REVIEW: CELL BIOLOGY

A Role for Lipid Shells in Targeting Proteins to Caveolae, Rafts, and Other Lipid Domains

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The surface membrane of cells is studded with morphologically distinct regions, or domains, like microvilli, cell-cell junctions, and coated pits. Each of these domains is specialized for a particular function, such as nutrient absorption, cell-cell communication, and endocytosis. Lipid domains, which include caveolae and rafts, are one of the least understood membrane domains. These domains are high in cholesterol and sphingolipids, have a light buoyant density, and function in both endocytosis and cell signaling. A major mystery, however, is how resident molecules are targeted to lipid domains. Here, we propose that the molecular address for proteins targeted to lipid domains is a lipid shell.

A domain is a region distinctively marked by some physical feature that distinguishes it from the surrounding landscape. A domain in a cellular membrane, therefore, is a region with physical features that differentiate it from the contiguous membrane—for example, the clathrin-coated pit. Coated pits can be identified in thin-section electron micrographs by the presence of a cytoplasmic fuzzy coat, and so are easily distinguished from the surrounding membrane. Their other properties offer important clues about the rules that govern the construction and maintenance of all membrane domains (*1*).

One obvious rule is that the molecular composition of a membrane domain differs from that of the surrounding membrane. The domain can be enriched in both peripheral and integral molecules. One set of enriched molecules has a structural function. The structural molecules of a coated pit, for example, are the peripherally associated clathrins and clathrin adaptors that form the polygonal coat structure. These molecules are recruited to the plasma membrane in a stepwise process that depends on interactions between the adaptor proteins and both membrane phosphoinositides (*2*, *3*) and integral membrane proteins (*4*). The presence of the lattice on the membrane locally organizes lipids like cholesterol (*5*) and serves as a platform that attracts a variety of integral and peripheral membrane proteins (*6*). Therefore, the molecular composition of this domain is determined by both the coat structure and the molecules it attracts.

Molecules that collect in membrane domains do so because they contain a specific molecular address for that domain. Most of what is known about the addresses for coated pits comes from the study of transmembrane receptors that mediate the uptake of molecules. Addresses come in two basic forms. Either the cytoplasmic tail of the protein contains a binding site that recognizes one or more coat proteins or it recognizes an adaptor protein that, in turn, has a binding site for a coat protein (*7*, *8*). The address, therefore, is encoded by an amino acid sequence in the cytoplasmic tail of the protein. Elimination or modification of the address motif prevents the molecule from accumulating in coated pits (*9*). This raises the possibility that molecular addresses can be dynamically regulated so that a molecule with the proper address spends only a portion of its functional life in its target domain.

Coated pits have taught us that membrane domains are dynamic structures with molecules entering and leaving according to specific rules. They are constructed with specific cellular machinery, and without constant maintenance, coated pits would rapidly dissipate into the surrounding membrane. On the basis of these considerations, cholesterol-sphingolipid–rich lipid domains must have unique physical features, upper and lower size limits, functionality, and a system for removing and adding specific molecules.

Lipid Domains

Lipid domains were first detected in human and hamster fibroblasts as a detergent-insoluble glycoprotein matrix (*10*) rich in glycosphingolipids (*11*). Simons and Van Meer (*12*) postulated that microdomains of similar lipid composition in the Golgi apparatus mediated sorting of sphingomyelin and glycosphingolipids in polarized epithelial cells. A relation between these observations was established with the discovery of caveolin/VIP21 (*13*, *14*). Independent studies showed that this protein is a marker both for plasmalemmal caveolae at the cell surface and apically targeted vesicles produced in the Golgi apparatus of polarized epithelial cells. Both of these membranes are detergent insoluble, rich in sphingomyelin and glycosphingolipids, and appear to contain similar molecules. Their function also depends on cholesterol (*15*–*17*).

Caveolae. The caveola is a lipid domain that was first described over 40 years ago as a membrane invagination on the surface of gallbladder epithelial cells (*18*) and endothelial cells (*19*). Their unique physical features can include a distinctive membrane coat composed of caveolin-1 (*16*), an absence of intramembrane particles in freeze fracture images (*20*), cholesterol concentrated around the rim of the domain (*20*, *21*), and a flask-shaped, invaginated morphology during internalization. The lower size limit appears to be the diameter of a flask-shaped caveola (50 to 80 nm); the upper limit is more variable. The cytoplasmic coat can occupy an area up to ~150 nm in diameter (*16*). Moreover, caveolin is associated in some cells with tubular invaginations that extend several micrometers into (*22*), and even across (*23*), the cell. A functional role for caveolae in endocytosis and signal transduction has been established (*24*). Finally, specific molecules have been identified that are dynamically associated with this membrane domain, including receptor tyrosine kinases (*25*) and glycosylphosphatidylinositol (GPI)–anchored proteins (*26*).

Rafts. The term "lipid raft" (*27*) is defined operationally by the procedures used to isolate a population of membranes from cells. Thus, rafts are a collection of membranes characterized by insolubility in nonionic detergents at 4°C; a special lipid composition that is rich in cholesterol, sphingomyelin, and glycolipids like GM1 ganglioside (*28*); and a light buoyant density (LBD) on sucrose gradients (*27*). One group of membranes that have this property are caveolae (*29*). Some cells, however, do not express caveolin and appear to lack typical flask-shaped membrane invaginations (*30*). LBD fractions from these cells are enriched in many of the same molecules found concentrat-

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ed in the caveolae of caveolin-expressing cells (*31*). It is not clear if noncaveolae rafts share a common origin with caveolae or how they function differently. The inability to identify unambiguously noncaveolae rafts by using morphologic techniques complicates the study of these domains.

Molecular addresses for lipid domains. The caveola is the only membrane in the LBD fraction that has been characterized at the ultrastructural level in cells. Noncaveolae rafts must also occupy space in the membrane, but even with the use of sophisticated microscopic techniques, a clear consensus about the size, shape, and location of noncaveolae rafts has not emerged (Table 1). Regardless of their actual size, these studies document that certain proteins are dynamically associated with lipid domains. For example, direct immunofluorescence (*32*) and immunoelectron microscopy (*33*) have shown that GPI-anchored proteins are uniformly distributed on the cell surface. These proteins, however, become clustered when cells are exposed to cross-linking antibodies. Importantly, the simultaneous addition of two antibodies that recognize different GPI-anchored proteins (e.g., the folate receptor and alkaline phosphatase) causes coclustering of the two proteins (*33*), whereas separately each antibody clusters only the protein it is directed against. Electron microscopy (EM) measurements have shown that these clusters are ~100 nm in diameter (*34*). In cells expressing caveolin-1, clusters colocalize with caveolin-rich regions of the cell surface (*34*) that often display flask-shaped morphology in EM images. The same numbers of invaginated caveolae are seen in cells before and after the addition of antibody (*34*), suggesting that the cross-linking antibodies cause the protein they recognize to migrate to preexisting caveolae. Why then do these proteins migrate to caveolae and not to some other membrane domain?

Protein-targeting theory (*35*) predicts that proteins collect in caveolae, coated pits, and other membrane domains because they contain specific molecular addresses that direct them to these locations. Many transmembrane proteins have critical tyrosine residues or dileucine motifs in their cytoplasmic portion that function as molecular addresses (*6*). Molecules that cluster in caveolae/rafts, however, often do not extend into the cytoplasm, and those that do will collect in caveolae/rafts in the absence of this part of the molecule (*36*). This suggests that the molecular address that directs membrane molecules to caveolae/rafts is in the membrane anchor, the extracellular region, or both. Another property of proteins that are targeted to caveolae/rafts is their light buoyant density before they are induced to cluster in caveolae/rafts by exposure to cross-linking antibodies (*32*, *37*). In other words, these proteins have a light buoyant density without being in a lipid domain containing thousands of molecules.

Lipid Shells

We believe that the LBD property of these molecules is an important clue about why they cluster in caveolae/rafts. They have a light buoyant density not because they are in a lipid domain, but because they are encased in a shell of cholesterol and sphingolipid. A typical protein that collects in caveolae/rafts is the GPI-anchored, 25-kD Thy-1. It has a density of 1.37 g/ml, which would cause it to migrate at the bottom of the 38 to 5% sucrose gradient used to purify Triton X-100–insoluble caveolae/rafts. The association of just 80 molecules of cholesterol-sphingolipid (lipid density 1.035) with this protein, however, would sufficiently reduce its density so that it floats in these gradients. Most likely, the Triton X-100 in the fraction will bind to the lipid shell and further lower the buoyant density of the protein. The estimated diameter of a cholesterol-sphingolipid–rich shell containing 80 lipid molecules is ~7 nm. The lipid shell is conceptually analogous to solvent shells, such as the hydration shells that surround proteins or ions in aqueous solution and mediate the structural transition from the solute to the bulk solution. Thus, lipid shells and the proteins they surround need not form a separate lipid phase but exist as mobile entities in the plane of the membrane. We hypothesize that lipid shells are thermodynamically stable structures that have an affinity for preexisting caveolae/rafts. Hence, they target the protein they encase specifically to these membrane domains.

Condensed complexes. We propose that shell formation is a dynamic process that depends on the self-assembly of cholesterol-phospholipid complexes and on the propensity of certain proteins to associate with these complexes (Fig. 1). The ability of sphingolipids to cluster appears to depend on hydrogen bonding between themselves and cholesterol (*38*, *39*). Thompson and Tillack (*40*) first realized that sphingolipids could potentially form small clusters in the plane of the membrane. More recent studies (*41*) have found that the thermodynamically nonideal mixing behavior of cholesterol-phospholipid monolayers at relatively low surface pressures can be accounted for by the presence of "condensed" complexes of cholesterol and sphingolipid. Each complex contains 15 to 30 molecules, usually in a stoichiometric ratio of 2 sphingolipids to 1 cholesterol. Importantly, these complexes may or may not phase separate in biological membranes. In other words, condensed complexes need not exist as a discrete phase while populating a relatively cholesterol-poor phase in the membrane. Condensed complexes may only form a defined "macrophase" under certain conditions, such as when clustered in lipid domains (Fig. 2).

We believe that interactions between specific proteins and condensed complexes differ in important ways from interactions between proteins and boundary lipids, which were postulated over 20 years ago to surround integral membrane proteins (*42*). While there may be circumstances in which boundary lipids might function as lipid shells, we believe that in general they are less stable. Boundary lipids are detected on electron spin resonance but not nuclear magnetic resonance time scales, suggesting that the residence time for a lipid in the boundary layer is no more than ~10-fold longer than its resi-

Table 1. Methods used to determine the size of lipid rafts give different results. The conventional fluorescence resonance energy transfer (FRET) results could mean that there are no rafts (0 size) or that the density of the marker protein is too low to support intermolecular FRET (*65*). 5′NT, 5′ nucleotidase; FR, folate receptor; DAF, decay accelerating factor; PALP, placental alkaline phosphatase; HA, hemagglutinin; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine.

Method	Cell	Marker	Size (nm)	No. of molecules	Ref.
Conventional FRET	MDCK HeLa NRK FAO	GM1 5′NT FR CD59	0	0	(*37*, *65*)
Molecular cross-linking	BHK	DAF	>4 nm	15	(*66*)
Laser trap	BHK	PALP HA	~50 nm	3000	(*67*)
Depolarization FRET	CHO	FR	<70 nm	6000	(*68*)
Single-particle tracking	C3H	GM1	~200 nm	50,000	(*69*)
Single dye tracing	HASM cells	Thy-1 DMPE	~700 nm	600,000	(*70*)

dence in the surrounding bilayer. By contrast, we speculate that a lipid shell is formed by long-term interactions between specific lipids and selected proteins in the membrane. A study of the biophysical properties of condensed complexes bound to a membrane protein might reveal important relationships between boundary lipids and lipid shells.

Interaction of proteins with lipid complexes. An impressive literature has accumulated documenting the lipid-binding properties of a variety of integral and peripheral membrane proteins (Table 2). Many of these proteins interact with specific lipids. For example, caveolin-1, synaptophysin, prominen, α-hemolysin, and NAP22 interact with cholesterol, whereas molecules such as MARCKS interact with phosphatidylserine (PS). Because lipid-interacting proteins are found both in membranes and in the cytosol, we expect there to be both peripheral and integral membrane proteins that are capable of forming lipid shells. The potential for various proteins to strongly interact with lipids is undeniable and, thus, is the crucial underpinning of the shell hypothesis.

We surmise that GPI-anchored proteins use multiple lipid-lipid, glycan-lipid, and protein-lipid interactions to stabilize their association with condensed complexes (Fig. 1A). The GPI-anchored proteins of all higher eukaryotic cells contain at least one ethanolamine phosphate moiety attached to the glycan, which could ion-pair with the phosphoryl head group of sphingomyelin. The glycan is thought to have an extended conformation along the plane of the membrane covering up to 6 nm^2 in the case of variant surface glycoprotein of trypanosomes (*43*, *44*), which may further stabilize cholesterol-sphingolipid complexes by providing an "umbrella" that protects cholesterol from water (*45*). Another consideration is that GPI-anchored proteins usually have longer, more saturated acyl chains that would preferentially associate with the longer, more saturated acyl chains in the sphingolipids (*46*) present in condensed complexes, thereby ameliorating the hydrophobic mismatch between the GPI-anchor and the bulk bilayer. Finally, recent studies suggest that a structural motif common to a GPI-anchored protein mediates binding to sphingolipids (*47*). Combinatorial extension methods have revealed that the V3 region domain of HIV-1 gp120 is also present in β-amyloid and prions. All three proteins bind to sphingolipids, and a mutation in this region of prion protein abrogates binding to sphingolipids. Furthermore, prions will bind to cholesterol-sphingolipid–rich synthetic membranes (*48*). As yet, there is no experimental evidence that GPI-anchored proteins can associate with cholesterol-sphingolipid complexes, but it has recently been shown that Thy-1 incorporated into dimyristoyl-phosphatidylcholine vesicles will organize about 50 lipid molecules (*49*).

There may be multiple ways in which shell-forming transmembrane proteins interact with cholesterol-sphingolipid complexes. One way is through the transmembrane region (Fig. 1B). An example of such a protein is the platelet-derived growth factor receptor (PDGFR), which appears to be associated with cholesterol and sphingolipids when it is in caveolae (*50*). Another example of such a protein is influenza haemagglutinin. Although a direct association of this transmembrane protein with membrane lipids has not been demonstrated, the protein has a light buoyant density (*51*) and an ability to collect in caveolin-rich membranes (*52*) that is abolished by changing the amino acid sequence of the transmembrane domain (*51*). There appears to be a requirement for hydrophobic residues to be in contact with both the exofacial leaflet of the bilayer and cholesterol. Thus, formation of lipid shells around transmembrane proteins may involve direct interactions between cholesterol-sphingolipid complexes and specific amino acids in the transmembrane domain.

Another potential way in which shells might form is through electrostatic interactions between charged amino acids in a protein and the oppositely charged head groups of phospholipids (Fig 1C). An example of a protein that interacts electrostatically with lipids in membranes is MARCKS (*53*). MARCKS is a myristoylated membrane protein that contains a polybasic effector region between amino acids 151 and 175. The NH_2-terminally attached myristate causes the protein to weakly associate with the membrane and position the effector region so that it can bind acidic phospholipids. MARCKS bound to acidic lipids like PS can be desorbed from phosphatidylcholine:phosphatidylserine (PC:PS) liposomes by calmodulin as well as by phosphorylation, which suggests that the binding of MARCKS to lipids can be regulated. In membrane monolayers, cholesterol can form condensed complexes with PS in the same way that cholesterol-sphingolipid complexes form (*41*). Thus, electrostatic interactions offer a mechanism whereby proteins can reversibly acquire a lipid shell by binding preformed cholesterol-PS complexes

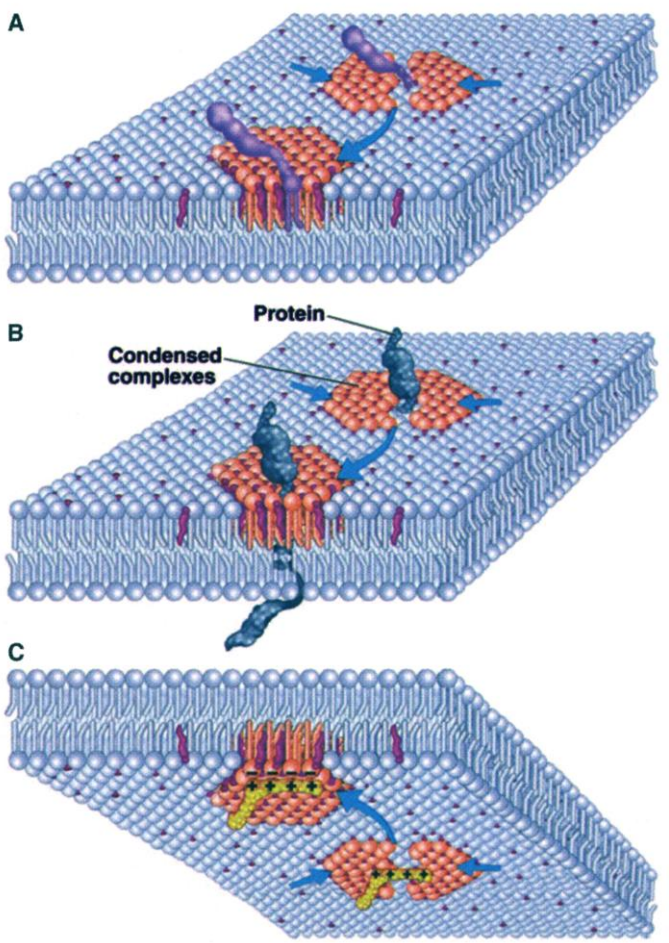

Fig. 1. Mechanism of lipid shell formation. Cholesterol (purple) and sphingolipids (orange) self-assemble to form cholesterol-rich complexes in the membrane. The orange head groups of the sphingolipids project above the phospholipid head groups owing to the longer fatty acyl chains on the sphingolipids. The model proposes that each protein interacts with at least two complexes (right and left blue arrows). (**A**) The mechanism of shell formation around GPI-anchored proteins (purple) can involve a combination of physical interactions with the condensed complexes, including hydrophobic mismatch, water exclusion from cholesterol, glycan-sphingolipid interactions, and protein-sphingolipid interactions. (**B**) Specific transmembrane proteins (blue) have the ability to dynamically associate with two or more condensed complexes, thereby becoming encased in a shell of cholesterol-sphingolipid (curved blue arrow). (**C**) On the inner membrane surface, the predominant condensed complex is composed of cholesterol and PS. Shell formation around polybasic proteins like MARCKS (yellow) occurs when they electrostatically interact with these negatively charged condensed complexes.

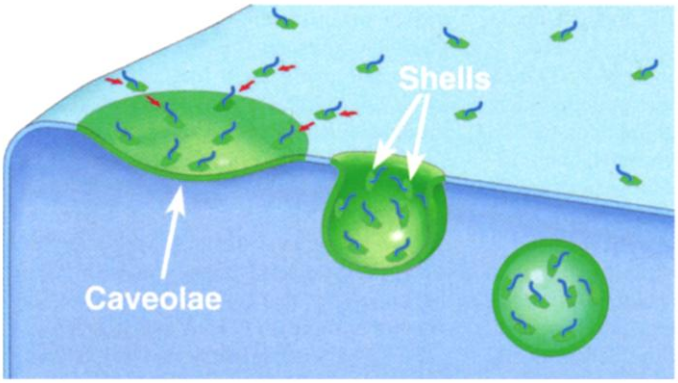

Fig. 2. Sorting of lipid shells by caveolae/rafts. A scale model of the relation between shelled proteins and caveolae/rafts. Once a protein associates with condensed complexes (green polygons), it is targeted (red arrows) to lipid domains like caveolae/rafts (green invaginations). Caveolae/rafts enriched in shelled proteins can bud from the membrane and carry their cargo to specific locations in the cell.

in the membrane. This mechanism of shell formation and stabilization may be particularly relevant to the inner monolayer of the plasma membrane, which is rich in PS and contains cholesterol as well as a number of acylated proteins with polybasic domains (*54*).

Targeting of Lipid Shells to Lipid Domains

Lipid shells are the missing link in a membrane-sorting process postulated over 10 years ago to be essential for normal cell polarity (*55*). Lipid shells may not form a separate lipid phase, but are mobile in the plane of the membrane. Because of the lipid nature of the shell, they are attracted to lipid domains that are formed by specialized cellular machinery (Fig. 2). The detergent-insoluble property of caveolae/rafts has been attributed to the liquid-ordered (l_o) phase of cholesterol-sphingolipid–rich membranes (*46*). We postulate that molecules surrounded by a lipid shell have an affinity for membranes that have a l_o phase because of the molecular compatibility between lipids of the shell and the l_o phase. Thus, there are at least two potential steps wherein the attraction of a protein to a caveola/raft might be regulated. The first is when the protein associates with condensed complexes to form the lipid shell. The second is when the shelled protein associates with caveolae/rafts. Once attracted to caveolae/rafts, protein and carbohydrate interactions between the shelled molecule and resident molecules in these domains may increase the length of time the shell remains at this site. In addition, the movement of shelled molecules into or out of caveolae/rafts may be further influenced by specific ligands. For example, the binding of epidermal growth factor to epidermal growth factor receptor stimulates the receptor to move out of caveolae (*25*), whereas cross-linking antibodies favor the clustering of the folate receptor within caveolae (*34*). One might expect that shelled molecules on opposite sides of the membrane would be targeted to lipid domains independently of each other. At least in one case, however, antibody-induced clustering of a GPI-anchored protein results in coclustering of doubly acylated src-like kinase *fyn* (*32*). This raises the possibility that transbilayer interactions occur between lipid shells situated on opposite sides of the membrane (*39*).

Sorting of transmembrane proteins like the low density lipoprotein (LDL) receptor depends on interactions with adaptor proteins that bind the cytoplasmic portions of each receptor (*1*). A special function of the lipid shell, therefore, is to mediate the sorting of molecules independently of transbilayer interactions. A lipid-based sorting mechanism offers a wide range of possible sorting modalities that may be crucial for the biogenesis of membrane compartments in the cell (*55*). For example, caveolae/rafts in the Golgi apparatus and at the cell surface may play a crucial role in sorting molecules marked with lipid shells and moving them in vesicles to specific locations in the cell (Fig. 2). Much remains to be learned about the intracellular traffic of these and other lipid domains.

Future Directions

If lipid shells surround individual proteins and target them to caveolae/rafts measuring 50 to 200 nm in diameter, the challenge is to detect these entities and distinguish them from higher levels of lipid lateral organization. We surmise that at the steady state a protein capable of being targeted to caveolae/rafts will be either unshelled, shelled, or clustered in the domain. Several methods can distinguish between these three species. Molecular dynamics computations have the potential to determine whether the interactions we postulate to occur between a protein and the lipid components of a shell lead to enhanced stability of the structure. In addition, rotational diffusion measurements that are sensitive to the size of the diffusant can distinguish whether a protein is surrounded by a shell of very small diameter or, instead, is embedded in a membrane domain with the dimensions of a caveola or raft (*56*). Another potential way to determine whether a protein is in a shell or a raft is by fluorescence

Table 2. Cholesterol and sphingolipid binding proteins and their putative functions. SP-A/B, surfactant proteins A and B; HE1, human epididymis; PDGFR, platelet-derived growth factor receptor; PG, phosphatidylglycerol; ER, endoplasmic reticulum.

Protein	Lipid	Function	Ref.
Caveolin	Cholesterol, fatty acids	Lipid transport, caveolae internalization	(*71*, *72*)
Prominin	Cholesterol	Structure of microvilli	(*73*)
ApoB	Phospholipids	Assembles LDL lipids in ER	(*74*)
Lipocalins and calycins	Multiple	Bind lipid ligands	(*75*)
Proteolipids, Lipophilins	Multiple	Myelinogenesis, secretory proteins	(*76*)
Synaptophysin	Cholesterol	Synaptic vesicle traffic	(*77*)
SP-A/B	PG, PC	Assembles surfactant lipids	(*78*, *79*)
HE1	Cholesterol	Lipid transport from lysosomes	(*80*, *81*)
NAP22	Cholesterol	Organizes cholesterol in synthetic membranes and regulates neurite outgrowth	(*82*, *83*)
PDGFR	Cholesterol Sphingolipid	Signal transduction	(*84*)
Mal/BENE/Plasmolipin	Galactolipids	Binds multiple membrane lipids	(*85*)
Hemolysin	Cholesterol Sphingolipids	Bacterial toxin	(*86*)
MARCKS	PS	Signal transduction	(*53*)
Prion	Cholesterol Sphingolipids	Unknown	(*48*)
β-Amyloid peptide	Sphingolipids	Unknown	(*87*)
HIV-1 gp120	Sphingolipids	Viral attachment to membrane	(*87*)

correlation spectroscopy (*57*). This technique gives information about the lateral diffusion coefficient as well as the absolute number of proteins that are diffusing. Moreover, when identical proteins bearing different fluorescent labels cluster in caveolae/rafts, they will exhibit cross-correlated motions (*58*). Finally, the molecular brightness distribution can be used to distinguish individual from clustered proteins with photon-counting histograms (*59*). After appropriate calibration of monomer brightness in model membranes, photon-counting histograms can provide information about the relative number of proteins that are monomeric versus clustered in domains.

The shell hypothesis should be able to explain existing data on the behavior of proteins that associate with caveolae/rafts. The hypothesis provides a nice explanation of recent single-particle tracking data on the diffusion of CD59 in biological membranes (*60*). Fine-grained analysis of membrane protein diffusion suggests that biological membranes are segregated into corrals of ~100 nm that are separated by diffusion barriers called picket fences (*61*). For a protein to transit between adjacent corrals, it must pass through the fence. The GPI-anchored CD59 exhibits the same diffusion properties as a simple lipid probe. It is difficult to explain this type of movement if CD59 is in a caveola/raft because the size of the domain would be bulky and impede movement across the barrier. If, instead, CD59 is surrounded by a lipid shell and is not embedded in a lipid raft, then it would be small enough to pass through the adjacent pickets at a rate similar to that of the lipids.

In conclusion, we propose that lipid shells are the smallest aggregate in a hierarchy of laterally organized lipids that exist within the bilayer of biological membranes. They surround individual proteins and target them to caveolae/rafts measuring 50 to 200 nm in diameter. We believe that the biogenesis and maintenance of caveolae/rafts depend on specialized cellular machinery, but little is known about this process. Caveolae/rafts are able to aggregate to form lipid domains of even larger dimension. For example, fibroblasts and endothelial cells have patches of caveolae containing hundreds of units that occupy several square micrometers of the surface. Although the function of caveolae in potocytosis (*62*) and cell signaling (*28*) is well documented, recent studies suggest that aggregates of caveolae/rafts are able to engage bacteria through interactions with GPI-anchored proteins and internalize them (*63*, *64*). The internalized bacteria remain alive and eventually are exocytosed from the cell without harm. Thus, each level of lipid lateral organization adds new functionality for cell membranes.

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