the same vesicular morphology and marker proteins are obtained regardless of whether cells express detectable caveolin-1 (8, 35).

Dynamics of Caveolae Molecules

Biochemical and morphologic techniques have identified a number of molecules that appear to be concentrated in caveolae relative to the surrounding membrane (Table 1). Approximately 35% have been localized by both morphological and biochemical methods (see check marks). The list does not take into consideration the purification method used to identify the molecule. Taken as a whole, the list indicates that caveolae have a specific lipid composition and are enriched in lipid-modified proteins. They are also rich in receptors and signal-transducing molecules.

GSL, SPH, and cholesterol, which form the lipid core of caveolae, govern the phase properties of the lipids in this domain. The detergent insolubility of caveolae at low temperature (45) is a characteristic of the liquid-ordered phase (referred to as the β phase) (46, 47). The detergent-insoluble properties of caveolae have been reconstituted using liposomes composed of cholesterol and sphingomyelin (48, 49). In general, the lateral and rotational mobilities of phospholipids are high in β phase membranes (50), whereas in

sphingomyelin/cholesterol-rich membranes, the fluidity appears to be lower than that of the bulk cell membrane (48).

The GSL/SPH/cholesterol lipid core plays an important role in attracting lipid-modified membrane proteins to caveolae (Table 1). Proteins modified with either GPI or fatty acids are found to be enriched in caveolae fractions obtained by most methods of purification (7, 8). Mutations that abolish either the GPI-anchor addition (51, 52) or fatty acylation (53, 54) shift the protein to other fractions, which suggests that the lipid moiety is required for targeting to caveolae. These two different covalent modifications, therefore, are responsible for targeting proteins with a wide range of biochemical activities to opposing surfaces of the same membrane domain. Because the acyl chains on these proteins intercalate in the lipid bilayer, they probably collect in caveolae as a result of a slowed lateral mobility upon encountering the β phase lipids (55). Perturbing the β phase with cholesterol-sequestering drugs such as filipin (56) disperse GPI proteins in the plane of the membrane (57, 58). Protein-protein and protein-lipid (59, 60) interactions within caveolae influence how long the molecules remain at this site. The phase properties of the core lipids therefore play a major role in generating the complex molecular environment found in caveolae.

GPI proteins are dynamically associated with caveolae: They spontaneously insert into membranes of living cells (61). When the GPI-anchored complement inhibitor CD59 is inserted into the promonocyte cell U937, it is initially dispersed in the membrane but becomes clustered after a brief time (62). The clustered molecules are active in cell signaling, whereas unclustered CD59 is inactive. The GPI-anchored folate receptors are also mobile in the membrane. Ordinarily about 60–70% of the receptors in the monkey kidney cell MA104 are recovered in caveolae fractions (58), and indirect immunogold shows ~80% in discrete clusters (63). Surprisingly, incubation of these cells in the presence of a monoclonal anti-receptor immunoglobulin (Ig)G shifts receptors to the noncaveolae fraction (58), and by direct immunofluorescence with the same antibody, they appear dispersed in the membrane (64). These and other experiments (55) show that GPI proteins are not static on the cell surface. They are constantly moving, potentially accessing many different membrane compartments during their lifetime. In some cases, protein ligands for GPI proteins shift the protein from caveolae to other compartments, where they may become tethered by a resident protein (65–68). A relatively fast lateral mobility combined with a natural attraction for β phase lipids allows GPI proteins to shuttle information among different membrane compartments.

A persistent problem in caveolae research has been the conflicting reports on the native distribution of GPI proteins. Electron microscopy (EM) histochemistry clearly shows that GPI alkaline phosphatase (AP) is clustered both

on the surface and within invaginated caveolae (69–71). Immunocytochemistry, by contrast, shows AP either clustered or diffusely distributed, depending on (*a*) the primary antibody used (72, 73), (*b*) whether a second antibody (or protein A) is applied (74), and (*c*) the fixation conditions (56, 64, 74). Other GPI proteins, as well as GSL and SPH, appear diffuse on the surface of cells after direct antibody labeling but clustered when indirect labeling methods are used (75). The clustered GPI proteins visualized after antibody additions are not randomly distributed. Instead they are always nearby (63) or inside (74, 76) invaginated caveolae or tightly co-localized with caveolin-1 (64, 75). In contrast to morphology, both detergent-dependent (7) and independent (8, 31) purification methods find GPI proteins concentrated in caveolae fractions. Only when membranes are pretreated—with antibodies against the protein (58), with cationized silica (77), or with pH 9.5 Tris buffer (36)—do they appear in noncaveolae fractions. Obviously these molecules cannot be clustered and diffuse at the same time! The most likely explanations for these discrepant results is that GPI proteins remain mobile in the plane of the membrane after weak aldehyde fixation and that their distribution is easily altered by physical agents such as antibodies. The majority of the morphologic and functional data supports the conclusion that GPI proteins tend to cluster and associate with caveolae.

Caveolin-1 interacts with GPI proteins as well as several other proteins and lipids enriched in caveolae. Immunoprecipitates of caveolin-1 from cells exposed to insulin (78) or platelet-derived growth factor (PDGF) (79) contain different sets of tyrosine-phosphorylated proteins. Anticaveolin IgG precipitates can also contain endothelial nitric oxide synthase (eNOS) (80), Ras (36, 81), P75(Ntr) (82), bradykinin receptors (83), and both endothelin and endothelin receptor subtype A (84). Immunoprecipitates of alpha integrin (85), dystrophin (86), and the GPI urokinase receptor (87) contain caveolin-1, whereas specific caveolin-1 peptides bind heterotrimeric Gα (88), Ras (81), and Src (81) in vitro. A photoreactive derivative of GM1 ganglioside binds caveolin-1 after it is inserted into cells (89). Despite the proposal that caveolin-1 is a scaffolding protein (21), there is still no direct experimental evidence that any of these interactions with caveolin-1 are required for targeting to caveolae. To the contrary, both eNOS (90, 91) and nonreceptor tyrosine kinases (53, 54, 92) lacking fatty acids are not concentrated in caveolin-rich caveolae. GM1 gangliosides (35), GPI proteins (35, 76, 93), heterotrimeric G proteins (93), and nonreceptor tyrosine kinases (93) are concentrated in caveolae fractions that do not contain detectable caveolin-1. Furthermore, addition of the K-Ras consensus sequence for prenylation to Raf-1 targets the kinase to caveolae (94), which suggests that farnesylation plays a role in targeting Ras to caveolae.

Reconciling the Biochemical and Morphological Caveola

Not all membrane domains with the biochemical and physical characteristics outlined above have a flask shape. Synaptic plasma membranes, which have numerous membrane invaginations of unspecified origin (95), yield fractions with the properties of caveolae (35). Many cells have tubular invaginations lined by flask-shaped out-pocketings (76) or terminating in clusters of typical flask-shaped membrane. Similar structures appear to form into T tubules during skeletal muscle cell differentiation (96). A tubular intracellular compartment (71) rich in detergent-insoluble GPI proteins (97) is prominent in neutrophils. GPI proteins also co-localize with tubular invaginations in placental epithelial cells (76). Clustered GPI proteins in the neuronal cell line N2A are associated with membrane invaginations that have variable morphology (76) but apparently lack a striated coat (98).

Thus, the shape of a caveola in thin-section EM is variable, not all flask-shaped membranes have detectable caveolin-1, and a striated coat is not always visible. The common features of this domain are a light buoyant density, a GSL/SPH/cholesterol lipid core in the liquid-order phase, a high concentration of lipid-anchored proteins and signaling molecules, and a discrete size. The only viable conclusion is that patches of membrane with these properties are dynamic domains that assume different shapes depending on their activity in the cell. Flat caveolae that contain caveolin-1 and have a striated coat, for example, become flask shaped during internalization (99). Modifiers such as flat, invaginated, tubular, and vesicular should be used to indicate more precisely which caveolae shape is being considered. In cases where the native shape is unclear, the term caveolae-like is a suitable identifier.

BIOGENESIS AND MAINTENANCE OF CAVEOLAE

The building of caveolae is a multistep process that involves both an initial assembly step and a mechanism for actively maintaining the structure so that it can function properly at the cell surface (Figure 3).

Initial Assembly

The detergent-insoluble, GSL/SPH/cholesterol lipid core of the caveola membrane forms in the transitional region of the Golgi apparatus (Figure 3*A*) (24, 43). GPI proteins and caveolin-1 (24), arriving from the endoplasmic reticulum (ER) after synthesis, are then incorporated to complete the initial assembly step. Anticaveolin-1 immunoprecipitation and chemical cross-linking experiments indicate that other proteins associate with caveolin-rich membrane at this point (24). Caveolae are shipped to the cell surface embedded in the membrane of exocytic vesicles (Figure 3*A*) (100). There is no direct evidence that they bud off and migrate as independent vesicles, although this is a formal possibility.

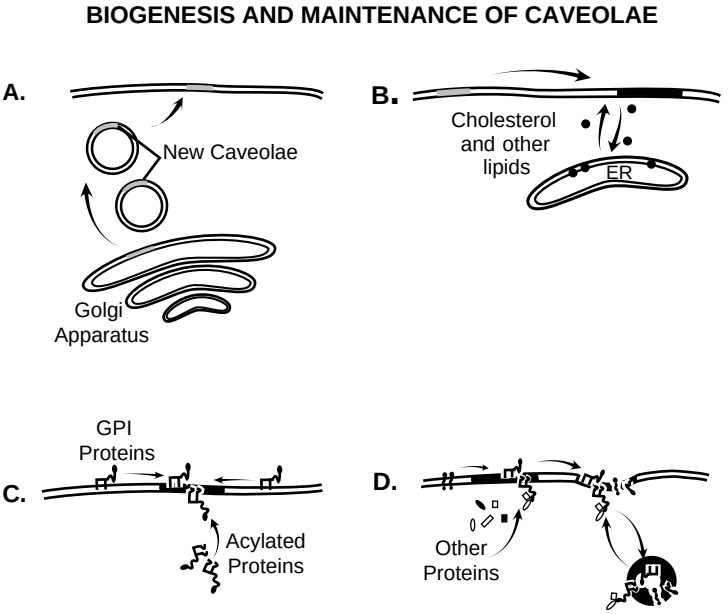

Figure 3 Biogenesis and maintenance of caveolae. (*A*) Caveolae biogenesis begins with the formation of glycosphingolipid (GSL)/sphingomyelin (SPH)/cholesterol-rich domains in the Golgi apparatus. Here the phase transition takes place in which the detergent-insoluble properties of this membrane are created. This is also where caveolin-1, glycosylphosphatidylinositol (GPI)-anchored proteins, and other proteins initially join the membrane. (*B*) New caveolae are shipped to the cell surface, where the lipid shuttle (*solid circle*) begins transporting cholesterol and other lipids from the endoplasmic reticulum (ER). (*C*) The lipid shuttle maintains the liquid-order phase of the caveolae core lipids, which is essential for concentrating GPI and acylated proteins migrating in and out of the domain. (*D*) Protein-protein interactions between lipid-anchored proteins in caveolae and nearby peripheral and transmembrane proteins bring additional molecules to the domain. Once the assembly process is completed, caveolae internalize molecules and deliver them to specific locations in the cell.

A natural outcome of the assembly process is the sorting of specific proteins and lipids away from bulk membrane components. Extensive work in polarized epithelial cells has documented how sorting by GSL/SPH/cholesterol domains—sometimes referred to as rafts (101) or DIGS (102)—contributes to the overall membrane polarity of the cell (37, 38).

The biogenesis of caveolae points out a major difference between this membrane domain and those coated with peripheral proteins such as clathrin. Caveolae are assembled in the Golgi apparatus and then shipped to other locations while clathrin-coated membrane is assembled de novo at sites of vesicle formation (103). Therefore, caveolae-like domains may exist in all membranes

that traffic to and from the cell surface (104). They behave as coherent patches of membrane (47) immersed in the lipid bilayer, like icebergs floating in a sea (101, 105, 106). Surprisingly, the core lipids do not melt into the surrounding bilayer. In part, this is because the lipid composition of the domain is continuously maintained (Figure 3*B*,*C*).

Maintenance

Both cholesterol and SPH contribute to the lipid β phase (107). Cholesterol, however, is constantly fluxing out of the cell (108). Several immediate consequences result when caveolae cholesterol levels get too low. GPI proteins no longer cluster properly in caveolae (27). The striated coat disassembles (6), and the number of invaginated caveolae declines (27). Eventually internalization by caveolae ceases (27). Pharmacologic agents that block cholesterol transport to the cell surface have exactly these effects (19), which suggests that cholesterol is continuously transported to caveolae. A novel transport system has been identified that appears to be necessary for maintaining the proper level of cholesterol, and maybe other lipids, in caveolae.

Cholesterol moves bidirectionally between the ER and the plasma membrane (109). Transport of newly synthesized cholesterol to the cell surface is rapid (110, 111) and occurs in a light membrane fraction that does not appear to pass through the Golgi apparatus (110, 112–115). Newly synthesized cholesterol that has accumulated in the ER at 14°C is rapidly transferred to caveolae upon shifting the temperature to 37°C (19). This process suggests that the light membrane fraction is related to caveolae. The arrival of new cholesterol in caveolae is followed by the immediate movement of the sterol to noncaveolae membrane and possibly out of the cell (19). Caveolae have also been identified as intermediates in the cellular efflux of both newly synthesized cholesterol and cholesterol delivered to the cell by low-density lipoprotein (LDL) (116, 117). Therefore, cholesterol, and possibly other lipids, are constantly flowing through caveolae.

Bidirectional ER-to-caveolae transport of cholesterol appears to involve caveolin-1. The caveolae fraction from cells expressing caveolin-1 has a cholesterol-to-protein ratio that is four- to fivefold higher than that of noncaveolae fractions (19, 25). Selective oxidation of caveolae cholesterol with cholesterol oxidase causes caveolin-1 to move from caveolae to the ER and eventually accumulate in the Golgi apparatus (25). After the enzyme is removed, caveolin-1 reappears in caveolae at the same time that the cholesterol levels return to normal. Caveolin-1 also leaves the surface and accumulates in internal membranes when cells are exposed to progesterone (19), a condition that depletes caveolae cholesterol. Finally, transfection experiments show that caveolae fractions become enriched in cholesterol when caveolin-1 is expressed. Expression is

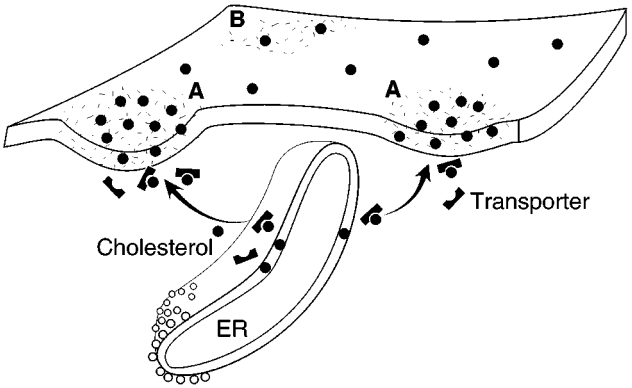

Figure 4 Endoplasmic reticulum (ER)-caveolae lipid shuttle. Cells have both high-cholesterol (*A*) and low-cholesterol (*B*) caveolae. The cholesterol level is maintained by a lipid transporter system that in some cells uses caveolin-1 as the cholesterol carrier. The molecular details of this system remain to be elucidated. Most likely the transporter can work in both directions and therefore is able to transport cholesterol from the plasma membrane to the ER. It may also transport lipids other than cholesterol.

accompanied by a four- to fivefold increase in the rate of cholesterol transport to the surface (19).

Caveolin-1 binds cholesterol (28), preferentially incorporates into cholesterol-containing membranes in vitro (28, 29), and moves between caveolae and internal membranes. High cholesterol levels in cells also cause an increase in caveolin-1 mRNA (118). Therefore, caveolin-1 appears to be part of an intracellular lipid transport system capable of moving sterols between ER and caveolae (Figure 4). The proteolipid MAL/VIP17, which is found in caveolae fractions (119, 120), may also be part of this system. The ER in all cells is near the plasma membrane, sometimes entwined with invaginated and vesicular caveolae (Figure 1*A*). Thus these molecules can easily move between the two membranes. Cholesterol acceptor/donor molecules such as high-density lipoprotein (HDL) influence the net direction of cholesterol movement. Recently the high-affinity HDL receptor SR-B1 was localized to caveolae (121), placing it in a position to facilitate the transfer of cholesterol between HDL or LDL and caveolin-1.

Caveolae contain different levels of cholesterol (and may be other essential lipids), depending on the activity of the lipid shuttle (*A*, Figure 4). High-cholesterol domains perform the full range of caveolae functions such as maintaining clustered proteins and lipids (Figure 3*C*) or internalizing molecules (Figure 3*D*). Low-cholesterol domains (*B*, Figure 4) perform only a subset of

these duties. Cells containing low levels of caveolin-1 have reduced cholesterol transport, so formation of invaginated caveolae is suppressed (122). Conversely, cells expressing high levels of caveolin-1 like adipocytes (123) transport more cholesterol and as a consequence have greater numbers of invaginated caveolae (124).

INTERNALIZATION BY CAVEOLAE

Caveolae are involved in endocytosis. The underlying mechanism of invagination, budding, and vesicle trafficking differs significantly from the coated pit pathway.

Transcellular Transport

Space precludes a critical evaluation of the extensive literature on transcellular transport in endothelial cells. Both morphologic (4, 125, 126) and biochemical (127, 128) evidence supports the view that caveolae are the source of vesicles that move between the two surfaces of the cell. The process is inhibited by *N*-ethylmaleimide (NEM) (129, 130) and filipin (127) and may require the hydrolysis of GTP (128). Vesicles appear to move directly without merging with an intermediate compartment (125, 126, 131). Serial section and dye penetration show that many caveolae vesicle profiles in endothelial cells are actually open to the cell surface (132). Endothelial cell caveolae can fuse to form transcellular channels that allow the passage of small molecules across the cell (126). The vesiculo-vacuolar organelle (VVO) in the cytoplasm of the endothelial cells lining tumor microvessels may also be channels formed by the fusion of multiple swollen caveolae (133). Topical application of vascular endothelial growth factor (VEGF) rapidly induces the swelling and fusion of caveolae (134), which indicates that the formation of caveolae channels is under hormonal control. The swelling might depend on the water channel aquaporin-1 (135). Therefore, the formation of tubule-shaped caveolae appears to be part of a regulated transendothelial transport pathway. Tubular caveolae in muscle cells, neutrophils, and placental epithelia may have a similar origin.

Potocytosis

The GPI-anchored folate receptor provided the first biochemical clues that caveolae could mediate the uptake of molecules and ions in a variety of cells. The cardinal features of this pathway were discovered in MA104 cells (5). These cells express a limited number of clustered receptors that often appear to be associated with invaginated caveolae and are not detected in the coated pit pathway (63). Folate receptors internalize bound 5-methyltetrahydrofolate (136) while maintaining a constant ratio of internal and external receptors. Internalized 5-methyltetrahydrofolate dissociates from its receptor in response to an

acidic environment (136, 137) and diffuses directly into the cytoplasm of the cell (138). Accumulation of folate in the cytoplasm plateaus even though the receptor continues to internalize (137). A minor population of folate receptors appear in the coated vesicle pathway of cells expressing high numbers of receptors (139). But when chimeric receptors are targeted specifically to coated pits, folate delivery is inefficient and unregulated (52). The process was named potocytosis (5) to emphasize the special ability of caveolae to concentrate and move molecules or ions into the cell.

Potocytosis was confirmed and extended by the discovery that caveolae mediate the delivery of molecules to the ER. The first molecule found to travel this route was caveolin-1 (25, 26). Viral pathogens also appear to reach the ER by caveolae (140–142). Membrane-bound simian virus 40 (SV40) becomes trapped in tight-fitting membrane invaginations that have a light buoyant density (141), are detergent insoluble (141), and contain caveolin-1 (141, 142). From monopinocytic vesicles (142), viruses next appear in smooth, tubular membrane extensions of the ER. A variety of molecules and ions may be delivered to the ER during potocytosis. These include lipids such as cholesterol, Ca^{2+} (see Table 1) as well as other ions, and ligands bound to receptors in caveolae. Opportunistic molecules such as cholera toxin (143) possibly reach the ER by this pathway too.

Complementary studies on the internalization of alkaline phosphatase (74), the folate receptor (99, 144), and cholera toxin (74) indicate that membrane recycling occurs during potocytosis. The mechanism of recycling appears to depend on the activity of protein kinase Cα (PKCα) and a serine/threonine phosphatase activity present in purified caveolae (144). During folate uptake, a constant pool of internal and external receptors is maintained. Cells depleted of PKCα no longer internalize receptors; instead, the internal pool returns to the cell surface (99). By contrast, the phosphatase inhibitor okadaic acid causes a decline in the number of invaginated caveolae and the intracellular accumulation of cholera toxin–positive vesicle and tubular caveolae profiles (74). More of these profiles appear when coated pit uptake is blocked. Okadaic acid also causes alkaline phosphatase to leave the surface, consistent with a block in the recycling of internalized enzyme. The effects of okadaic acid are prevented by staurosporine, an inhibitor of PKCα. The internalization of GPI CD59 by lymphocytes (145) and SV40 by fibroblasts (141) also appears to depend on PKCα.

If these two enzymes control opposing limbs of a caveolae recycling pathway, then inactivation of PKCα would inhibit sequestration by caveolae, and inhibiting the phosphatase(s) would cause the accumulation of caveolae vesicles. Several studies indicate that the internalization cycle can be regulated at these two sites. Histamine binding to H_1 receptors transiently inhibits the

internalization of clustered folate receptors (144) by inactivating PKCα. Exposure of MA104 cells to indomethacin prevents both internalization and externalization of folate receptor by an unknown mechanism (146). Tubular caveolae in unstimulated neutrophils sequester three GPI proteins [alkaline phosphatase, decay accelerating factor (DAF), and CD16] away from the surface, but all three rapidly reappear when cells are stimulated with chemotactic peptide (71, 147–149). Up to 20% of the cholecystokinin receptors are sequestered by caveolae after ligand binding, but when the coated pit pathway is blocked, nearly all the receptors become sequestered (150). Ligand binding also stimulates caveolae sequestration of bradykinin receptors (83) and possibly endothelin (151). Therefore, caveolae vesicles may be used regularly as compartments for storage, processing, and rapid deployment of molecules to the surface (152).

Besides a requirement for cholesterol (27) and possibly caveolin-1, little is known about how caveolae invaginate and bud from membranes (Figure 3*D*). Based on sensitivity to various treatments, however, they use a mechanism that is different from the one used by coated pits. Inhibitors of caveolae vesicle formation include cholesterol-binding drugs (127, 141, 153), cytochalasin D (74, 145), and in some cases PKC inhibitors (141, 154). Clathrin-coated vesicle formation is selectively (74, 150) blocked by hypertonic treatment (155) and K^+ depletion (156). Both processes are sensitive to NEM (129, 157). Even though several proteins implicated in vesicle trafficking have been localized to caveolae fractions (158), it is not clear whether tubular or vesicular caveolae ever fuse with endosomes from coated pits. Cholera toxin, for example, can reach endosomes (74, 159, 160), but quantitative studies (160) indicate it is only a subfraction of the total surface-bound toxin and could easily have arrived by rapidly recycling coated pits. In fact, neither labeled toxin (74) nor cholecystokinin (150) are usually found in typical endosomes when the coated pit pathway is blocked.

Caveolae and clathrin-coated pits are specialized to internalize different types of molecules. Therefore, potocytosis and receptor-mediated endocytosis (157) are parallel, but not redundant, endocytic pathways. Molecules internalized by potocytosis follow one of four distinct intracellular routes (Figure 5). From the cell surface they travel to the cytoplasm (Figure 5*A*), the endoplasmic reticulum (Figure 5*B*), the opposite cell surface (Figure 5*C*), or a caveolae-derived tubular/vesicular compartment (Figure 5*D*). Rarely do receptors or ligands appear in an intermediate compartment during ligand delivery. That is, the carrier vesicles retain many of the morphological and biochemical properties of caveolae. As a consequence, vesicular compartments generated during internalization can transform into exocytic vesicles that carry molecules back to the surface. This type of compartmentalization most likely has a variety of special uses in the cell (152).

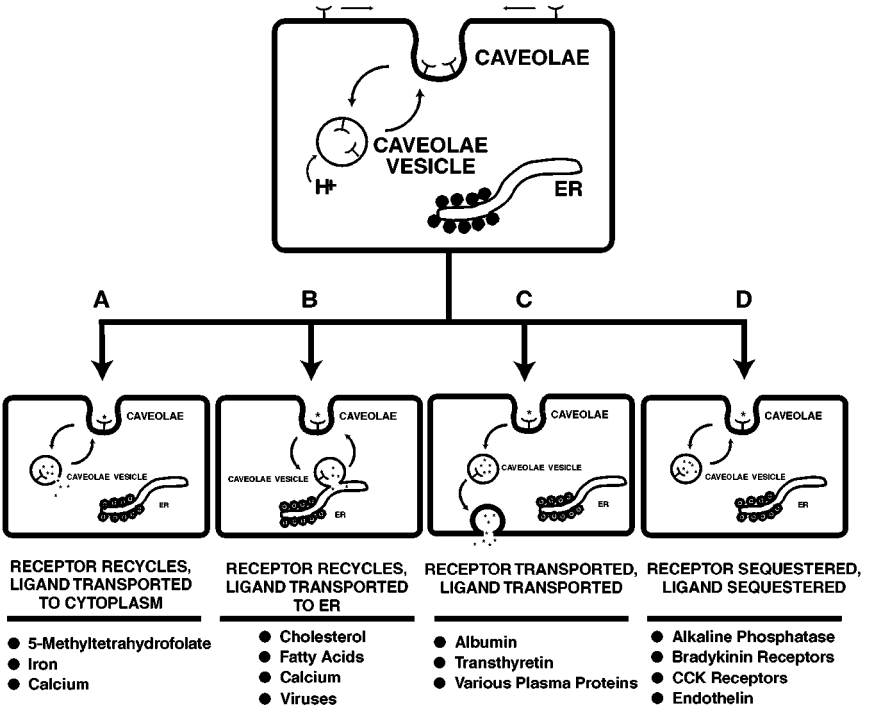

Figure 5 Multiple pathways of potocytosis. Molecules and receptors internalized by caveolae can have one of four fates: (*A*) The ligand is delivered to the cytoplasm while the receptor recycles back to the surface. (*B*) The ligand is delivered to the ER while the receptor recycles back to the surface. (*C*) The ligand is transported across the cell, and the receptor recycles. (*D*) Both the ligand and the receptor remain in a vesicular caveolae compartment. Examples of molecules that follow these routes are listed.

SIGNAL TRANSDUCTION FROM CAVEOLAE

The original potocytosis model (5) predicted that caveolae were involved in cell signaling at the plasma membrane (152, 161). No one was prepared, however, for the variety of signaling molecules that have now been found concentrated in this domain.

Tyrosine Kinases

Receptor and nonreceptor tyrosine kinases (PTK) are reliable markers for caveolae (Table 1). With one exception (34), all the purification methods find PTKs to be substantially enriched in caveolae isolated from a variety of cells and tissues. Moreover, the immunoprecipitation of multiple GPI proteins coprecipitates

PTKs (162). Immunoblotting, enzymatic activity, and immunocytochemistry all indicate that this is a major location for PTKs. For example, by immunofluorescence nearly all of the PDGF receptor in quiescent fibroblasts colocalizes with caveolin-1, and the majority of the receptor is recovered in isolated caveolae (79).

The first indication that PTKs were in caveolae can be traced to the paradoxical finding that antibodies directed against GPI proteins stimulated PTK activity (162–164) and PTK-dependent cell functions (164–166). A complex of GPI proteins was then found to be associated with nonreceptor PTKs (167–169). The complexes exhibited several properties of caveolae, including detergent insolubility, richness in glycolipids (170), a light buoyant density (171), and a size of ~100 nm. These studies were also the first to show that multiple kinase substrates are enriched in caveolae-like membranes and that phosphorylation of these substrates can occur in vitro. PTK activity has now been localized to invaginated caveolae by immunocytochemistry (172). A major substrate for PTKs is caveolin-1 (173–175). Tyrosine phosphorylation of caveolin-1 is stimulated by insulin (78), oxidants (176), sulfonylurea (177), and cell transformation (173, 178). A peptide sequence in caveolin-1 (amino acids 82–101) that interacts with c-Src possibly modulates PTK activity (81). PDGF stimulates the phosphorylation of multiple caveolae proteins, both in vivo (79) and in vitro (172), and its receptor is linked to a preassembled mitogen-activated protein (MAP) kinase module (172). Both PDGF (79) and epidermal growth factor (EGF) (179) stimulate the recruitment to caveolae of multiple signal-transducing molecules as well as the migration of the respective receptor out of caveolae.

GTP-Binding Proteins

A long-standing issue in signal transduction is whether receptors, G proteins, and effectors are organized or randomly distributed at the cell surface (180). A combination of cell fractionation and immunocytochemistry has now documented that all three classes of molecules are enriched in caveolae (Table 1). G proteins, for example, are found in most caveolae preparations. Receptors for endothelin (84), cholecystokinin (150), m2 acetylcholine (181), and bradykinin (83) are dynamically associated with these membranes. Receptors appear to be functionally connected to effectors in caveolae. Histochemistry has localized isoproterenol-stimulated adenylyl cyclase activity to membrane invaginations resembling caveolae (182–184). Isolated caveolae fractions contain a significant proportion of the total adenylyl cyclase activity (185). Isoproterenol-stimulated cyclic AMP formation cofractionates with the enzyme. Caveolae-like fractions from *Dictyostelium* are highly enriched in chemoattractant receptors as well as in adenylyl cyclase and phosphodiesterase

(186). Finally, bradykinin stimulates the recruitment of $G_{\alpha q}$ and $G_{\alpha i}$ to caveolae (83). Several studies suggest that caveolin-1 has a role in recruiting G proteins to caveolae as well as in modulating their activity (12, 36, 81, 88). The functional organization of the cyclase in S49 cells, however, is not dependent on caveolin-1 (185).

Calcium

A model of excitation-contraction coupling mediated by caveolae was proposed in 1974 (187). Since then, considerable evidence has accumulated that caveolae are sites of calcium storage and entry. For example, pyroantimonate precipitates of calcium are present in caveolae of relaxed smooth-muscle cells (188). Stimulation of contraction generates a diffuse distribution of the precipitate in the myoplasm, consistent with a movement of calcium into the cell. A rich collection of morphologic observations documents smooth-muscle–cell caveolae interacting with smooth ER, just as sarcoplasmic reticulum interacts with T tubules in skeletal muscle. Indeed, caveolae play a direct role in the biogenesis of the T-tubule system (96). Ca^{2+} ATPase (33, 189), IP3 receptors (33, 190), and calmodulin (90)—key molecular components of calcium transport—have all been localized to caveolae. These findings suggest a role for ER-caveolae interactions during calcium signaling.

Lipid Signals

Some of the lipids and lipid-anchored proteins incorporated into caveolae in the Golgi apparatus (see Biogenesis and Maintenance of Caveolae, above) are important sources of signaling intermediates. Sphingomyelin, phosphatidylinositol 4,5, bisphosphate, and GPI proteins/lipids are substrates for enzymes that release ceramide (82, 191), inositol trisphosphate (IP3) (192, 193), and inositolphosphoglycans (IPG) (194, 195), respectively. Each is produced in caveolae after a specific stimuli. Ceramide increases after Il-1β (191) or neurotrophin (82) stimulation. IP3 is released after exposure to either bradykinin or EGF (193), and IPG forms in response to insulin (196). These responses appear to be specific because neither ceramide nor IP3 is generated in noncaveolae fractions, and the IPG released on the extracellular side of the membrane is internalized, presumably by caveolae. All three molecules elicit characteristic cellular responses. These are just three examples of what must be a general mechanism whereby lipids sorted to caveolae become the source of critical signaling intermediates.

Compartmentalized Signaling

Caveolae compartmentalize enzymatic reactions at the surface that are important for signaling. Immunocytochemistry (90, 197), cell fractionation (90, 91, 197–199), and immunoprecipitation (200) show that the majority of cell surface

endothelial nitric oxide synthase (eNOS) is located in caveolae. This finding suggests that caveolae are the site of nitric oxide (NO) production. A unique tubular compartment in neutrophils with the biochemical properties of caveolae (71) produces superoxide ($O_2^{\cdot -}$) in response to chemotactic peptides (201), and NAD(P)H oxidase has been localized by histochemistry to invaginated caveolae (202). GPI 5′-nucleotidase targeted to caveolae may convert extracellular 5′-AMP to adenosine where it locally activates receptors (203). Finally, caveolae are likely to be the site where α_7 integrin is ribosylated by a GPI ADP-ribosyltransferase (204). Integrins have recently been found to interact with caveolin-1 (85), and integrin function is regulated by the urokinase receptor (87).

Signal Integration

With so many different signaling molecules in one location, caveolae are the logical place to look for signal integration. Integration refers to the feedback interplay between two or more signaling processes that result in a reciprocal modulation of the interacting pathways. The stimulation of GPI proteins in endothelial cells is used to illustrate the concept. GPI proteins can activate PTKs and generate a Ca^{2+} influx (62, 164). PTKs phosphorylate eNOS, thereby inhibiting the enzyme and promoting its interaction with caveolin-1 (200). But the released Ca^{2+} will bind calmodulin, which activates eNOS. Any NO produced will stimulate the MAP kinase pathway through Ras (205), in synergy with PTKs (172). All the underlined components are in endothelial cell caveolae (Table 1), allowing the cross talk between pathways to occur at one site on the plasma membrane. Furthermore, the ability of caveolae to sequester molecules provides an opportunity for locally produced or imported molecules to modulate these signaling events.

CAVEOLAE AND HUMAN DISEASE

A number of human diseases appear to involve the caveolae membrane system. The system is the target of several pathogens and becomes altered during cell transformation.

Prion Diseases

Prions are a class of proteins that cause fatal encephalopathies in humans and other animals. The posttranslational conversion of cellular prion (Pr^C) to the scrapie isoform (Pr^{Sc}) is the mechanism of transmission. GPI Pr^C has been localized to invaginated caveolae in both fibroblasts and neuronal N2A cells (76) and fractionates with caveolae (35, 206–208). A caveolae localization appears to be necessary for conversion of Pr^C to Pr^{Sc} because replacement of the GPI

anchor of Pr^{C} with a coated pit targeting sequence prevents conversion (209). Lowering cellular cholesterol, which disperses GPI proteins in the membrane (27), also inhibits conversion (210). Accumulation of Pr^{Sc} may impair many different caveolae functions.

Pathogens

Caveolae appear to be the cellular entrance point for pathogens as well as molecules produced by pathogens. The internalization of SV40 by caveolae has already been discussed (see Potocytosis, above). *Campylobacter jejuni* (211) may enter cells by caveolae too. A portion of the surface-bound cholera toxin is internalized by caveolae (74), but it is not known whether the entering A subunit reaches the cytoplasm by this route. Finally, because GPI proteins are able to spontaneously insert and cluster in caveolae (62), they may be the target for GPI proteins shed by parasites such as *Plasmodium*, *Trypanosoma*, and *Leishmania* (212, 213). Purified GPI proteins from these organisms simultaneously activate macrophage PKC and $p59^{hck}$ (213).

Cancer

Invaginated caveolae are substantially reduced in many types of transformed cells (214). The loss of these invaginations is correlated with the tyrosine phosphorylation of caveolin-1 (173, 214) and its loss from the cell (214). Although caveolin-1 was originally discovered as a PTK substrate in v-Src transformed cells (173), other oncogenic viruses have the same effect (214). Caveolin-1 may be a tumor suppresser because expression of the cDNA in transformed cells reverses anchorage-independent growth in soft agar (215). Expression of caveolin-1 could be essential for normal signal transduction from caveolae. Alterations in the permeability of tumor blood vessels resulting from VVOs (133) suggest that caveolae also have an indirect role in tumor formation.

Cardiovascular Disease

Caveolae are abundant in most parenchymal cells of the cardiovascular system. Having key roles in calcium metabolism, cell signaling, blood clotting, and cholesterol transport, caveolae are vulnerable sites in these cells. Caveolae are sensitive to oxidized cholesterol and contain receptors that bind HDL, LDL, and oxidized lipoproteins (121). This raises the possibility of a direct link between damage by oxysterols, inappropriate activation of multiple signaling pathways in caveolae, and cell proliferation during atherogenesis.

CONCLUSION

The rapid growth in caveolae research has brought with it a changing view of this membrane domain. Caveolae constitute a membrane system equal in

complexity to any cellular compartment or organelle. Specific diseases have been identified that can attack this system, making it important to learn more about its normal biology. From another perspective, caveolae are an important research tool. They clearly contain a variety of signal-transducing molecules that interact in characteristic patterns after cell stimulation. The ease of caveolae isolation makes it possible to study at the molecular level how the natural organization of these molecules imparts cell function. Compartmentalized signal transduction is a growing area of research, and caveolae promise to provide many new insights.

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