THE STATE OF LIPID RAFTS: From Model Membranes to Cells

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Abstract Lipid raft microdomains were conceived as part of a mechanism for the intracellular trafficking of lipids and lipid-anchored proteins. The raft hypothesis is based on the behavior of defined lipid mixtures in liposomes and other model membranes. Experiments in these well-characterized systems led to operational definitions for lipid rafts in cell membranes. These definitions, detergent solubility to define components of rafts, and sensitivity to cholesterol deprivation to define raft functions implicated sphingolipid- and cholesterol-rich lipid rafts in many cell functions. Despite extensive work, the basis for raft formation in cell membranes and the size of rafts and their stability are all uncertain. Recent work converges on very small rafts <10 nm in diameter that may enlarge and stabilize when their constituents are cross-linked.

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INTRODUCTION

“...it is not as sure as both chymists and Aristotelians are wont to think it, that every seemingly similar or distinct substance that is separated from a body... was pre-existent in it...”

Robert Boyle, The Sceptical Chymist, 1661 (13)

Domain models of cell membranes, which view lipid bilayers as more mosaic than fluid, have long been proposed based on the properties of lipids in liposome membranes (54, 63). Indeed, even the model that summed up early work on membrane organization and emphasized bilayer fluidity, the fluid-mosaic model of Singer & Nicolson (129), considered the possibility of small membrane domains (∼100 nm at most) in the fluid cell membrane bilayer. However, all models of cell membranes comprising domains were rather general and did not focus on specific biological functions that required domain formation.

Lipid rafts were conceived as functional lipid microdomains less than 15 years ago (128, 143). They were proposed as the solution to a particular biological problem, the selective delivery of lipids to the apical and basolateral surfaces of morphologically polarized epithelial cells. The lipid raft microdomain model drew on the physical chemistry of model lipid membranes to create a mechanism for segregating and sorting newly synthesized lipids for traffic to the cell surface. In turn, it suggested experiments in models to test aspects of the raft model.

This review begins with a history of the way in which physical chemistry, biochemistry, and cell biology came together to create and expand the raft model for lipid trafficking to encompass other areas of membrane biology, including signaling and invasion by intracellular pathogens. This survey of the early work in several areas maps some of the streams that feed the lipid microdomain model. These streams have disappeared in successive reviews of the field, but they are important to understanding the prospects and limits of lipid raft microdomain models. Other streams, in particular that of caveolae and caveolin, are the subject of excellent recent reviews (1, 2) and are not mapped here.

The fruitful interplay between results in model membranes and those on cell membranes continues to this day. Accordingly, after the historical introduction, this review will cover data from model membranes, monolayers, supported bilayers and liposomes, and cell membranes. We will see that the results with models demand rethinking of many of the procedures defining lipid rafts in cell membranes. My reading of this literature is that procedures create artifacts that do not reflect the state
of lipids in native membranes. On the other hand, both model and cell experiments appear to be converging on the scale of lipid raft domains in native membranes and on mechanisms for organizing lipid rafts. I expect that further work will show that protein-lipid interactions rather than lipid-lipid interactions are important in organizing membrane lipids for trafficking and in signaling.

PREHISTORY AND EARLY HISTORY OF LIPID RAFTS

Epithelial Cells and Membrane Sphingolipids

“...in the plane of the membrane there are domains enriched in sphingomyelin and cholesterol (or both)” (47).

Epithelial cells define and separate functional compartments in tissues and organs. They are morphologically and functionally polarized. The apical surface of a typical epithelial cell is chemically and functionally distinct from the basolateral surface. The demarcation between the two surfaces is a band of tight junctions binding adjacent cells in the epithelial sheet. This band of tight junctions allows selective fractionation of apical from basolateral plasma membranes. Apical lipids of apical membrane fractions are enriched, relative to basolateral membranes, in glycosphingolipids (GSL). This enrichment was confirmed using enveloped viruses to sample apical or basolateral host cell membranes (111, 142) and by other approaches (132). This difference in composition appeared to be due to the sorting of proteins and lipids in the Golgi complex and trans-Golgi membranes and directed trafficking of these molecules to the cell surface. Simons and van Meer (128, 143) based the lipid microdomain model for these cell biological observations on model membrane and other physical chemical studies that showed a high propensity for hydrogen bonding between GSL (11, 94), as well as on observations that neutral GSL, but not charged GSL, were found in small clusters, about 15 molecules maximum, in model and native bilayer membranes (108, 140). Thus in the proto-lipid raft model, trafficking microdomains were seen as clusters of hydrogen-bonded GSL, with associated proteins, either trapped in the hydrogen-bonded sphingolipid phase or specifically associated with sphingolipids. The proteins were presumed to carry the sorting signals that would result in trafficking entire microdomains to the apical surface.

Lipid-Anchored Proteins

Glycosylphosphatidylinositol (GPI)-anchored membrane proteins were found to concentrate at the apical surfaces of polarized epithelial cells both in vivo and in vitro (71). Their distribution was often more highly polarized than that of GSL, and the GPI-anchor appeared to be the sorting tag. Substituting a GPI modification sequence for the transmembrane region and cytoplasmic tail of a basolateral membrane marker redirected it to the apical surface (14, 70).
The parallel between polarity of GSL and that of GPI-anchored proteins suggested that the two classes of membrane molecules trafficked in the same unit, the lipid microdomain of van Meer and Simons. A scattering of biochemical data indicated that sphingolipids (1, 36) and GPI-anchored proteins (48) were insoluble in the neutral detergent Triton X-100, especially when cholesterol was present. These data were pulled together in a systematic study by Hooper & Turner (50) that showed that GPI-anchored ectoenzymes of renal epithelial apical membranes were insoluble in 5.9 mM Triton X-100 at 4°C, but soluble in low concentrations of other detergents. In a landmark paper, Brown & Rose (15) used this insolubility criterion to follow the membrane organization of a GPI-anchored alkaline phosphatase as the protein traveled through the endoplasmic reticulum and Golgi complex on its way to the apical surface of MDCK cells. Ninety percent of nascent alkaline phosphatase molecules were Triton X-100 insoluble by the time their N-oligosaccharide chains had matured, but had not been sialylated. This placed the point of their conversion from detergent-soluble to detergent-insoluble forms and entry into lipid microdomains for sorting somewhere prior to the medial Golgi complex. When detergent extracts were fractionated by density, mature alkaline phosphatase was found in lipid-rich, low-density fractions, whereas a control transmembrane protein fractionated to lipid-poor, high-density fractions.

The low-density fractions enriched in alkaline phosphatase appeared to be membrane vesicles of varied sizes, but typically hundreds of nanometers in diameter. Relative to whole cell lipids, the vesicles were depleted of the glycerophospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and enriched in cholesterol and sphingolipids. The molar ratio of glycerophospholipid:sphingolipid:cholesterol was 34:36:32 or ~1:1:1. This ratio has become the canonical “lipid raft” mixture—the composition used for model membrane studies of raft properties.

Two other observations further defining a sorting lipid raft followed that of Brown & Rose (15). Le Bivic and colleagues (32) used a brief extraction (5 min in 1% Triton X-100, rather than the 20 min used by Brown & Rose) and reported that 50%–60% of marker GPI-anchored proteins were Triton X-100 insoluble. Although it was expected from the composition of detergent-insoluble rafts, and from some observations on clustering of GPI-anchored proteins (112), Le Bivic et al. found no effect of cholesterol-binding drugs on insolubility of GPI-anchored proteins. Another group, using a longer extraction time (30 min), did find that GPI-anchored alkaline phosphatase was almost all soluble if cells were treated with saponin, to complex cholesterol, before extraction (20).

Together, the experiments summarized above redefined lipid rafts as requiring both sphingolipids and cholesterol. They also created two operational definitions for lipid rafts. One definition, for constituents of rafts, was fractionation of proteins of interest to a lipid-rich complex insoluble in cold Triton X-100. A second definition, for raft requirements in cell function, was perturbation of some cellular process by cholesterol depletion, either acute or chronic. These two operational definitions opened the way for a vigorous pursuit of lipid rafts in
trafficking newly synthesized lipids and proteins to cell surfaces. A consensus emerged that cholesterol/sphingolipid-rich membrane rafts are central to this traffic (126); however, this consensus is strained and challenged by the plasticity and range of lipid-sorting patterns in a variety of cells (149) and by the adaptability of cells to cholesterol or GSL depletion (37, 93).

**Signaling Tyrosine Kinases and Lipid Rafts**

Cross-linking GPI-anchored proteins can activate lymphocytes. Because activation is signaled through a cascade of intracellular kinases, signaling by GPI-anchored proteins raises the same formal problem that is raised by their vectorial transport, i.e., communication between the outer leaflet of the membrane bilayer and the cell cytoplasm. In three closely linked papers, Horejsi, Stockinger, and their colleagues showed that GPI-anchored proteins of human lymphocytes could be isolated in cold Triton X-100–insoluble complexes that also contained src family tyrosine kinases (22, 134, 135). These papers are the basis of a large literature that generally shows association of ligated receptors, signaling kinases and phosphatase, and G proteins with raft fractions to form signaling complexes (127). The three types of receptors most explored are the so-called IgE receptor (FceR1) of mast cells and basophils (124), the T-lymphocyte receptor for antigen (150), and the B-lymphocyte receptor for antigen (95). Although the case for some involvement of rafts or raft lipids in receptor-mediated signaling and in regulation of this signaling (57) is compelling, work in this area is bedeviled by a lack of quantitative precision. The model for the function of rafts in T cell receptor signaling has also received a vigorous challenge from a detailed consideration of the biochemistry, rather than the physical chemistry, of receptor signaling (33).

More recently, operational definitions have pointed to lipid rafts as sites of entry and exit for intracellular pathogens (141). These may prove quite useful in sorting out raft function because they offer the possibility of using mutant viral and bacterial proteins as probes of native cell membranes.

**THE LIPID RAFT RUBRIC**

Together, the data of the last section give us the rubric—the outline and direction—for the rest of this review, a discussion of model membranes, lipid monolayers and bilayers, and cell membranes. First, we examine the ways in which sphingolipids, cholesterol, and glycerolipids behave in model membranes to gain an understanding of the way they could interact to form rafts in cell membranes. Models that begin as rather general investigations into the properties of a class of lipids, for example, studies of pure sphingolipid monolayers, can evolve into highly specific systems, such as mixtures of 1:1:1 phosphoglycerolipids:sphingolipids:cholesterol, which approximate the composition of detergent-insoluble domains isolated from cells. Most of these studies are consistent with the inferences about lipid rafts in intact
cells. They also shed new light on the way in which bilayer leaflets could be coupled in lipid raft–mediated signaling and on the minimum size of cholesterol/lipid complexes. On the other hand, we will see that some studies of the solubilization of membranes by Triton X-100 may subvert the operational definition of lipid rafts in terms of detergent insolubility.

I also argue on cell biological grounds that the second operational definition of lipid rafts, cholesterol depletion, is also unsound. This leaves us with the problem of probing for lipid rafts in native cell membranes, without biochemical modification or detergent extraction. Optical methods, such as fluorescence resonance energy transfer (FRET), single molecule and single particle tracking, and laser trapping, have all proved useful here. These optical methods appear to be converging on a size for a unit or core lipid raft that is small indeed, consisting of at most half a dozen molecules, a far cry from the 100-nm rafts first defined as detergent-resistant membrane vesicles.

Although core lipid rafts shrink to a few molecules, we are left with the problems in cell biology that led to the raft model in the first place. These imply the reorganization of core rafts into larger structures. The trafficking of lipids, formation of signaling complexes, and virus interaction with cells all report segregation and concentration of raft lipids. I suggest that the common denominator in all these events is protein interaction with lipids, either by recruiting subsets of lipids to clustered transmembrane domains or by clustering of lipids interacting with protein exodomains apposed to the membrane surface. In short, proteins recruit lipids, rather than the opposite.

**INTERACTION OF PHOSPHOLIPIDS, GLYCOLIPIDS, AND CHOLESTEROL IN MODEL MEMBRANES**

**Model 1: Sphingolipids**

As we noted earlier, the lipid raft model was based on studies of purified sphingolipids. As a class, the acyl chains of sphingolipids are more saturated than those of glycerolipids. Typical plasma membrane glycerolipids bear saturated acyl chains at the glycerol sn-1 position and unsaturated acyl chains at sn-2, for example, 16:0/18:1. In contrast, the sphingoid backbone of a sphingolipid is 18:0, and the acyl chain is also saturated, 18:0 to 24:0 (16, 106). A mixture of fluid glycerophospholipid and sphingolipid might be expected to segregate sphingolipid-rich domains, optimizing acyl chain packing. This tendency is enhanced by the presence of both hydrogen bond donor (hydroxyl) and acceptor (fatty acyl carboxyl) groups in sphingomyelin (SM). It is opposed by sphingolipid headgroups, the phosphocholine of SM, and the neutral or charged oligosaccharides of GSL. The bulk and charge of these headgroups limit the packing density of pure sphingolipid species, particularly of the negatively charged gangliosides (76). On the other hand, hydrogen bonding between sugars of the headgroups can stabilize glycolipid interactions. Indeed, as noted earlier (108, 140), clusters of neutral glycolipids may be present in native membranes. In contrast, clustering of gangliosides is not
observed, although they can be induced to cluster by Ca$^{2+}$ or by multivalent toxins, such as cholera toxin (35).

Mixtures of sphingolipids with different size headgroups segregate from glycerophospholipids, forming complexes that can locally develop into gel phases in a liquid crystalline phospholipid matrix. Thus the gel to liquid crystalline phase transition temperature of either 16:0/16:0 PC or SM in a matrix of fluid native PC (16:1, 18:0) is raised significantly by the addition of 10% ceramide (acylated sphingoid base) to the mixture. The ceramide has a larger effect on the melting temperature of SM than on that of dipalmitoylphosphatidylcholine (DPPC), presumably because it can interact with SM through both acyl chain interactions and hydrogen bonding (78). In this system, where the acyl chain length of all lipids was similar, there was no evidence of formation of ceramide-rich domains. Only interactions with other lipids led to segregation of a gel domain. The author speculates that local changes in ceramide concentration, due to local sphingomyelinase activity, could create small gel domains in cell membranes, perhaps changing local permeability and activating membrane-associated enzymes.

SM also forms complexes with glycolipids, consistent with the co-isolation of these molecules in raft fractions (79). The preferential segregation of GSL and SM from glycerophospholipids, independently of other lipids or cholesterol, may be important in organizing the apical surfaces of renal and intestinal epithelial cells, which stabilizes them against breakdown by the hostile fluids (for example, bile salt–containing intestinal fluids) that bathe them. This is suggested by the observation that apical brush border lipids form detergent-resistant domains even in the absence of cholesterol (38, 85). This is also suggested by recent work comparing the permeability of apical and basolateral membrane vesicles from guinea pig colon (18). In some sections of the colon, the cholesterol:phospholipids ratio is twofold higher in basolateral membrane vesicles than in apical membrane vesicles, yet the permeability of the latter is much lower than that of the former.

Model 2: Cholesterol-Containing Lipid Mixtures

“...in biomembranes containing various phospholipids species one would expect that certain lipids (saturated species for example) would tend to accumulate in the vicinity of cholesterol...” (53).

The lipids of mammalian cell plasma membranes are roughly one-third cholesterol. This rigid sterol molecule intercalates between acyl chains of phospho- and glycolipids whether these are in the gel or the liquid crystalline phase. In the former, because the rigid sterol cannot conform to pack with all-trans acyl chains, cholesterol decreases acyl chain order and this is reflected in the mixture melting at a slightly lower temperature and over a much broader range than the pure lipid. In the liquid crystalline phase—the matrix of cell membranes—cholesterol orders acyl chains, decreasing the area per molecule. It has been well put that “sterols appear to have evolved to fill the flickering spaces among acyl chains in membrane bilayers” (92). The great range of cholesterol interactions is evidenced
both by model membrane studies and by the finding that cell metabolism of cholesterol and choline phospholipids, both glycerolipids and SM, is coordinately regulated. These interactions are discussed in an excellent recent review (92). Here we focus on cholesterol-containing membranes whose composition is modeled on lipid rafts, as defined by detergent extraction. As noted above, raft lipid composition is approximately 1:1:1 phosphoglycerolipids:sphingolipids (both SM and GSL):cholesterol. In rafts prepared with detergent, the phospholipid population is enriched in saturated acyl chains relative to the average for whole cell phospholipids (30, 97) [although this is not the case for sphingolipid-rich raft fractions prepared without detergent (9)]. This finding converged with studies of cholesterol interactions with saturated chain PCs, particularly DPPC, to suggest that the lipids of rafts, as defined by detergent insolubility, were in a liquid-ordered phase, \( l_o \). This phase was defined in theoretical work based on five different experimentally determined phase diagrams for DPPC cholesterol (52). It is one in which the unfavorable free energy of cholesterol solvation in an ordered gel phase is balanced by specific short-range interactions of cholesterol with the conformationally ordered acyl chains of this phase. The result is decoupling of acyl chain conformational order from PC crystalline order. Thus, while acyl chains are largely in the all-trans conformation, rotational and translational diffusion of lipid molecules is almost equal to that in a liquid crystalline phase. Moderate (~10–20 mol %) concentrations of cholesterol result in a mixture of gel and liquid-ordered phases below the PC melting temperature (41°C) and a mixture of two immiscible liquid crystalline phases, liquid ordered and liquid disordered, above the melting temperature. Further theoretical work has reinforced the view that cholesterol and related sterols with a smooth, unbroken hydrophobic surface (but not sterols such as lanosterol with this surface made “bumpy” by methylene groups) are uniquely suited to interact with acyl chains to create a liquid-ordered state (84).

Not only is the lipid composition of detergent-insoluble lipid rafts consistent with the existence of a liquid-ordered state, but liposomes made of a binary mixture of DPPC and cholesterol 2:1, proportions that are in the liquid-ordered region of the phase diagram, were completely insoluble under the conditions used to define lipid rafts by detergent insolubility. Liposomes with compositions approximating that of lipid rafts, rich in cholesterol and sphingolipids, were also largely detergent insoluble (121). In a series of experiments comparing lipid mixtures capable of forming liquid-ordered domains with those that are not, excellent correlations were made between detergent insolubility, ordering of acyl chains, and formation of ordered domains detected by dequenching a fluorescence probe. The probe, diphenylhexatriene (DPH), could partition into more ordered phases, whereas the short-range quencher, a spin-labeled lipid, partitioned in disordered phases. Hence, increases in fluorescence were taken to indicate at least partial segregation of the fluorescent probe into liquid-ordered domains. These experiments are summarized in a recent review (72). They speak to lateral variation in the surface of liposomes of appropriate composition but only explore the phase diagram of the systems studied to a limited extent. They also do not speak to the size of the liquid-ordered domains, an
issue of paramount interest in studying native cell membranes. Short-range quenching could be impeded if donor fluorophores were in small domains and separated from the quencher by a few phospholipid radii, as well as if the fluorophores were in large domains and sequestered many molecule diameters away from the quencher.

Fluorescence resonance energy transfer (FRET) measurements can be used to estimate the size of lipid domains, if the apparent partition coefficients of donors or acceptors determined by FRET can be compared with the partition coefficients measured by some distance-independent method. Prieto and colleagues (74) have taken this approach with the classic liquid-ordered/liquid-disordered model membrane, DPPC/cholesterol. They find that liquid-ordered domains dispersed in liquid-disorder phase are small, \( \leq 20 \) nm, the \( R_o \) for their donor-acceptor fluorophore pair.

The interpretation of the DPPC/cholesterol phase diagram in terms of immiscible liquid phases \( l_o \) and \( l_d \) is not unique. In another view, these mixtures comprise immiscible phases, one of which is rich in specific complexes of cholesterol with phospholipids. Complex formation is treated as a reversible reaction characterized by an equilibrium constant, \( K_{eq} \), with a stoichiometry that appears to be \( \sim 1:5 \) cholesterol/glycerophospholipid or sphingolipid in monolayers (104) and 1:12 cholesterol:phospholipid in vesicle bilayers [(3), reviewed in (82)]. A complex-rich phase separates from cholesterol-rich and liquid crystalline phases [of dimyristoylphosphatidylcholine (DMPC), which does not form complexes efficiently] in monolayers at low pressures (82). At higher pressures, comparable to those in a bilayer membrane, the complexes appear to be miscible with the other phases.

The cholesterol/lipid complex model can be used to interpret thermodynamic phase diagrams for mixtures of cholesterol and disaturated acyl chain phospholipids, for example, dipalmitoylphosphatidylethanolamine (DPPE), which were used to infer the coexistence of \( l_o \) and \( l_d \) phases, without specifying a liquid-ordered phase or indeed specifying anything about the high-cholesterol region of the phase diagram, except that it contains cholesterol/phospholipid complexes. It contrasts with the \( l_o/l_d \) model, but each model has a different emphasis and different uses. A membrane model with immiscible \( l_o \) domains is useful for thinking about the ways bilayer leaflets can be coupled by cholesterol and interdigitating acyl chains (116). It also sets a direction for modeling immiscible liquid-state lipid phases and for interpreting cell experiments where cross-linked membrane receptors change their association with particular lipids. This sort of model tempts one into thinking about \( l_o \) phases as stable regions of cell membranes, i.e., available to cross-linked receptors. It also neglects the chemical activity of cholesterol, i.e., its availability for specific chemical reactions with proteins and lipids.

A membrane model in which cholesterol is in a stoichiometric complex with either phospho- or glycolipids emphasizes the regulation of cholesterol activity in membranes and points to the possibilities of rapid local remodeling of cell membranes by available cholesterol. Changes in cholesterol activity can in turn lead to changes in membrane enzyme activity (in response to small changes in bilayer thickness or chain order) (73, 136), changes in activity of lipases (118)
and accessibility of their substrates, and changes in receptor and antigen display, as well as global responses by cells.

The canonical raft lipid mixture and its variants yield multimicrometer-diameter domains in both monolayers and supported bilayers below the melting temperature of all their component lipids. Overall, studies of these domains yield convincing evidence for the possibility of liquid/liquid immiscibility in cell membranes and also highlight the mechanical and physical properties of \( l_0 \) phases. One group emphasized the mechanics and dynamics of domains excluding R-DOPE (dioleylphosphatidylethanolamine), showing that properties of these dark domains are those of fluids, not gels. The behavior of the domains under shear and the dynamics of their fusion may speak to the way small, functional \( l_0 \) domains form in cell membranes. Their behavior when the bathing solution was stirred indicated that they were thicker than the surrounding bilayer, consistent with atomic force microscopy (AFM) and X-ray diffraction measurements (31). Another group explored variables of probe, form of model membrane, and composition. The probes for raft domains in monolayers and supported bilayers were a series of headgroup-tagged DPPE molecules: NBD-PE, fluorescein (FL)-PE, and Texas Red-PE. NBD-PE, the probe with the smallest headgroup, entered \( l_0 \) domains in supported monolayers at lateral pressures of \( \sim 30 \) dyne/cm similar to those inferred for cell membrane bilayers. However, when supported bilayers were probed, FL-PE partitioned about equally well into \( l_0 \) and \( l_d \) phases. The \( l_0 \) domains could not be resolved after cholesterol depletion and reappeared when cholesterol was added back to the depleted bilayer.

When monolayers were formed from renal epithelial cell brush border lipids, the domains were often ovoid with rough contours rather than circular shapes with smooth perimeters, as expected for a liquid phase. Similar shapes were seen for dark regions (excluding TR-DPPE) in monolayers prepared from cholesterol-depleted brush border lipids. This suggests the possibility that the domains are gel, not \( l_0 \). Indeed, although the dark domains in complete brush border lipids appeared to allow some penetration of TR-DPPE, the rough-contoured domains in cholesterol-depleted brush border lipids were impermeable to TR-DPPE, consistent with their being in the gel state.

Lipid monolayers were also used to visualize two other important parts of the lipid raft model: (a) partition of GPI-anchored proteins into \( l_0 \) domains and (b) the effects of cross-linking on partition of a probe molecule between \( l_0 \) and \( l_d \). Results for both visualizations were not as clear-cut as might have been expected. The GPI-anchored Thy-1 molecule was present in both the \( l_d \) matrix lipids and the \( l_0 \) domains of dioleoylphosphatidylcholine (DOPC)/SM/cholesterol or brush border lipid monolayers, with more intensity in putative \( l_0 \) domains. A similar result for bilayers was reported for GPI-anchored alkaline phosphatase (117). Surprisingly, less Thy-1 partitioned into \( l_0 \) if a small amount (<1 mol %) of ganglioside GM1 was present. Because rafts defined by detergent extraction are enriched in Thy-1, gangliosides, and other glycolipids, it is not clear why Thy-1 and GM1 did not colocalize.

Another surprise was found when FL-DOPE or FL-DPPC was cross-linked. FL-DOPE partitioned mainly into the \( l_d \) matrix lipids of the monolayer regardless
of whether it was cross-linked, consistent with its unsaturated acyl chains, and FL-DPPE partitioned into \( l_d \) domains only after cross-linking by antifluorescein antibody. The partition of FL-DPPE between \( l_d \) and \( l_o \) is different in supported bilayers than in monolayers. This suggests that subtle changes in molecular interactions could shift it more toward the \( l_o \) phase even in monolayers. It would be interesting to know if TR-DPPE also partitions into the \( l_o \) phase after cross-linking.

Although \( l_d \) and \( l_o \) domains can be detected by microscopy, wide-angle X-ray diffraction studies detected only a single lamellar phase in multilamellar vesicles of 1:1:1 DOPC:SM:cholesterol. Gel and fluid phases could be distinguished in 1:1 DOPC:SM vesicles in which SM forms a gel phase (31). The authors discuss possible reasons for this difference, pointing out variations in boundary conditions and composition among the model membranes used in References 24, 25, 31, and 114, though citing other explanations as well.

**Model 3: Triton X-100 and the Solubility of Model Lipid Mixtures**

Membrane lipid raft domains are often defined operationally in terms of detergent insolubility in cold Triton X-100, although not exclusively (for example see Reference 26 and a discussion of lipid solubilization by different classes of detergent in Reference 7). However, few of these experiments explore a wide range of Triton concentrations or follow the timecourse of membrane solubilization by detergent. Indeed, much of the published work on detergent-resistant domains in cell membranes does not even specify the ratio of detergent to membrane lipid. A few experiments on model membranes do explore a large sample of the variable space. These show that SM/cholesterol monolayers and bilayers are more resistant to solubilization by Triton X-100 than are pure SM membranes. Hydrogen bonding between cholesterol and SM may be important for this. However, Triton X-100 at low concentrations penetrates SM-containing membranes more readily than chain-matched phospholipid DPPC membranes, and SM membranes are solubilized at lower concentrations of detergent than are phosphoglycerolipid membranes [see (90) and references therein].

The modeling work also suggests that detergent-resistant lipid domains are created when low concentrations of Triton X-100 induce membrane blebbing and fusion, possibly scrambling membrane lipids (47, 131). Addition of small, sublytic amounts of detergent to vesicles made of raft lipids increases their light scattering, implying an increase in size, owing to bilayer fusion. Electron microscopy of vesicles treated this way shows that multilayer vesicles are indeed converted to larger structures containing ramified branched bilayers (47), as are cell membranes (130). Furthermore, it appears that inclusion of GSL in model membranes reduces the detergent concentration required for membrane solubilization. The authors note that this is consistent with the tendency for GSL to form micelles in water.

A recent study suggests that the detergent could induce domain formation in otherwise miscible lipid mixtures (45). NMR and three different types of
calorimetric measurements— Isothermal titration, differential scanning, and pressure perturbation—were used to follow micellization of lipids, an indication of membrane solubilization, as a function of temperature and Triton X-100 concentration and, in parallel, thermal transitions. When Triton X-100 was titrated into a POPC:SM:cholesterol raft model membrane system, a sharp change in heat of transfer occurred at detergent:lipid ratios that did not solubilize membrane. This was interpreted as a detergent-driven transition within the lamellar state. The author argues that the observed changes represent the balance between enthalpically favored associations of SM and cholesterol and entropically driven dispersion of SM and cholesterol in the liquid-disordered membrane phase. When detergent:lipid ratios reach some critical value, in this system about 0.3 Triton X-100/POPC, the enthalpic penalty of mixing becomes so high that SM and cholesterol segregate from the POPC and form detergent-resistant domains. These results suggest that the large SM/cholesterol-rich domains isolated from detergent do not reliably report on the organization of these lipids in the cell membrane.

MODELS MEET CELLS AND RAISE PROBLEMS

Problem 1: How Are the Two Monolayers of a Cell Lipid Bilayer Membrane Coupled to Form a Trans-Bilayer Raft?

One might expect that rafts would form independently in each leaflet. In the event, the two leaflets appear to be coupled in both gel/liquid glycerophospholipid mixtures (4, 64) and raft mixtures (24, 25). This might be due to interdigitation of longer acyl chains (106) and/or cholesterol (116) of the two monolayers. However, understanding the coupling of raft lipids between the inner and outer leaflets of cell plasma membranes is vexed by a number of factors, such as the high degree of unsaturation of inner leaflet phospholipid acyl chains, the asymmetry of SM distribution, with only 15% of the total in the inner leaflet, and the unresolved distribution of cholesterol between the inner and outer leaflet. Estimates for cholesterol distribution range from about 65% in the outer leaflet (23) to 75%–80% in the inner leaflet (120). Thus, the global ratio of cholesterol to sphingolipids may be as high as 10:1 in the inner leaflet and as low as 0.3:1 in the outer leaflet, with a consensus ratio of 2:1. There is also evidence of multiple pools of membrane cholesterol extractable with methyl-β-cyclodextrin (43, 91) or accessible to cholesterol oxidase (28, 99). It is not clear what fraction of membrane cholesterol can associate with sphingolipids to form rafts.

In a model mixture of PCs with unsaturated (20:4 and 18:0) acyl chains, 3H NMR spectra suggest that cholesterol segregates into cholesterol-rich regions (17); however, these are not likely to be in a raft-like l_α state. No l_α domains are detected in bilayers made of cholesterol and lipids characteristic of the inner leaflet, PS and PE with unsaturated acyl chains (147). The authors speculate that any inner leaflet rafts must be organized by factors, such as proteins, extrinsic to the inner leaflet. Immiscible liquid phases do form in monolayers made from erythrocyte membrane
inner leaflet lipids, about 5% in SM (60), but these could be cholesterol/lipid-complex-rich domains, not \( l_0 \) domains.

Coupling of inner and outer leaflets in native cell membranes has been shown by qualitative (41, 55) and quantitative (103) colocalization (at the resolution of the light microscope) of receptors, GPI-anchored proteins, or glycolipids clustered in the outer leaflet with acylated cytoplasmic signaling and other proteins found in detergent-defined raft fractions. The range of such lipidated proteins is summarized in the introduction to a recent study of the importance of lipidation for their colocalization with membrane cholesterol and gangliosides (81). Green fluorescent protein (GFP) fused to specific sequences from Yes, Fyn, Lck, Src, and other proteins that contained acylation sites and, in some cases, a polybasic sequence colocalized well with cholesterol labeled by fillipin, and also with GM1 labeled by cholera toxin. This is a useful model, although incomplete, because it is clear that the isoforms and conformations of lipidated proteins play a role in localizing them to cholesterol-rich, raft-like regions of the inner leaflet (89, 101).

Raft localization of cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) fused to consensus sequences for acylation or prenylation has also been detected in terms of FRET between the fluorophores (151). The FRET data were analyzed using an approach that can distinguish FRET in a randomly distributed population of fluorophores from FRET among clustered fluorophores (61) by the relationship between FRET efficiency (\( E \)) and acceptor concentration (\( A \)) (measured in terms of YFP fluorescence). The data are extremely scattered and noisy, but the change in \( E \) versus \( A \) after cholesterol is extracted with methyl-\( \beta \)-cyclodextrin is consistent with cholesterol-dependent clustering of doubly acylated GFP. The fluorescence quenching method described earlier (72) also showed that dual acylation is sufficient to associate peptides with \( l_0 \) fractions in model membranes (146).

Once directed to the bilayer by acylation, a protein may explore it, rather than localize to one lipid environment. The cytosolic signaling protein Raf, a serine/threonine kinase, has an affinity for both anionic lipids and cholesterol (46), opening the possibility of its moving from one region of membrane to another. If this sort of dynamic is true of all or most associations of lipidated proteins with the inner leaflet, they could sample a number of local lipid environments, with their dwell time in a given environment dependent upon local biochemical reactions (81, 122). As nicely put in a recent review, lipidated proteins are in “a perpetual search for short relationships” (51).

Problem 2: What Do Detergent Extraction and Insolubility Tell Us About the State of Cell Membranes?

As we have seen, solubility in cold Triton X-100 detergent is a principal biochemical definition of lipid rafts. Results with model membranes and cell membranes weaken these operational definitions of lipid rafts.

The model membrane and early cell membrane experiments cited in an earlier section make the case that cell membranes are vesiculated and fused by low,
sublytic concentrations of Triton X-100. This suggests that raft components defined in detergent-resistant membranes came together by vesicle fusion rather than because they were proximally associated in cell membranes. The careful analysis of the way in which detergent interacts with raft mixtures (45) suggests that detergent can drive reorganization of lipids to form detergent-insoluble phases. This suggestion is reinforced by comparison of the lipid composition of membrane rafts isolated with or without detergent (97). The former are rich in lipids with saturated acyl chains, whereas the latter are rich in highly unsaturated acyl chains. If the rafts defined in detergent extracts existed prior to detergent addition, then we might expect that the lipid compositions of raft fractions isolated in different ways would be grossly similar.

A review of the large literature on lipid rafts as detergent-insoluble, lipid-rich membrane fractions also highlights the imprecision of most extractions. Detergent:lipid ratios are not well controlled and often not defined. Also, partition of proteins of interest between detergent-insoluble and detergent-soluble fractions is seldom quantified and rarely absolute, with all of a protein of interest in one fraction or the other. Even the starting material for extraction varies considerably. Comparing papers on the association of lipidated cytoplasmic proteins with raft fractions highlights the possible importance of controlling this factor. Thus McCabe & Berthiaume (81) extracted monolayers of intact COS-7 cells with 1% Triton X-100 at 4°C for 20 min and found that GFP with the Lck-derived N-terminal acylation sequence (myristate + palmitate) was totally soluble in detergent, while Wang et al. (146) found that 60% of the same construct, also expressed in COS-7, was detergent insoluble when isolated membranes, rather than whole cells, were extracted. The same extent of insolubility was found for GFP with the Lyn acylation sequence, when MDCK membranes were extracted (46). Another discordance is between two reports on the effects of brefeldin A treatment on the detergent insolubility of GPI-anchored proteins (in the lumenal leaflet of the Golgi complex) compared with that of G proteins (associated with the cytoplasmic leaflet of the Golgi complex). Brefeldin A treatment results in collapse of the Golgi into the endoplasmic reticulum. One group finds that GPI-anchored proteins of the Golgi complex are detergent soluble after brefeldin A treatment (20). Another group finds that a detergent-insoluble membrane fraction containing G protein subunits and caveolin (but not probed for GPI-anchored proteins) could be isolated from whole cell extracts after brefeldin A treatment (34). Still another discordance is the recent observation that although the IL-1 receptor β chain and GPI-anchored proteins are co-isolated in detergent-insoluble membrane fractions, they traffic separately from the cell surface (113).

**Problem 3: How Directed Are the Effects of Cholesterol Depletion on Cell Function?**

Cholesterol is required to form $l_o$ domains to maintain clusters of GPI-anchored proteins and caveolae and for formation of detergent-insoluble fractions in cell and
membrane extracts. However, cell requirements for cholesterol extend far beyond this. I have already noted the effects of membrane lipids (including cholesterol) on the function of membrane proteins and that levels of membrane cholesterol affect turnover of other lipids, including SM, and the activity of the cholesterol storage and synthetic pathways (29, 67, 92). These may come into play when growing cells in LDL-deficient media chronically depletes cell cholesterol. Because most studies of cholesterol’s role in raft function involve acute depletion with either cholesterol oxidase or nonionic surfactant methyl-β-cyclodextrin, which forms inclusion complexes with cholesterol [briefly reviewed in (91)], it might be expected that they would be specific for rafts. However, acute cholesterol depletion disrupts the clusters of snares required for exocytosis (66), blocks formation of clathrin-coated endocytic vesicles (109, 137), and, perhaps most important of all, delocalizes a plasma membrane signaling phospholipid, phosphatidylinositol(4,5)-bisphosphate [PIP(4,5)P2], from the plasma membrane (98). PIP(4,5)P2 is a major regulator of the actin cytoskeleton (19, 56, 105) and is intimately involved in endocytosis (12). Hence, cholesterol depletion, dispersing PIP(4,5)P2 from sites of functional interaction with cell proteins, can disrupt many cell functions. Indeed, recently, we have found (65) that cholesterol depletion, like sequestration of PIP(4,5)P2, alters cell actin organization and inhibits lateral diffusion of membrane proteins. Overall, it would seem that a correlation between reduction in cell cholesterol, loss of detergent-resistant membrane fractions, and loss of a particular function, for example, virus budding or receptor-mediated signaling, cannot necessarily be taken to show that lipid rafts are directly involved in the cellular function that is measured.

CELLS INSIST ON LIPID RAFTS

Lipid Rafts and Function

Despite great reservations about the interpretation of “classical” operational definitions of lipid raft components and functions, we are left with cells stubbornly insisting that lipids, lipid-anchored proteins, and acylated cytoplasmic signaling proteins are selectively trafficked and associated, and left with viruses that selectively sample host lipids to enrich their envelopes with sphingolipids and cholesterol. In the signaling field, perhaps the most striking finding is that receptors found in raft fractions of mature B cells are not found in these fractions from B cells at an earlier stage of development (133) and that raft localization of receptors is different in functionally different T cells (5). There are also a number of examples of viruses selectively incorporating GPI-proteins, cholesterol, and sphingolipids in their membranes (8, 44, 58, 87, 119).

There is abundant evidence that lipids and lipid-anchored proteins are segregated along the endocytic pathway. It was early noted that GPI-anchored proteins are internalized in a pathway separate from that for transmembrane proteins (6, 59) and, more recently, that fluorescent lipid analogs, dialkylindocarbocyanines (DiIs)
with unsaturated or saturated acyl chains, were sorted away from each other after endocytosis with the unsaturated chain DiI going to recycling endosomes, returning to the plasma membrane, and saturated chain DiI going to late endosomes (86). In the past few years, focus has returned to the trafficking of GPI-anchored proteins and sphingolipids. Although SM analogs enter by both clathrin-dependent and clathrin-independent pathways (102), GPI-anchored proteins and glycolipids are internalized by a nonclathrin fluid-phase endocytic process (88, 102, 113). Once internalized, GPI-anchored proteins return to the surface more slowly than other recycling membrane components (80). This appears to be because they are retained in recycling endosomes by association with components of lipid rafts; their retention is sensitive to depletion of cell cholesterol. Recycling time is shortened if cell cholesterol or SM levels are reduced (21, 80). Recently, the Pagano laboratory (123) has pushed the limits of resolution in the light microscope and imaged the segregation of GSL analogs in the early endosome membrane. Separate domains of high and low concentrations of lactosylceramide form in endosomes. The high-concentration domains, about half the total, recycle to the cell surface, while the low-concentration domains traffic to the Golgi complex. It will be interesting to connect these observations to the cholesterol-dependent domains recently visualized on the surface of cultured cells (39).

**Detecting Lipid Rafts in Intact Cell Membranes**

The raft model implies that component molecules are concentrated and clustered. The lipid composition defined for rafts implies a higher viscosity for raft lipids than for the membrane average. The first implication has been tested by cross-linking and FRET measurements; the second by a variety of diffusion measurements. Measurements through 2000 are summarized in a recent review (2). They converge on raft diameters <100 nm. Some FRET measurements (61) detected no clustering at all, implying that rafts were vanishingly small or that only a fraction (<20%) of the molecules of interest were in rafts. The most refined recent diffusion measurements used a laser trap to confine molecules labeled with beads to small areas (<100-nm diameter) and then followed the thermal motion of the beads around their position (100). Local membrane viscosity was inferred from this motion; the distribution of viscosities appeared to report on the diffusion of proteins within a raft and, as well, on the diffusion of an entire raft. The average raft diameter was estimated as 26 ± 13 nm with a raft lifetime of >1 min. Another group using the same logic, but conventional fluorescence recovery after photobleaching (FRAP) for measuring diffusion, was unable to detect diffusion of rafts as opposed to individual components of rafts (62). However, it is hard to compare the two results because of the difference in time and spatial scales of the methods used.

Fresh approaches to FRET (S. Mayor, personal communication) and single particle tracking techniques (138) have yielded a new picture of lipid rafts in unperturbed membranes. Mayor and colleagues (144) had earlier used fluorescence polarization as a measure of energy transfer between labeled GPI-anchored
proteins. In their new work, they returned to this technique but combined it with time-resolved anisotropy measurements and theoretical modeling of the way in which the relationship between fluorescence anisotropy and fluorophore concentration would vary as a function of raft size. Using this analysis they concluded that units containing GPI-anchored proteins, probably associated with cholesterol, are <10 nm in diameter and contain perhaps five molecules of GPI-anchored protein. This size converges with the cholesterol/sphingolipid complexes discussed earlier (3, 82, 104).

Kusumi and colleagues (138) used high-speed particle tracking to compare the Brownian motion of GPI-anchored proteins with a di-unsaturated phosphatidylcholine, DOPC, which should not enter rafts. With a time resolution of 25 µs they found that GPI-anchored proteins and DOPC both diffused freely within compartments ~110 nm in diameter, hopping to a new compartment every 25 ms. From the identical behavior of the two classes of molecules, they concluded that at steady state these core lipid rafts consist of only a few molecules and persist for ~1 ms or less. However, if the GPI-anchored protein CD59 was cross-linked with antibody or its native ligand, larger rafts developed with a diffusion coefficient about eightfold smaller than that of core rafts. Cross-linked CD59 was frequently immobilized, and these immobilized rafts were associated with actin and signaling kinases. Thus membrane rafts appear to be small and unstable, but they are capable of developing into larger functional structures when their constituents are ligated.

**HOW DOES IT ALL WORK?**

Model membrane data and the stubborn insistence by cells that raft lipids can be organized and segregated into membrane domains can be integrated if we consider the ways in which proteins can interact with raft lipids and lipid-anchored proteins. Proteins can either recruit raft lipids to their transmembrane domains or bind their exodomains to the membrane surface [reviewed in (77, 148)]. A recent review emphasizes the formation of lipid shells around proteins integrated into the bilayer (2) and as well lists a baker’s dozen of proteins that specifically bind cholesterol or sphingolipids.

An important interaction of raft components with cytoplasmic proteins is with actin (40), which is implicated in raft-mediated signaling (42, 49, 110, 138). This interaction is coming into new focus as important in surface organization of rafts (27) and for the mechanical properties of cell membranes (139). Actin does not bind directly to membranes, but rather to proteins, such as annexins, and polybasic proteins, such as MARCKS and GAP43. It has been suggested that annexin binding to raft lipids would hinder assembling of signaling proteins in a raft [though it would order lipid acyl chains, promoting association of raft lipids (25, 147)]. However, another model was developed recently (83) in which polybasic proteins with single acyl chains bind negatively charged PIP(4,5)P2, creating an oligomer with multiple saturated acyl chains that could recruit cholesterol and SM and so
consolidate raft components with a signaling lipid that has highly unsaturated acyl chains. This can be generalized to a mechanism in which patches of charges on monoacylated proteins bind counter-charged lipids (PE and PS) and create a region of ordered acyl chains that can recruit SM and cholesterol. Chains might also be ordered by insertion of hydrophobic regions of a protein; this is suggested by the finding that prion protein binds negatively charged lipids by a combination of electrostatic and hydrophobic interactions (115). Still another possibility is that local sphingomyelinase activity could create small foci of highly ordered lipids (78). Whatever the mechanism for clustering inner leaflet lipids, it could be coupled to the outer leaflet and so recruit raft components such as signaling kinases and G proteins in response to receptor cross-linking.

Binding of proteins in the outer leaflet may organize lipids there. An energy transfer study of the proximity of the exodomain of a GPI-anchored protein to the bilayer surface shows that the protein is close enough to the surface to interact with the bilayer lipids, suggesting two-way transmission of conformational changes in the interacting partners (69). More specific interactions between raft glycolipid components and exogenous toxins, for example, cholera toxin and shigatoxin, have long been recognized (35). There is also a hint of specific lipid binding in the fact that influenza membrane proteins found in rafts (hemagglutinin and neuraminidase) no longer target there if their cytoplasmic domains are truncated (152). Recently, a common sphingolipid-binding domain (which binds SM as well as a glycolipid) has been identified on Alzheimer β-amyloid protein, prion protein, and HIV-1 glycoprotein (75). This suggests another way in which membrane integral and peripheral proteins could interact with lipids to organize larger domains from core rafts. Interestingly, we have found the homologous sequence in a resident membrane integral protein, class I MHC (D. Fooksman, personal communication). Finally there is some evidence that a tetraspan protein, EMP2, modulates the surface expression of GPI-anchored proteins (145a), which hints at a function for EMP2 as an organizer of raft lipids.

CONCLUDING REMARKS

The interaction of proteins with membrane lipids to enlarge and stabilize transient rafts offers a perspective on the function of lipid rafts that combines physical chemistry and biochemistry. We can imagine cell membranes rich in raft components whose interactions with one another depend upon changes in the properties of small regions of membrane. Enzyme activation, receptor cross-linking, recruitment of lipids by proteins, and local changes in lipid concentrations could all work toward recruiting raft components into stable, relatively long-lived functional complexes, or the opposite, dispersing interacting components and quenching reactions. All this involves a mix of two-dimensional and three-dimensional diffusion as well. Model membranes can be used to investigate these interactions, but we should keep in mind the difference between the conveniences of scale required for observing the models and the actual scale of creating and dispersing functional signaling or
trafficking rafts. Whatever we learn from models should be tempered by the fact that the cells are always right.

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