# Membrane microdomains and caveolae

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Glycosphingolipid- and cholesterol-enriched microdomains, or rafts, within the plasma membrane of eukaryotic cells have been implicated in many important cellular processes, such as polarized sorting of apical membrane proteins in epithelial cells and signal transduction. Until recently, however, the existence of such domains remained controversial. The past year has brought compelling evidence that microdomains indeed exist in living cells. In addition, several recent papers have suggested that caveolae, which are considered to be a specific form of raft, and caveolins, the major membrane proteins of caveolae, are involved in the dynamic cholesterol-dependent regulation of specific signal transduction pathways.

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**Abbreviations**

| | |
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| **APP** | amyloid precursor protein |
| **CSD** | caveolin scaffolding domain |
| **DIG** | detergent-insoluble glycolipid-rich domain |
| **eNOS** | endothelial nitric oxide synthase |
| **ER** | endoplasmic reticulum |
| **FRET** | fluorescent resonance energy transfer technique |
| **GFP** | green fluorescent protein |
| **GPI-AP** | glycosylphosphatidylinositol-anchored proteins |
| **MAPK** | mitogen-activated protein kinase |
| **NPC** | Niemann-Pick type C |
| **Shh** | Sonic hedgehog |

## Introduction

According to a textbook view, the cell membrane is a two-dimensional liquid where membrane proteins are uniformly solubilized in the lipid solvent. This simple view has been seriously questioned in the past few years. Perhaps the most profound articulation of an opposing view is the raft hypothesis, which was proposed by Simons and co-workers [1,2]. This hypothesis postulates the existence of lateral assemblies (rafts) of glycosphingolipids and cholesterol, which associate with specific proteins while excluding others. The main forces driving the formation of rafts are lipid–lipid interactions, dependent on the biophysical characteristics of the lipid components. Rafts are viewed as platforms to concentrate signaling molecules or any other molecules with the same destination in the cell. Although very appealing, the hypothesis remained speculative because the methods for detection of rafts in living cells or their biochemical isolation were missing.

Caveolae — small invaginations on the surface of many cells — can be considered to be a specialized form of raft with a specific form. The role of caveolae and caveolins (protein components of caveolae) in cellular function is a long-standing question. In this review we discuss the latest data supporting the existence of microdomains. We then go on to consider the involvement of caveolins in signal transduction and cholesterol traffic and the intriguing interrelationship between these two apparently unrelated phenomena.

## Observation of rafts in living cells

The main evidence for the existence of rafts was based on biophysical studies of artificial lipid reconstitution systems and on the use of non-ionic detergent extraction of cells or membranes at low temperature. Using the latter technique, some lipids and proteins are found in a detergent-insoluble fraction, which can be isolated by sucrose gradient centrifugation. This fraction (called detergent-insoluble glycolipid-rich domain [DIG], or TIFF, GLS etc.), which is enriched in glycosphingolipids, sphingomyelin and cholesterol, was thought to represent microdomains (or even caveolae) in intact cells. As previously pointed out [2–4], however, this method could lead to artifacts. Many proteins identified in DIGs were not localized to caveolae and DIGs even contained proteins from different cellular organelles. It became clear that more sophisticated, non-invasive methods would be needed to visualize rafts.

A major advance in the detection of rafts in living cells in the past year came from studies of glycosylphosphatidylinositol-anchored proteins (GPI-APs). Members of this protein class are attached to membranes via their carboxy-terminal lipid modification. GPI-APs have been considered as constituents of rafts because of their insolubility in Triton-X-100 [5]; however, previous usage of conventional immunofluorescence techniques revealed no clusters of these proteins on the cell surface [6,7]. This observation could be explained either by non-existence of rafts or by the possibility that microdomains are smaller than the limit of detection of fluorescence microscopy (>300 nm).

Although a previous electron microscopic study favored the existence of microdomains containing GPI-APs at a submicron level [8], it is only through the use of different versatile techniques by several groups that a breakthrough in our understanding of rafts has been achieved. Using the elegant single-particle tracking method in one study [9], gold particles were attached to the components of interest and so-called transient confinement zones were determined. The authors demonstrated that a fraction of the

glycosphingolipid, GM1 — as well as Thy-1, a GPI-AP — were confined to zones of 200–300 nm in diameter.

A more direct demonstration that GPI-APs are clustered on the surface of the cell was provided by Varma and Mayor [10••] using a new variant of the fluorescent resonance energy transfer technique (FRET). With a random distribution of fluorophore, emission is depolarized because of energy transfer; in contrast, clusters of fluorophores should have a constant value of anisotropy in a wide concentration range. Indeed, when folate receptor (GPI-AP) on living cells was labeled with a fluorescent ligand, depolarization was constant over the entire concentration range. Only photobleaching or dilution of the ligand with a non-fluorescent analogue led to the change of anisotropy. In contrast, anisotropy values for folate receptor isoform with a transmembrane anchor were inversely dependent on fluorescence intensity. The authors calculated that GPI-anchored folate receptors should be clustered in domains of about 70 nm diameter; moreover, in agreement with the role for cholesterol in raft formation, depletion of cholesterol from the cell surface led to disaggregation of these clusters.

Another argument for clustering of GPI-APs came from the use of a newly discovered property of green fluorescent protein (GFP) to change the relative intensity ratio of green fluorescence emitted upon excitation by light at 395 nm and 475 nm wavelength at maxima of emission upon interaction of two GFP molecules [11•]. The ratio of fluorescence intensities at 410 nm/470 nm for GPI-anchored GFP, which was expressed in HeLa cells, was significantly lower then that of cytoplasmic GFP but much higher if the protein was cross-linked by antibodies against GFP. The authors concluded that although the clustering caused by GPI anchors was significant, it must be rather loose and/or transient.

It must be noted that the above data are somewhat contradictory to those obtained in a study using a conventional FRET approach [12] in which the distribution of 5′-nucleotidase (a GPI-AP) on the surface of MDCK cells was studied using antibodies (Cy3 and Cy5) labeled with two types of fluorophores. Applying different ratios of antibodies, no significant transfer of energy between donor (Cy3) and acceptor (Cy5) molecules could be detected suggesting that 5′-nucleotidase is randomly distributed on the cell surface. In contrast to the study by Varma and Mayor [10••], however, fixed cells were used. It is possible that either the fixation procedure or antibody molecules disrupt rafts.

Besides the above microscopic studies, there are also biochemical data supporting the clustering of GPI-APs in living cells [13••]. In this study, chemical cross-linking with a bifunctional reagent was used to show that GPI-APs are in spatial vicinity on the cell surface. When MDCK cells expressing a reporter protein bearing a GPI-anchor were subjected to cross-linking, oligomers consisting of up to 15 molecules were detected. The clustering was specific for the GPI-anchored form, as two transmembrane forms bearing the same ectodomain did not form oligomers. As in the study by Varma and Mayor [10••], depletion of cholesterol caused the unclustering of microdomains. In contrast, if cells were extracted by detergent prior to the cross-linking, more oligomers of higher molecular mass were visible.

Two main conclusions can be drawn from these studies. First, that rafts are rather small (of about 70 nm in diameter) and second that they are very dynamic. Future studies should clarify which biophysical properties of lipids and proteins determine their association with rafts. Also, the mechanisms that regulate their spatial distribution on the cell surface and their temporal stability need to be unraveled.

## Rafts in lymphoid cells

Under various conditions, small rafts tend to coalesce. This may not be simply an *in vitro* artifact of, for example, detergent extraction [13••] but might have very important physiological functions. It was recently demonstrated that upon cross-linking with antibodies smaller rafts form larger patches [14•]. These patches could accumulate filamentous actin and tyrosine-phosphorylated proteins and serve as centers for signal-transduction in lymphocytes [15].

Two recent papers [16••,17••] impressively demonstrate the importance of large raft formation for T cell activation. In order to be activated, T cell receptors need costimulatory molecule pairs like B7–CD28 or ICAM-1–LFA-1; however, the molecular mechanisms involved in this coactivation were not clear. Using beads covered with antibodies against surface proteins, two groups demonstrated that costimulation directs active transport of lipids and proteins to the site of cell–cell contact between the T cell and antigen-presenting cell. This process is dependent on cytoskeletal components (myosin and actin). Although the influence of cholesterol was not investigated in these papers, others illustrate that cholesterol plays an important role in compartmentalization of T cell receptor and downstream molecules [18•,19•].

## Caveolae: endocytosis, and mechanotransduction

Rafts appear to be a universal organizing principle of mammalian cells and clearly have important roles in concentrating signaling molecules. For example, signaling proteins sharing a simple lipid modification, such as multiple palmitoylation, could be brought together simply by virtue of their affinity for the raft domain [20]. The functions of rafts are more widespread than this, however, and it is clear that many cellular processes such as apical sorting in epithelia [21•] and possibly even the formation of the transverse-tubule system of muscle [22] may rely on similar organizing principles. This raises the question of what is the role of caveolae, specific forms of rafts, and the major proteins of caveolae, caveolins, in cellular function. We first consider the role of the caveolar invagination as distinct from the DIG domain.

The bulb-shaped caveolar pit is a characteristic, highly uniform, feature of many mammalian cells, which is apparently generated by caveolins [23]. Through their ability to bind cholesterol [24] and glycosphingolipids [25], caveolins have been suggested to stabilize and cause coalescence of DIG domains to form caveolae [2,23,26]. Whether caveolins physically deform the pit to form the caveolae or whether the caveolar shape is a result of a unique lipid composition generated by caveolins is as yet unknown. The caveolar invagination appears to be well suited as an endocytic vehicle. Consistent with an endocytic function of caveolae, two recent studies showed that dynamin, a large GTPase which is involved in budding of clathrin coated pits, is also a regulator of caveolae budding [27•,28•]. Indeed it has long been proposed that in endothelial cells caveolae endocytosis is a constitutive process involved in vesicular transport across the endothelial monolayer. In contrast, in fibroblasts this may be an infrequent, possibly regulated process [29]. The actual role of caveolae endocytosis in fibroblasts is, however, unclear. A model for transient closure of caveolae playing a role in the GPI-anchored protein-mediated uptake of small metabolites, termed potocytosis [30], has been questioned by recent studies of GPI-anchored protein trafficking [31•]. Caveolae budding may remove and inactivate a signaling event initiated at the cell surface, or may be required for activation of certain pathways, as shown for the clathrin coated pit pathway [32,33]. The latter is consistent with a recent study of the role of caveolae endocytosis in the Ras/mitogen activated protein kinase (MAPK) pathway [34].

Although caveolae have been implicated in endocytosis in endothelial cells, another endothelial cell function, which has recently gained attention, is a role of caveolae in mechanotransduction. Recent studies have suggested that caveolae can monitor blood flow over the endothelial cell surface and respond by activating specific signal transduction pathways, including endothelial nitric oxide synthase (eNOS) and Ras/MAPK, in a cholesterol-dependent manner [35•–37•]. This attractive model for the function of caveolar invagination provides an alternative or parallel role of endothelial caveolae, which may depend on the unique architecture of the caveolar invagination to act as a flow sensor. Of further interest is the finding that caveolae may represent a site of calcium signal generation in endothelial cells with calcium waves emanating from caveolae-rich domains [38].

In addition to the role of caveolae in normal cellular processes, it appears that caveolae endocytosis has been exploited by pathogenic agents as an entry route into animal cells. Cholera toxin requires caveolae, or caveolae-like domains [39,40], for endocytic uptake in a pathway that leads to the active subunit of the toxin reaching the endoplasmic reticulum (ER) and then the cytosol [41]. Simian Virus 40 (SV40) has apparently used a similar pathway to reach the ER [42–44]; it was proposed that recruitment of caveolin, and presumably associated DIG domains, around the virus leads to enclosure of the virus and thus internalization [44]. A quite different type of pathogen may have taken advantage of this pathway; FimH-positive *E. coli* bind to cell surface GPI-APs and are then internalized in a cholesterol-dependent process involving recruitment of caveolin-1 [45].

## Caveolae and caveolins; signaling, and cholesterol

In the past few years, caveolae have been implicated as organizing centers for signaling molecules. The list of signaling molecules apparently localized to caveolae has increased and includes Src family kinases, nitric oxide synthases, epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), phospholipase Cγ (PLCγ), protein kinase C (PKC)α, PKCβ, Ras, trimeric G protein Gα subunits, Mek1 and Erk2 (reviewed in [46•,47•]). Caveolin can directly interact with many of these signaling molecules via a conserved 20 amino-acid domain termed the caveolin scaffolding domain (CSD; residues 82–101 of caveolin-1) [48]. In the majority of cases the interaction with the CSD appears to hold the signaling proteins in an inactive conformation [46•]. Maybe the best-characterized example of this interaction and its role in signal transduction is the interaction of caveolin-1 with eNOS [49]. Interaction of caveolin with eNOS holds the enzyme in an inactive conformation, which is released upon activation by calcium–calmodulin. This type of mechanism appears important in both endothelial cells [49] and cardiac myocytes [50•].

These studies suggest a primary role for caveolin — and particularly the CSD — as a negative regulator of many signaling events; only release of inhibitory constraints through caveolin dissociation allows the signaling to take place. In some cases, however, this general principle has been seriously questioned by the finding that the signaling molecules (e.g. trimeric G protein α subunits [51,52] or Ras [53•]) are not all directly associated with caveolae and caveolins. This argues against a simple model in which the caveolin interaction is required to prevent the signaling event. Moreover, caveolin-1 can also have an activating role in signal transduction, for example in insulin signaling [54] and in the integrin-mediated activation of Ras-signaling pathways [53•]. Activation of integrins was shown to cause caveolin-dependent activation of fyn. The caveolin pool involved in the integrin-mediated signaling event constituted a Triton-soluble — presumably non-caveolar — form of the protein. This soluble pool was associated with integrins and with Fyn but not with Ras, c-Src or trimeric Gα subunits. Interestingly, the interaction with caveolin was proposed to be mediated through the transmembrane domain of the integrin α-subunit and therefore presumably with the caveolin intramembrane domain, in contrast to the interaction of other signaling proteins with the inhibitory CSD.

After summarizing these studies showing interactions of a wide range of proteins with caveolin-1, it is important to add a word of caution. The special properties of caveolins could lead to artifacts in co-immunoprecipitation experiments. The extraction procedures using detergents might retain lipids, leading to co-precipitation of rather large complexes (aggregates of DIGs) containing many proteins, not just directly interacting partners.

Indirect evidence for involvement of caveolin in signal transduction comes from studies of cellular transformation. Caveolin is downregulated and caveolae numbers are reduced in NIH3T3 cell lines transformed by v-Abl, Bcr–Abl and Ras [55]. Conditional expression of caveolin-1 in Ras and Abl transformed cells, at levels sufficient to induce formation of caveolae, abrogates anchorage independent growth [56]. Most interestingly, antisense inhibition of caveolin expression has the reverse effect causing activation of Ras/MAPK-dependent signaling pathways and cell transformation [57••]. Consistent with this study, analyses of several different types of tumors show a reduced caveolin expression [58] although increased caveolin expression has also been correlated with some tumor phenotypes [59].

While there is accumulating evidence to suggest that caveolin can interact with signaling molecules and can influence signaling pathways *in vitro* and *in vivo*, other features of the caveolar system should be taken into consideration when examining caveolin and caveolae function. In particular, the relationship of caveolin to cholesterol is of considerable interest [60]. Caveolin-1 binds cholesterol [24] and caveolin-1 expression is regulated at the transcriptional level by cholesterol [61,62]. Caveolin-1 transfection also increases ER to plasma membrane cholesterol transport [63,64]. These results suggest a primary role for caveolin, and presumably caveolae, in cholesterol regulation. Biochemical studies show that caveolae fractions are enriched in cholesterol and represent sites of cholesterol efflux to extracellular carriers [60]. The high density lipoprotein-binding protein SR-B1, which has been proposed to facilitate transfer of cholesterol between extracellular high density lipoprotein and the plasma membrane, is also concentrated in caveolae [65]; moreover, the efflux of cholesterol and the expression of caveolin are regulated during the cell cycle [66]. Caveolin also apparently cycles between internal compartments and the cell [21•,67,68].

How can the cholesterol related properties of caveolin be equated with a role for caveolin in signal transduction? In fact, it is now apparent that cholesterol plays a vital role in signaling in higher eukaryotes. Signaling pathways that are crucial for normal development are inhibited by cholesterol-disrupting teratogenic toxins *in vivo* [69]. The Patched protein, a surface receptor for Sonic hedgehog (Shh), has a putative cholesterol-sensing domain, which is crucial to its function [70•] and Shh itself is modified covalently by cholesterol [71]. A recent study provided more direct evidence for a key role of cholesterol-rich domains in signaling [72••]. In this study it was shown that cholesterol depletion directly activates Ras-dependent signaling pathways in the absence of growth factors. The authors suggested that the reduced plasma membrane cholesterol of transformed cells may trigger hyperactivation of signaling pathways involved in cell division and therefore contribute to cell transformation.

Two recent studies using different systems support this model and provide crucial links between these cholesterol effects and the role of caveolin *in vivo*. In the first study [73], a caveolin-1 deficient *Caenorhabditis elegans* was generated using RNA-interference methodology and phenocopies were analyzed. The effect of a loss of caveolin-1 was an acceleration of meiotic cell cycle progression through the earlier exit from the pachytene arrest. This process in *C. elegans* depends on Ras/MAPK pathway and genetic analysis confirmed a close link between the Ras and caveolin regulation of cell cycle progression. Intriguingly, cholesterol depletion caused an identical phenotype and the authors suggested that caveolin-1 links the cellular cholesterol level to the control of meiosis, an interesting concept in view of recent studies showing a role of caveolin and decreased cellular cholesterol in cell cycle progression [66].

The second study of interest in this context employed a mutational approach in an attempt to generate caveolin mutants that acted as dominant-negative inhibitors of caveolar function [74]. A caveolin truncation mutant completely blocked H-Ras-, but not K-Ras- mediated Raf activation. H and K-Ras differ in their plasma membrane localization motifs; both are farnesylated but only H-Ras is palmitoylated [75]. The mutant caveolin did not impair the plasma membrane localization of H-Ras, or the recruitment of Raf to the plasma membrane. The mutant protein was not detectable on the cell surface, but was localized to the Golgi complex and non-endocytic cholesterol-rich vesicles. These findings suggested that the caveolin mutant is unable to follow the normal caveolin cycling pathway but is held up in some cholesterol-rich compartment. Together with the known enrichment of palmitoylated proteins in cholesterol-enriched raft domains, this suggested that the mutant may inhibit H-Ras function through an effect on cellular cholesterol. A crucial experiment supporting this hypothesis showed that a short incubation of the mutant-expressing cells with cyclodextrin-complexed cholesterol rescued the block in Raf activation [74]. This strongly suggests that the caveolin mutant acts through an effect on maintenance or generation of the cholesterol-rich surface domains required for H-Ras function and supports the idea that maintenance of high cholesterol in caveolae (or DIG domains) is an active process requiring caveolin-1 [72••].

Taken together, these studies raise the possibility that caveolin regulation of cellular cholesterol may be equally

as important as direct interactions of caveolin with signaling molecules in the regulation of signal transduction. The studies show a crucial importance of caveolin in regulating intracellular cholesterol levels and thus maintaining and modulating the function of cholesterol-enriched surface microdomains.

## Many kinds of rafts?

As postulated previously caveolae are only one specific form of raft with a specific shape and rafts can exist in the absence of caveolins. Are there other molecules having similar properties to caveolin and do different kinds of lipid rafts exist?

Maybe the best candidates to form non-caveolar rafts are proteins of the MEC-2/stomatin family [76–78]. These proteins like caveolins have a very long hydrophobic amino acid stretch and similar membrane orientation and are also found in detergent-insoluble complexes. The reggie/flotillin family of proteins has also been suggested to associate with caveolae or non-caveolar detergent-insoluble domains [79,80]. A recent paper also presented evidence for a non-caveolar detergent-insoluble glycolipid signaling domain containing GM3, c-Src, cholesterol and sphingomyelin; this fraction could be separated from the caveolar domain containing caveolin, Ras, and higher levels of cholesterol [81].

## DIGs and disease

Over the past year it has become increasingly apparent that DIGs, caveolae, and caveolins are involved in several human disease states. Mutations in the predominantly muscle-specific isoform of caveolin, caveolin-3, cause a form of limb girdle muscular dystrophy [82•,83•]. Other disease states indirectly implicate caveolae-related processes in disease progression. Niemann-Pick type C (NPC) disease is characterized by an imbalance in cellular cholesterol. The underlying defect resides in NPC1, a multispanning membrane protein with a putative cholesterol-sensing domain [70•]. Homozygotes lacking the NPC1 protein show an aberrant accumulation of free cholesterol in the lysosomal system. In NPC1 heterozygotes, caveolin-1 protein levels are increased several-fold [84]. This is presumably a compensatory response as free cholesterol levels are maintained at near normal levels. In contrast, homozygotes show only slightly increased caveolin-1 protein levels despite greatly increased free cholesterol. This may indicate that the heterozygotes have the capacity to regulate caveolin levels in response to a cholesterol imbalance but that this mechanism is absent in the homozygotes completely lacking the NPC1 protein. This would indicate a close link between the NPC1 protein and caveolin-1 in regulating cellular free cholesterol [84,85].

Another important area in which DIGs, and possibly caveolae, have been implicated is the generation of the amyloid peptide from the amyloid precursor protein (APP), which occurs in Alzheimer's Disease. Generation of the β-amyloid peptide has been shown to occur in DIG domains in a cholesterol-dependent process [86–88]. Intriguingly, heterologous expression of caveolin in non-neuronal cells facilitates cleavage of the APP and is inhibited by antisense inhibition of caveolin expression [88]. While this may also be compatible with an indirect effect on cellular cholesterol, cross-linking experiments showed an association of caveolin and APP in the transfected cells. Clinical links between cholesterol disease and susceptibility to Alzheimer's disease [89] support the postulated importance of cholesterol in amyloid generation and emphasize the universal importance of cholesterol-rich domains in cellular function.

## Conclusions

Several papers in the last year have unambiguously demonstrated the existence of glycosphingolipid- and cholesterol-enriched microdomains, or rafts, within the plasma membrane of living cells. We now need to understand what biophysical properties govern the formation of rafts, and what factors regulate their stability or distribution on the cell surface. Distinct types of rafts may exist and the challenge is to dissect how these distinct microdomains form and function. Caveolae and caveolins remain an enigmatic subject with their apparently bewildering array of possible functions; however, the past year has produced key insights into the role of caveolins and cholesterol in the functional organization of specific signal transduction pathways and in particular the essential role of the caveolae system and cholesterol in the regulation of cell division.

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