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Lipid Rafts, Caveolae, and Membrane Traffic

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1.1
Introduction

Cell membranes are dynamic assemblies of a variety of lipids and proteins. They form a protective layer around the cell, but also mediate the communication with the outside world – that is, neighboring cells in a tissue, hormones and growth factors arriving with the blood supply, or pathogens trying to enter the system. The unique feature of cell membranes is that their lipid and protein constituents can self-assemble into 5 nm-thin, two-dimensional fluids composed of two apposing lipid monolayers that form a hydrophobic interior and two polar interfacial regions oriented towards the aqueous medium. This organizing principle – the lipid bilayer – is the oldest, still valid molecular model of biological structures. The first model that incorporated proteins was proposed by Danielli and Davson, and assumed that the bilayer was made up entirely of lipids and that proteins covered the two polar surfaces [1]. Some 40 years later, the fluid mosaic model of the cell membrane proposed by Singer and Nicolson [2] was a conceptual breakthrough. Amphipathic membrane proteins were recognized to reside within, and even span, the whole bilayer that was depicted as a dynamic structure, the components of which are laterally mobile. However, the view that the lipids in the bilayer mainly serve as a homogeneous solvent for proteins [2] has been proven to be too simplistic. Lipids are not only distributed asymmetrically between the two leaflets of the bilayer, but also within the leaflet they are heterogeneously arranged [3]. This chapter will recapitulate the history and recent advances in membrane biology including the lipid raft concept, and then summarize current views on the functions of rafts and caveolae in membrane traffic.

1.2
Basic Organization Principles of a Cell Membrane

The lipid bilayer is a two-dimensional fluid, where lipid molecules exchange slowly between leaflets but are mobile within the leaflet. This mobility consists of two parts:

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- the "translational freedom" of a molecule – that is, its lateral mobility; and
- the "configurational freedom" that is, the ability to flex parts of the molecule and to rotate bonds in its carbon backbone.

Synthetic bilayers change from a liquid state with high translational and configurational freedom into a rigid gel state at a characteristic freezing point. Cell membranes at physiological temperatures are almost always in the liquid state, but can contain regions with high configurational order, as will be described later. Importantly, the lipid bilayer of cell membranes is asymmetric, with a different lipid composition in the two leaflets. The main lipid components of cellular membranes are glycerophospholipids, with the most abundant species being phosphatidylcholine (PC) in the exoplasmic leaflet and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner leaflet, as well as sphingolipids with glycosphingolipids and sphingomyelin (SM) mostly localized to the exoplasmic leaflet. Sterols make up the third lipid class, and are present in both leaflets. Mammalian cell membranes contain only one sterol, namely cholesterol, but probably more than thousand different glyco- and sphingolipid species, emerging from the combinatorial propensity to assemble lipids from different backbones linked in different ways with two varying hydrocarbon chains and a vast number of headgroups. A large number of flippases and translocators tightly control the asymmetric distribution of all these lipids across the bilayer [4].

Lipids are differentially distributed between cellular organelles. The endoplasmic reticulum and the Golgi-complex contain mainly glycerophospholipids and only small amounts of sphingolipids, whereas the plasma membrane is relatively enriched in SM and glycosphingolipids [5]. Also within the membrane plane of one organelle, lipids are believed to be heterogeneously arranged. Caveolae – small invaginations of the plasma membrane – are enriched in glycosphingolipids [6], and phosphatidylinositol-3'-phosphate (PI(3)P) is concentrated in subdomains of early endosome membranes [7]. Recently, vacuole-fusion in yeast has been shown to be controlled by microdomains of ergosterol, diacylglycerol and phosphoinositide-3-and-4-phosphate [8]. Furthermore, membranes are differentially susceptible to extraction by detergents such as Triton X-100 or CHAPS at 4 °C, with some proteins and lipids being completely solubilized and others forming so-called "detergent-resistant membranes" (DRM; for a review, see [9]). These findings suggested that cell membranes contained microdomains in which lipids were more tightly packed and thus not accessible to the detergent, although it is widely accepted that DRMs do not have an exact *in-vivo* correlate but are defined by being formed during the detergent treatment [10]. These microdomains were later termed "rafts" and were described as sphingolipid-cholesterol assemblies containing a subset of membrane proteins [11]. Currently, the raft hypothesis is heavily debated [12–14], with the main discussion points being the methodologies to study rafts and the size of the domains (see below). The core of the raft concept is that cell membranes phase-separate into different domains and that this is a lipid-driven process. In light of the ongoing discussion in the field, the following sections will provide an overview about what is known about phase separation, first

discussing the studies conducted in model membrane systems and later in cell membranes.

1.3 Evidence for Phase Separation in Model Membrane Systems: Liquid-Ordered and Liquid-Disordered Phases

Various model membrane systems have been used by physicists and chemists to study phase separation in lipid mixtures. They are either monolayers or bilayers. Monolayers are either assembled at an air-water interface with the packing density of the lipids being adjusted by applying lateral pressure, or on a supporting lipid monolayer that is fixed to a solid support. Bilayers are used in the supported version as described above, or in the form of vesicles. The most commonly used vesicles are large or giant unilamellar vesicles (LUV or GUV, respectively) composed of only a single bilayer, but also multilamellar vesicles (MLV) are used. The basic principles were first established in simple binary lipid mixtures, but recently ternary mixtures which more closely mimic the composition of the cell plasma membrane have been used. The mixtures usually contain one lipid with a high melting temperature (T_m), one with a low T_m, and cholesterol. GUVs are probably the system closest to a cell membrane, because artifacts from a support are excluded. Still, cell membranes are asymmetric with different lipid compositions of the outer versus the inner leaflet, while the GUVs used so far were all symmetric. Since maintaining an asymmetric lipid distribution is energy-consuming, perhaps by reconstituting lipid translocators into liposomes this drawback can be overcome in the future. Although model membrane systems produce very simplified pictures of cell membranes, there are many examples of a close correlation with experimental data obtained in living cells [14].

Ipsen et al. were the first to describe the formation of a liquid-ordered phase by cholesterol and saturated phospholipids [15,16]. This phase can coexist with other lipid phases, and its characteristics are described as follows: the translational order of lipid molecules within the liquid-ordered phase is similar to that in a fluid bilayer state, whereas the configurational order of the hydrocarbon chains compares more to that in a gel state. The formation of the liquid-ordered phase was attributed to the unique chemical nature of cholesterol (for a review, see [17]), but later it was shown that all natural sterols promote domain formation and that also small amounts of ceramide (3 %) can stabilize domains formed in vesicles [18]. Leventis and Silvius showed that the interaction of cholesterol with different lipid species is dependent on the nature of their hydrocarbon chains and, to a lesser extent, also on their headgroup. The interaction preference decreases with SM > PS > PC > PE and with increasing unsaturation of the acyl chains [19]. Whereas the kink in unsaturated hydrocarbon chains is likely to hinder tight packing with the flat sterol ring of cholesterol, the reason for the preferential interaction of cholesterol with SM is still a debated issue.

The first visualization of “raft-like domains” in model membranes was achieved

by Dietrich et al. [20]. They visualized liquid ordered domains in supported bilayers and GUVs composed not only of synthetic lipid mixtures but also of lipid extracts from brush border membrane, the apical membrane of intestinal cells. Domain formation was cholesterol-dependent, since domains disappeared after treatment with the cholesterol-extracting drug methyl-β-cyclodextrin. Another big step forward was the establishment of a ternary phase diagram of SM/PC/cholesterol at the physiological temperature of 37 °C [21]. This predicts the coexistence of liquid-ordered and liquid-disordered phases for a wide range of compositions mimicking those occurring in the plasma membrane of cells. Most domains observed in model membranes are rather large (i.e., several micrometer in diameter) or they start small when they are being formed and then grow continuously by collision and fusion as the system reaches equilibrium [22]. Contrary to this, raft domains in cells are believed to be small, most likely because the cell membrane is not at equilibrium (see below). Interestingly, fluorescence resonance energy transfer (FRET) measurements on vesicles composed of a ternary lipid mixture mimicking the outer leaflet of the plasma membrane revealed heterogeneities (i.e., domains) of sizes in the tens of nanometer range at 37 °C [23]. Large domains were observed with the same lipid mixture only below 20 °C.

A slightly different interpretation of liquid-liquid immiscibility observed in model membranes was proposed by McConnell and colleagues. These authors argue for the formation of "condensed complexes" between cholesterol and SM rather than a liquid-ordered phase or domain. The name originates from the observation that cholesterol and SM occupy less surface area when mixed together compared to the sum of the areas occupied by each component alone before mixing. Such a complex is supposed to contain 15–30 molecules with a fixed stoichiometry of 2:1 (SM:cholesterol). These complexes could exist in quite high concentration without necessarily leading to a phase separation (for a review, see [24]). However, the condensed complex theory was developed on monolayer membranes and has not yet been validated for bilayers.

Taken together, there is clear evidence for lipid-driven domain formation in model membrane systems mimicking the outer leaflet of the plasma membrane. On the contrary, domain formation could not be observed in lipid mixtures mimicking the inner leaflet of the plasma membrane [25]. The intermolecular forces leading to phase separation are van der Waals interactions between saturated acyl chains and cholesterol, as well as forces such as hydrophobic shielding or the "umbrella effect", described for cholesterol filling the holes left between the acyl chains of glycosphingolipids with large headgroups [26]. However, none of the systems described so far has included proteins in their analysis, and the question remains whether proteins choose the domain they partition into, or whether they organize a domain around them.

Partitioning experiments have been performed, in which proteins were reconstituted into model membranes, and their phase distribution was analyzed. In this way, glycosyl-phosphatidyl-inositol (GPI)-anchored placental alkaline phosphatase (PLAP; [27,28] and Thy-1 [29] were shown to partition into the liquid-ordered phase, and the chain length of the GPI-anchor was shown to be important for

partitioning of the protein [30]. Similarly, peptides modified with prenyl groups were excluded from liquid-ordered domains, while peptides modified with cholesterol or palmityl chains partitioned significantly into the ordered phase [31]. Partitioning studies with synthetic transmembrane peptides revealed that longer transmembrane domains are incorporated better into liquid-ordered domains than shorter versions [32]. Another important determinant for the partitioning of a molecule is the size and orientation of its dipole moment [33]. The membrane dipole moment is stronger in ordered phases where the dipoles are better aligned. Only molecules displaying a dipole moment with the same orientation as the dipolar potential of the membrane, are predicted to be able to enter the ordered phase. Nevertheless, our knowledge about lipid-transmembrane protein interactions is still scarce and this area of research is a major challenge.

1.4 Evidence for Phase Separation in Cell Membranes: The "Raft Concept"

There are several indications for cell membranes being inhomogeneous fluids and for the existence of lipid-driven phase separation. One key finding was the selective co-clustering of certain membrane components and segregation from others upon application of antibodies to living cells. Co-clustering of lipids was first observed in lymphocytes, where one ganglioside species was capped with antibodies and another species was found to redistribute into the cap [34]. It was then shown that simultaneous addition of two antibodies against apparently homogeneously distributed surface antigens could, in selected cases, lead to their co-clustering and in other cases to their segregation [35]. These findings were explained by certain proteins residing in small raft domains that are below the light microscopic resolution in size, and others residing outside the raft domains. Upon cross-linking by antibodies the small raft domains coalesce into visible, stable clusters that contain several different raft proteins. The antigens that were previously in the non-raft environment are excluded from the coalescing domains and thus form separate clusters upon cross-linking. How these large-scale domains containing multiple raft components could be formed in a homogeneous membrane without the occurrence of phase separation is not obvious, and an alternative explanation for this phenomenon has not been put forward. Since then, two techniques have been used to directly assess liquid order in living cells. Gidwani et al. measured the steady-state anisotropy of the lipid-probe DPH-PC, which is sensitive to cholesterol-induced liquid order. With this approach, they found that approximately 40 % of the plasma membrane of mast cells is in a liquid-ordered state [36]. More recently, Gaus et al. were able to directly visualize liquid-ordered domains in living macrophages on the light microscopic level. They applied two-photon imaging of the amphiphilic dye LAURDAN, which changes its emission peak depending on the state of its lipid environment [37].

Other techniques have also been employed for assessing raft domains in living cells, most of them analyzing the distribution and dynamics of membrane pro-

teins rather than lipids. Pralle et al. measured the local diffusion of a bead attached to a single protein molecule in the plasma membrane of fibroblasts within an area smaller than 100 nm in diameter [38]. In this way, diffusion was not hindered by cytoskeletal constraints but was supposed to be free. Proteins previously shown to be resistant to detergent extraction diffused three times slower than detergent-soluble proteins. After cholesterol depletion, the former diffused as fast as the latter. The first group of proteins was thus assumed to reside in a raft environment and to diffuse together with the whole raft entity. After destruction of this entity by cholesterol extraction the proteins behaved as if they were diffusing in a non-raft environment. From the viscous drag and from the diffusion coefficient, the size of the raft entities was calculated to be approximately 50 nm in diameter. Extrapolated from average protein and lipid densities in cell membranes, one raft entity was calculated to contain roughly 3000 lipid molecules and 10–20 proteins.

Remarkably, Prior et al. come to a very similar size for raft domains formed in the cytoplasmic leaflet of the plasma membrane using a completely different technique [39]. They ripped plasma membrane sheets off adherent cells and labeled them with gold-coupled antibodies against H-Ras and K-Ras, supposed to reside inside and outside of raft domains, respectively. Statistical analysis of the distribution of the gold particles revealed that 35% of H-Ras labels were clustered in domains of roughly 44 nm diameter. These domains were cholesterol-dependent. Furthermore, cross-linking of GPI-anchored green fluorescent protein (GFP-GPI) in the exoplasmic leaflet resulted in co-localization of the H-Ras clusters with the formed GPI-patches, but did not change their size. However, 20% of the non-raft protein K-Ras was also found to be clustered in domains of 32 nm diameter, although these domains were cholesterol-independent. H- and K-Ras had been reported to occupy distinct domains in the plasma membrane before [40]. Recently, single molecule imaging of H-Ras revealed cholesterol and actin dependent domains as large as 250 nm [41].

An often-applied technique trying to visualize raft domains *in vivo* is that of FRET. Hetero-FRET, which detects energy transfer between two different fluorophores, has not proven successful [42–44], most likely because the probability that donor and acceptor are in the same microdomain is very low. Even cross-linking one raft marker by antibodies does not lead to appreciable recruitment of others [45]. Recently, Mayor and coworkers refined their previous analysis [46] using homo-FRET (i. e., energy transfer between two fluorophores of the same kind) to study clustering of GPI-anchored proteins in the plasma membrane [47]. By measuring the anisotropy decay over time, these authors found that 20–40% of the GPI-anchored proteins are present in small complexes of two to four molecules, while the remainder is randomly distributed as monomers. The limitation of FRET measurements becomes obvious in these studies. The technique provides information about "closeness" on a very small scale (5 nm), but is not suited for visualizing bigger entities.

The fact that raft domains are difficult to visualize *in vivo* has led to a number of alternative explanations, mostly describing smaller entities and, most importantly, describing the formation of these entities as a protein-driven, induced event. The

smallest entity was proposed by Kusumi and colleagues, who have pioneered single-particle tracking with ultra-high sampling frequencies of 40 000 Hz. The spatial resolution achieved with this frequency is 20 nm, meaning that if the domains were significantly larger and the probe resided either inside or outside the domain for several consecutive steps, then different diffusion behaviors could be observed. Since however raft and non-raft markers displayed the same diffusion characteristics, it was postulated that rafts are extremely small, namely molecular complexes of at least three membrane components, one of which comprises a saturated acyl chain or cholesterol. Stabilized raft domains accessible to diffusion measurements would only form by clustering following stimulation (for a review, see [48]). Anderson and Jacobson have put forward the lipid shell hypothesis, in which roughly 80 lipid molecules are supposed to surround a raft protein and form a shell of 7 nm diameter [49]. The shells would be thermodynamically stable structures resulting from specific binding interactions between proteins and lipids, and could target the protein into larger raft-domains. How the larger raft domains form and why the raft-protein must assemble a shell of raft-lipids around it before it can enter a raft-domain remain open questions.

The size of raft domains is heavily debated and, as a consequence of the different measurements, their existence is questioned. Consensus is reached in that the proposed domain sizes of 200 nm or larger based on single-particle tracking experiments [50,51] were most likely clustered rafts, formed and stabilized by the multi-valent beads used for the tracking. Also, the 50-nm raft calculated from the viscous drag experiments by Pralle et al. [38] could have been a stabilized raft in which the altered dynamics due to the optical trap led to enlargement of a previously smaller structure. This leaves us with a domain size between the 5 nm derived from the FRET measurements [47] and the <20 nm derived from the high-speed single particle tracking studies [52]. Better estimates will have to await the development of new methods which can finally assess the size of isolated raft domains *in vivo*.

In light of the co-clustering data [35], the visualization of distinct liquid-ordered domains in living cells [37], and the evidence that isolated cell membranes phase separate *in vitro* [20], it seems reasonable to assume that native cell membranes can display phase separation. One explanation for the formation of small and transient domains in the plasma membrane lies in its composition. In contrast to ternary lipid mixtures in model systems, the plasma membrane is composed of hundreds of different lipid species and, in addition to that, a variety of proteins. Viewed over a large scale, the complexity of the plasma membrane should counteract phase separation, buffer fluctuations, and in fact protect the cell against rapid phase transitions in response to small changes in the environment. If every fusion or budding event led to a phase transition, it would be difficult to prevent leakages through the bilayer and keep the membrane tight. Viewed on a smaller scale however, the picture can appear very different. Local impurities or changes in membrane composition can allow coalescence and separation of domains containing reaction partners and thus provide a regulatory principle.

1.5 Raft Domains are Clustered to Exert their Function

While the steady-state existence, size and shape of liquid-ordered domains in cells remains the subject of debate, agreement has been reached on the fact that raft domains coalesce upon cross-linking to form signaling and possibly also sorting platforms [53–55]. Cross-linking is achieved by multivalent ligands binding to surface receptors or by cytoplasmic scaffolding proteins. The initial cross-linking event is thought to increase the number of contact sites between raft proteins and lipids, which leads to a potentiation of the formerly weak interactions. The previously small raft domains coalesce and form large, more stable entities. It is the clustered state in which rafts are accessible to microscopy.

Cross-linking of raft antigens not only leads to co-clustering of raft components within one leaflet, but also influences the organization of the opposing monolayer. Cross-linking of the exoplasmic GPI-anchored PLAP led to partial co-clustering of the src-kinase fyn in the cytoplasmic leaflet of Jurkat cells [35, 56]. Cross-correlation analysis revealed co-distribution of an inner leaflet raft protein with FceRI transmembrane receptors that were cross-linked by binding of their multivalent ligand IgE, as well as with antibody cross-linked raft markers of the exoplasmic leaflet, such as the GPI-anchored protein Thy-1 or the ganglioside GD_{1b} [57]. The finding that clustering not only leads to lateral coalescence of small raft domains in the exoplasmic leaflet, but also in the cytoplasmic leaflet, strengthens the hypothesis that clustered raft domains provide a platform for bringing together signaling complexes and propagating signals into the cell (reviewed in [58]). Interestingly, also in symmetric model bilayers, liquid-ordered domains have always been observed to coincide in both leaflets [20, 59]. How the connection of the inner leaflet and the outer leaflet is achieved, remains an open question. Interdigitation of the often long fatty acid chains of glycosphingolipids has been proposed to enforce a higher order also in the cytoplasmic leaflet. Alternatively, or additionally, transmembrane proteins could mediate transbilayer coupling.

Many signaling processes have been proposed to depend on the clustering of raft domains [60,61] (see also Chapter 7), the T-cell synapse being the prime example [62,63]. According to a recent study by Douglass et al., the initial stage of signaling complex assembly does not require rafts but is rather dependent on protein-protein interactions [64]. Studies by Magee et al., on the other hand, have shown that raft clustering independent of protein-protein interactions can activate signaling pathways downstream of the T-cell receptor [65]. These authors observed that incubating T cells at 0 °C leads to coalescence of raft components into visible domains on the plasma membrane. At the same time, chilling activates the signaling cascade, leading to increased tyrosine phosphorylation and ERK activation. The cold-induced, protein-independent coalescence of raft domains is a clear indicator for a phase separation phenomenon, since it is well established that the phase-separated domains are larger at lower temperature and fragment at higher temperature due to the increase in Brownian motion [66]. However, it is not yet clear which role this raft coalescence would play in T-cell signaling under physiological conditions.

The formation of large, clustered raft domains is easiest imagined to occur by coalescence of pre-existing, small rafts. However, a recent study on model membranes of different compositions argued that phase separation can be induced by cross-linking one component in a previously homogeneous membrane [67]. GUVs composed of PC, SM and cholesterol exhibit phase separation into a liquid-ordered and a liquid-disordered phase, depending on the ratio of the components. When a small amount of the ganglioside GM1 is included in the vesicles, its cross-linking with the pentavalent cholera toxin B subunit leads to coalescence of the GM1-containing phase into larger, visible domains. Hammond et al. showed that domains can not only be formed at GUV compositions that displayed phase separation prior to clustering, but also at compositions very close to the phase transition boundary in which no previous phase separation was detected [67]. The local increase in GM1 concentration following the cross-linking might have been enough to cross the boundary and cause the membrane to phase separate.

1.6
The Apical Membrane of Epithelial Cells: A Percolating Raft Membrane at 25 °C

Columnar epithelia lining the kidney, intestine or pancreas are composed of a single layer of polarized cells. They have evolved to create stable apical and basolateral membrane domains, which are sealed off from each other by a tight junction barrier. While the basolateral domain of columnar epithelia faces the underlying extracellular matrix and the blood supply, the apical membrane is the one facing the lumen of the renal tubules, of the intestine, or of the pancreas. It has long been known that apical and basolateral membrane domains have a distinct protein composition [68,69]. However, lipids are also distributed differently between the apical and the basolateral membrane. The lipids found in the basolateral membrane resemble those found in the plasma membrane of an unpolarized cell, whereas the apical membrane contains much more glycosphingolipids [70]. In the brush border membrane of the intestine, glycosphingolipids account for more than 30% of the total lipid amount [71]. Considering that they reside exclusively in the exoplasmic leaflet, more than 50% of the lipids in the exoplasmic leaflet should be glycosphingolipids, and together with cholesterol they should leave very little space for glycerophospholipids. Glycosphingolipids mainly contain two long, saturated hydrocarbon chains, as opposed to glycerophospholipids which usually contain unsaturated acyl chains [72], and have been proposed to form a liquid-ordered phase together with cholesterol. It was this segregation of raft lipids in the outer leaflet of the apical membrane from the more phosphatidylcholine-enriched basolateral membrane that prompted Simons and van Meer to postulate the existence of lipid platforms involved in the biogenesis of the apical membrane [70] and has led to the formulation of the raft hypothesis [11].

Recently, we have experimentally explored the domain organization of the apical membrane of epithelial cells in comparison to that of a fibroblast plasma membrane by measuring long-range diffusion of several fluorescent membrane pro-

teins using fluorescence recovery after photobleaching (FRAP) [73]. By using this technique, the diffusion of millions of proteins can be examined at the same time in a noninvasive manner. As previously reported [74], all proteins display free diffusion with 100% recovery in the fibroblast plasma membrane. In the apical membrane of epithelial cells, however, we could distinguish two populations of proteins on the basis of their distinct diffusion characteristics at 25 °C. One group displayed free diffusion with recoveries close to 100%, whereas the other group displayed anomalous diffusion [75, 76] with limited recovery. This is indicative of a phase-separated system, in which there are (at least) two coexisting phases – one which has a mass fraction just high enough to be continuous (percolating) over the entire membrane surface, and the other being present in isolated domains [77]. Within the percolating phase, long-range diffusion is unconstrained, results in complete recovery, and can be described with a single apparent diffusion coefficient [78, 79] – as observed for the first group of proteins. In the non-percolating phase, proteins will be obstructed in their long-range diffusion, resulting in either incomplete or extremely slow recovery [78, 79] – as observed for the second group of proteins. Strikingly, all proteins falling into the first group have been proposed to reside in rafts, while all members of the second group have been proposed to reside outside of rafts. This may suggest that at 25 °C the apical membrane of epithelial cells is a percolating raft phase with isolated non-raft domains.

Phase separation likely exists also in fibroblasts, with the domain organization of the two membranes being inverted. The fact that in the fibroblast plasma membrane the raft and non-raft proteins diffuse with the same kinetics does, however, not contradict the existence of phase separation. Rather, the results can be explained on the basis of partition coefficients. From all we know, a limited set of proteins has the features required to be accommodated in the ordered lipid environment of a raft domain. While non-raft proteins that lack these features are largely excluded from rafts – that is, non-raft proteins have a low propensity to partition into the surrounding raft phase in the apical membrane of epithelial cells – raft proteins might have a preference for raft domains, but can easily partition into a less-ordered, non-raft environment – that is, raft proteins are not limited to raft domains in the plasma membrane of fibroblasts [28, 80, 81]. With the additional notion that raft domains in fibroblasts are believed to be small and highly dynamic, the differences between the long-range diffusion paths of raft and non-raft proteins in the fibroblasts plasma membrane become too small to be accessible to FRAP measurements.

1.7
Caveolae: Scaffolded Membrane Domains Rich in Raft Lipids

Caveolae were first defined morphologically by Palade, who observed plasma membrane invaginations in endothelial cells under the electron microscope [82]. He later named them “plasmalemmal vesicles” [83], implying that they would shuttle molecules across the cell. The name “caveolae” (little caves) was however

coined two years later by Yamada, who described invaginations on the surface of gallbladder epithelial cells [84]. Although he did not distinguish between coated and uncoated invaginations, the name "caveolae" was later specifically attributed to flask-shaped invaginations of 50 to 100 nm diameter that were devoid of the clathrin-coat, but instead displayed a characteristic striated coat [85]. While research on clathrin-coated pits and vesicles was rapidly progressing, caveolae long remained elusive.

This was changed when, almost 40 years after the morphological description, caveolin was identified as the major protein constituent of caveolae [86, 87]. Subsequently, two additional caveolin genes were cloned, so that the original caveolin was from then on referred to as caveolin-1. Caveolin-2 was co-purified with caveolin-1 from adipocytes [88], and its expression pattern overlaps with that of caveolin-1. The two proteins are most abundant in endothelial cells, fibroblasts and adipocytes, and they form stable hetero-oligomeric complexes *in vivo* [89]. Caveolin-3 shows a high degree of sequence similarity with caveolin-1, but its expression is restricted to muscle cells in which there is low caveolin-1 expression [90]. Both caveolin-1 and -2 have a smaller β-isoforms in addition to the full-length α-isoform. Caveolin-1 assumes an unusual topology in that it is an integral membrane protein [91] but does not span the bilayer. Instead the central hydrophobic domain is thought to form a hairpin structure which inserts into the cytoplasmic leaflet, leaving both the N- and C-terminus in the cytoplasm [87].

A characteristic feature of caveolins is their propensity to form high molecular-weight homo- and hetero-oligomers. Highly stable caveolin-1 oligomers of 14 to 16 monomers, dissociating only upon harsh detergent treatment at elevated temperatures, were found to be assembled relatively rapidly after synthesis of caveolin-1 in the endoplasmic reticulum and prior to Golgi exit [92]. The domain responsible for the oligomerization was mapped to the N-terminus [93]. The N-terminus has also been shown to target caveolin-1 to caveolar invaginations at the plasma membrane, since its absence results in Golgi retention [94, 95]. This ensures that only caveolin oligomers, not monomers, are transported to the plasma membrane. In addition to homo-oligomerization, caveolin-1 can form similarly stable hetero-oligomers with caveolin-2, which are localized mainly to plasma membrane caveolae [89]. In the absence of caveolin-1, caveolin-2 is not able to oligomerize and is retained in the Golgi in the form of monomers and dimers [96–98], again indicating that only the oligomeric form is transported to the plasma membrane.

The fact that caveolin-1 immunostaining decorated the striated coat around plasma membrane caveolae [86], together with the observation that it self-assembled into filaments *in vitro* [92] indicated that it indeed was an integral coat component. Since then, the function of caveolae became very closely linked to the function of caveolin, and it was shown that formation of the stable plasma membrane invaginations depended on caveolin expression. Cells not expressing caveolin-1 (e. g., lymphocytes) lacked cell-surface caveolae, and the expression of caveolin-1 in these cells was sufficient to induce their formation [99]. Quantification of the number of caveolin-1 molecules per caveolae by fluorescence intensity distribution measurements revealed that the uniform size of caveolae as seen by electron mi-

croscopy results from a quantal assembly mechanism in which 144 ± 39 caveolin-1 molecules are incorporated into a single caveola [100]; caveolin-2 was not assessed in this study. Caveolin-1 filaments had previously been proposed to assemble from heptamers, measuring 10 nm in diameter [101]. If this model were true, then 144 caveolin-1 molecules would form a filament of roughly 200 nm length, enough to surround an invagination of 50–100 nm diameter with a circumference of 150–300 nm once. The structure and composition of the caveolar coat are far from being understood (see also Chapter 2) but, most likely, caveolin-1 is not the only coat component. Other open questions are, where is the coat assembled and what is the assembly mechanism?

Caveolin-1 has been shown to bind cholesterol and the ganglioside GM1, both *in vitro* and *in vivo* [102, 103]. Cholesterol-binding occurs with high affinity, resisting even harsh detergent treatments [103]. The lipid composition of caveolae is thus similar to that of lipid rafts, and it can be extrapolated that the caveolar membrane should also display properties of a liquid-ordered phase. However, a detailed lipid composition of isolated caveolae is still lacking. The strong interaction with two *bona fide* lipid raft components predisposes caveolin-1 for the role as a raft-clustering agent. Similar to clustered rafts, caveolae have been proposed to function as signaling platforms [104] (see also Chapters 5, 6, and 11). The clear parallels in lipid composition and the partial co-purification of lipid raft and caveolar components in DRMs [105, 106], or in membranes of low buoyant density [107], has often led to an equation of the two membrane systems. However, we will continue to refer to caveolae as plasma membrane invaginations scaffolded by the caveolin-coat. The stable membrane curvature of caveolae could be a result of two contributions. Curvature could be induced by: (1) the high cholesterol concentration [108]; and (2) the insertion of caveolin-1 into the cytoplasmic leaflet of the bilayer, which would increase the surface area of the cytoplasmic leaflet relative to that of the exoplasmic leaflet and thus promote inward bending of the membrane. This stabilization of a curved membrane structure and the presence of caveolins would distinguish caveolae functionally from lipid rafts.

1.8
Caveolae and Lipid Rafts in Membrane Traffic

Membrane traffic mediates the exchange of components between the different cellular organelles. Membrane proteins and lipids are synthesized in the endoplasmic reticulum and from there are transported to their subcellular sites of action [109, 110]. While peripheral membrane proteins as well as single lipids bound to lipid transfer proteins can shuttle between different membranes via the cytoplasm or through contacts between membranes [4], most membrane turnover is mediated by vesicular traffic. Directed vesicular transport involves several regulated steps:

- lateral sorting of membrane components according to their destination (i. e., the concentration of cargo following the same pathway and its segregation from cargo following different pathways);

- stabilization of a membrane domain destined for trafficking;
- bending of the membrane domain into the shape of a vesicle or tubule;
- pinching off from the donor compartment;
- traffic through the cytoplasm by passive diffusion or motor-protein-mediated transport along microtubules or actin filaments;
- fusion with the acceptor compartment; and
- release of the cargo.

The best-understood sorting mechanism for transmembrane proteins employs recyclable protein coats, such as clathrin-, COPI- or COPII-coats [111, 112] (Fig. 1.1, left panel). In this case the cargo proteins contain specific sorting signals in their cytoplasmic domains, which are bound by adaptor molecules, to which the coat proteins are recruited. Oligomerization of the coats leads to bending of the membrane domain into a vesicle, which is pinched off by the action of the GTPase dynamin and released into the cytosol. Here the coat disassembles, enabling the vesicle to fuse with its target membrane. This protein-driven mechanism operates by active inclusion of certain components and is not very efficient at excluding.

For other sorting events in membrane traffic, the lipid bilayer itself has been proposed to play the decisive role, and proteins only regulate what lipids can do on their own [113]. From theoretical considerations and model membrane studies it is known that if phases with different properties coexist in the same membrane, then the mismatch of interactions at the phase boundary leads to the so-called "line tension" – the two-dimensional equivalent of surface tension. Multiplied with the length of the phase boundary it gives rise to the "line energy". One way to minimize line energy is therefore to minimize the contact between phases. In the case of domains in cell membranes, this can be achieved by fusion of many small domains into one large domain, and bending the domain out of the surrounding bulk membrane [114]. The bending energy needed to curve the membrane as the domain buds out counteracts the line energy. As the bending energy increases and the line energy decreases, the domain reaches a stable curvature when the sum of the two energies is minimal. For small domains this can be when the domain is still connected to the bulk membrane, but above a critical domain size budding becomes energetically favorable. This mechanism is termed "domain-induced budding" (Fig. 1.1, right panel) and is initially achieved purely by lipid-driven phase separation [114]. However, in order to attain directionality in the budding process (i.e., budding towards the cytoplasm in most cases in cells) and also kinetics that are compatible with the cell's needs, proteins will have to control this process.

The fact that lipids are unevenly distributed between the two surfaces, the apical and the basolateral membrane domains, of epithelial cells [70] together with the finding that newly synthesized glucosyl-ceramide upon leaving the Golgi complex becomes two- to three-fold enriched in the apical versus the basolateral plasma membrane [115], has led to the proposal that lipids are also sorted by vesicular traffic. Interactions between glycosphingolipids and apical proteins were postulated to aid the assembly of sphingolipid microdomains in the Golgi that would

concentrate apical cargo as the first step in vesicle formation [70]. This mechanism has two important features which distinguish it from the coat-mediated sorting:

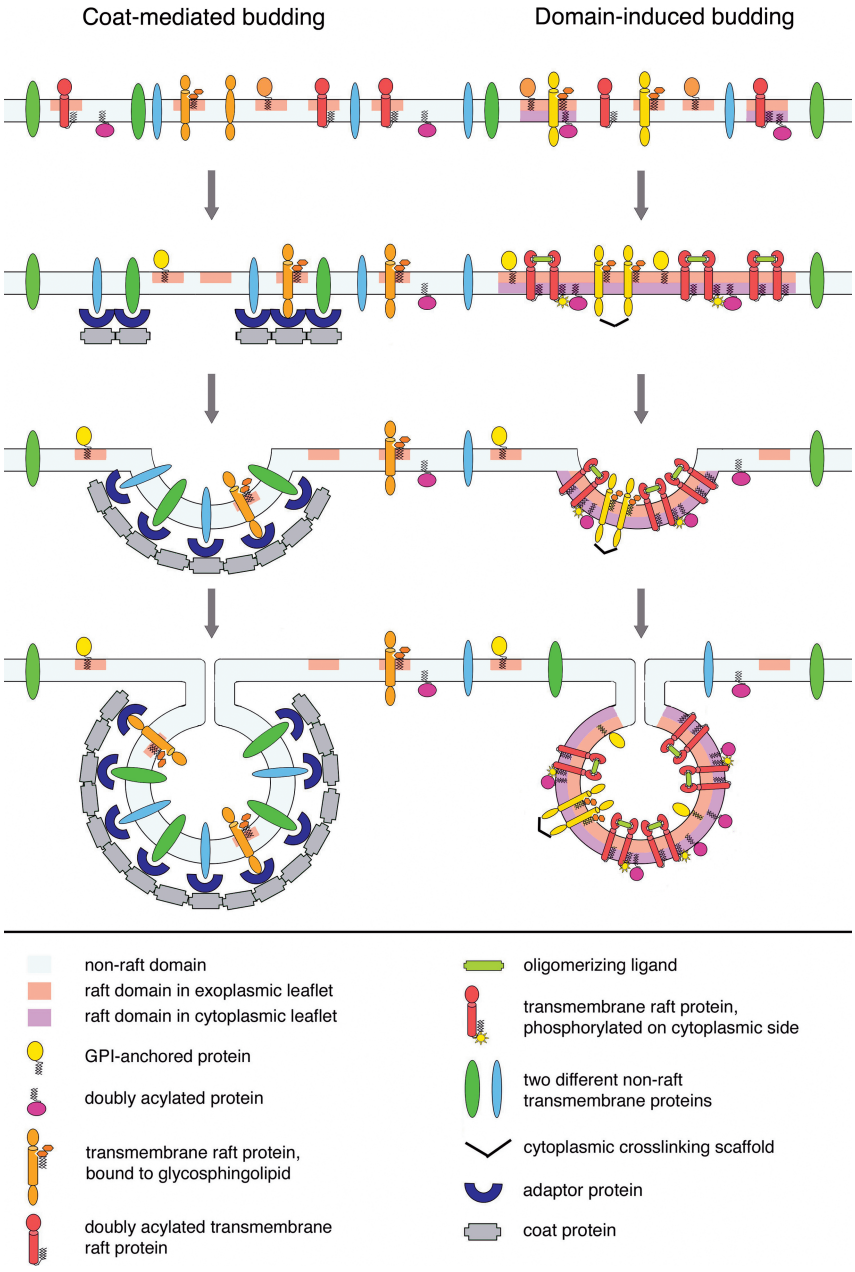
- It also allows for the sorting of lipids.
- It works by actively excluding cargo that does not belong into the pathway and thus prevents the transported membrane from being diluted with inadequate material.

It has been shown previously, that basolateral proteins are excluded from the apical membrane [116], whereas the converse is not true [69, 117]. Physiologically this is sensible, since the apical membrane facing the lumen of an organ must be extremely resistant to external aggression by bile salt detergents, digestive enzymes or low pH, and its composition must therefore be tightly controlled. Whilst it is known that basolateral delivery depends on the interaction with adaptor proteins [118], domain-induced budding seems to be a mechanism ideally suited for delivery to the apical membrane.

Since these microdomains, or rafts, are believed to be small and dynamic, they must be clustered by proteins such as multivalent ligands or caveolin in order to be able to form a bud and later a vesicle or tubule. In apical raft delivery this has been postulated to be mediated by lectins or other multivalent cargo receptors [119, 120]. Raft and caveolar endocytosis is triggered by multivalent cargo, the best described being Simian virus 40 and cholera toxin [121–124], both of which bind several GM1 molecules [125]. Here, the caveolar coat is not necessary for the membrane bending or vesicle formation, since rafts can endocytose upon clustering by a virus or toxin and be delivered to specific destinations in the cell without caveolin [124]. In fact, the internalization has been shown to be faster in the absence of caveolin [126]. Caveolin might thus not be necessary for the endocytic event as such, but rather add another level of regulation to this pathway, which is required for the efficient sorting of some ligands [127].

Indeed, caveolae membrane traffic does display special features that set it apart from other membrane traffic mechanisms. Caveolae were previously believed to be static structures [128], simply increasing the cell-surface area and keeping raft

Fig. 1.1 Two paradigms of cargo sorting and vesicle formation in membrane traffic: inclusion due to sorting signals followed by coat-driven budding (left), or exclusion due to phase separation and domain-induced budding (right). In the left column, proteins containing the appropriate cytoplasmic sorting signals (regardless of if they are residing in a raft or non-raft domain) are bound by adaptor proteins, on which the coat proteins assemble. For the clathrin-coat, membrane bending and subsequent budding is believed to be driven by a conformational change in the coat protein. In the right column, raft proteins are clustered by oligomerizing ligands or cytoplasmic scaffolding proteins, thereby excluding the group of non-raft proteins. Membrane bending and budding is driven by the need to minimize the line energy acting at the domain boundary. ▶


Coat-mediated budding
Domain-induced budding
non-raft domain
raft domain in exoplasmic leaflet
raft domain in cytoplasmic leaflet
GPI-anchored protein
doubly acylated protein
transmembrane raft protein, bound to glycosphingolipid
doubly acylated transmembrane raft protein
oligomerizing ligand
transmembrane raft protein, phosphorylated on cytoplasmic side
two different non-raft transmembrane proteins
cytoplasmic crosslinking scaffold
adaptor protein
coat protein

membrane available on the cell surface. Recently, it became evident that even in unstimulated fibroblasts and epithelial cells, 30% of the caveolae undergo local kiss-and-run cycles with the plasma membrane in which they pinch off and fuse again close to the original site [100]. Upon receiving a trigger for endocytosis, caveolae switch from this short-range cycling to long-range cycling, resulting in an intermixing of cell-surface and intracellular caveolar vesicle pools and transport to caveosomes or endosomes [100]. During the trafficking event, the caveolar coat seems to stabilize the clustered raft domain within the bilayer, so that it stays intact even after fusion with the acceptor compartment and can be re-used for multiple rounds of membrane trafficking [127]. Cargo release at the target compartment must therefore also follow different principles than in the clathrin-coated vesicle traffic where the coat disassembles before fusion. Caveolae apparently keep their cargo sequestered, until its release is triggered by a compartment-specific cue. Cholera toxin is released upon encounter of a low pH environment in early endosomes, but stays sequestered in caveolae in the neutral environment at the plasma membrane or in caveosomes. This type of membrane traffic seems especially suited for the sorting of non-membrane spanning cargo, in particular glycosphingolipid-binding ligands [127].

The vesicle fusion machinery on the target compartment also has been proposed to be organized into domains of different lipid composition. The apical t-SNARE syntaxin 3 was proposed to reside in raft domains [129]. More recent investigations have claimed that indeed different SNAREs are compartmentalized in the plasma membrane with the help of lipid domains, with syntaxin 3 residing in raft domains, syntaxin 2 being excluded from raft domains, and syntaxin 4 being equally distributed between the two [130]. In polarized epithelial cells, syntaxin 4 resides on the basolateral surface, whereas syntaxin 2 and 3 are localized to the apical surface [131]. The data would thus imply, that there could be two pathways trafficking to the apical side of epithelial cells – one raft- and one non-raft pathway. Indeed, it was previously observed that two different apical proteins, sucrase-isomaltase and lactase-phlorizin-hydrolase, use separate containers for transport to the apical membrane of Madin-Darby canine kidney (MDCK) cells, and the existence of two different pathways was proposed [132].

Research on rafts and caveolae is entering a new phase. The technologies that have been used to study these membrane domains are being revised, and new technologies must be developed. If rafts are small and dynamic, many of the standard techniques that have been employed to visualize them (e.g., FRET, single particle tracking, FRAP in most cases) can not provide anything else but negative results because they are not suited for the size and time resolution needed. Another critical point is the purification methods used to isolate rafts or caveolae. The two were often confused with each other since they were supposed to co-fractionate when isolated based on detergent insolubility or light buoyant density. It is now accepted that these fractions are useful to obtain information about the proteins found in them, but since they form during the purification process, they can not be assumed to represent an equivalent of any pre-existing cellular domain, neither rafts nor caveolae [10]. Instead, new approaches have been taken – for

example, to isolate plasma membrane fragments with small antibody-coated beads [133]. Techniques such as this must be developed in order to obtain pure raft and caveolae fractions that can be used to analyze their lipid and protein composition. With the new mass spectroscopic techniques it should then be possible to compare the lipidome of rafts and caveolae with each other to determine how similar they actually are, and also to compare them with the lipidome of the plasma membrane. Only then will we have a chance to assess properly the involvement of lipids in processes such as raft dynamics, raft clustering, and to address the special functions of caveolae.

Abbreviations

DRM	detergent-resistant membrane
FRAP	fluorescence recovery after photobleaching
FRET	Förster's resonance energy transfer
GFP	green fluorescent protein
GPI	glycosyl-phosphatidyl-inositol
GUV	giant unilamellar vesicles
LUV	large unilamellar vesicles
MDCK	Madin-Darby canine kidney
MLV	multilamellar vesicles
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI(3)P	phosphatidylinositol-3'-phosphate
PLAP	placental alkaline phosphatase
PS	phosphatidylserine
SM	sphingomyelin
T_m	melting temperature

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