Lipid Rafts As a Membrane-Organizing Principle

Daniel Lingwood and Kai Simons*

Cell membranes display a tremendous complexity of lipids and proteins designed to perform the functions cells require. To coordinate these functions, the membrane is able to laterally segregate its constituents. This capability is based on dynamic liquid-liquid immiscibility and underlies the raft concept of membrane subcompartmentalization. Lipid rafts are fluctuating nanoscale assemblies of sphingolipid, cholesterol, and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking. Here we review the evidence for how this principle combines the potential for sphingolipid-cholesterol self-assembly with protein specificity to selectively focus membrane bioactivity.

The lipid raft hypothesis proposes that the lipid bilayer is not a structurally passive solvent, but that the preferential association between sphingolipids, sterols, and specific proteins bestows cell membranes with lateral segregation potential. The concept has long suffered assessment by indirect means, leading to questions of fact or artifact (*1*). The resistance of sphingolipid, cholesterol, and a subclass of membrane proteins to cold detergent extraction (*2*) or mechanical disruption (*3*) has been widely used as an index for raft association with little or no regard for the artifacts induced by these methods. Though the acquisition of resistance to disruption may point to physiologically relevant biases in lateral composition (*4*), this disruptive measure tells us little about native membrane organization. Support from light microscopy was also missing because, with the exception of organization into specialized membrane domains such as caveolae or microvilli, putative raft components—specifically glycosylphosphatidylinositol (GPI)–anchored proteins, fluorescent lipid analogs, raft transmembrane (TM) domains, and acylated proteins—often show a homogeneous distribution at the cell surface (*5*). Moreover, early investigations into submicron membrane organization often yielded conflicting evidence regarding the distribution or motion of these constituents in the living cell (*1*). Today, however, the advancement of technology has produced compelling data that self-organization of lipids and proteins can induce subcompartmentalization to organize bioactivity of cell membranes.

Origins of the Lipid Raft Concept

Biochemically, it is clear that lipids are sorted within the cell (*6*). This is particularly notable in polarized epithelia where glycosphingolipids (GSLs) are enriched at the apical surface (*7*). Lipid rafts were originally proposed as an explanation: Self-associative properties unique to sphingolipid and cholesterol in vitro could facilitate selective lateral segregation in the membrane plane and serve as a basis for lipid sorting in vivo (*7*). This proposal for compartmentalization by lipid rafts suggested a nonrandom membrane architecture specifically geared to organize functionality within the bilayer. This function was initially thought to be membrane trafficking; however, rafts could influence organization of any membrane bioactivity (Fig. 1). Here, we highlight advances in technology that point to the existence of raft-based membrane heterogeneity in living cells and discuss the levels of preferential association underlying dynamic domain structure and biological function(s).

Lipid Interactions in Model Membranes

Assembly into two-dimensional liquid crystalline biomembranes is a fascinating property characteristic of lipids. Long thought to be incapable of coherent lateral structure (*8*), it is now apparent that principles of lipid self-association can also confer organization beyond nonspecific measures of fluidity. An important advance in model-membrane systems was the discovery of phase separation in wholly liquid bilayers (*9*, *10*). It is a cholesterol-dependent lateral segregation, wherein the planarity (molecular flatness) of the rigid sterol ring favors interaction with straighter, stiffer hydrocarbon chains of saturated lipids and disfavors interaction with the more bulky unsaturated lipid species (*11*). Interaction with cholesterol also forces neighboring hydrocarbon chains into more extended conformations, increasing membrane thickness and promoting segregation further through hydrophobic mismatch (*12*). In purified lipid systems, the combined effect is a physical segregation in the membrane plane: A thicker, liquid-ordered, Lo phase coexists with a thinner, liquid-disordered, Ld phase (*13*). Sphingolipids tend to display longer and more saturated hydrocarbon chains, thus potentiating interdigitation between leaflets (*14*) and favoring interaction with cholesterol. Moreover, unlike glycerophospholipids, the region of chemical linkage between the head group and sphingosine base contains both acceptors and donors of hydrogen bonds, thus increasing associative potential, both with cholesterol and other sphingolipids (*11*). Other explanations for cholesterol selectivity include the proposed umbrella effect, in which cholesterol hydrophobicity is preferentially shielded by the strongly hydrated head groups of sphingolipid (*15*) or stoichiometric, but reversible, complex formation between cholesterol and sphingolipid or saturated glycerophospholipid (*16*).

Immiscible liquid phase coexistence in vitro has been suggested as the physical principle underlying rafts in vivo (*17*). Of central importance is the demonstration of selectivity in association between certain lipids. However, phase separation in simple systems at thermodynamic equilibrium in vitro cannot be translated into proof for membrane domain formation in living cells (*1*). Instead, model-membrane work emphasizes the fact that certain lipids exhibit preferential association and provides a framework for understanding how heterogeneity in cell membranes may arise (*18*). In this respect, the terms Lo and Ld should not be applied to the living cell, as they refer only to the liquid-ordered and liquid-disordered phases of model-membrane systems where parameters relating to translational order (lateral diffusion) and conformational order (trans/gauche ratio in the acyl chains) can be accurately measured (*11*).

Glimpses of Nano-Assemblies in Living Cells

Currently, lipid rafts are viewed as dynamic nanoscale assemblies enriched in sphingolipid, cholesterol, and GPI-anchored proteins (*19*) (Fig. 2A). To reach this viewpoint, membrane research has had to contend with the observer's effect, akin to Heisenberg's uncertainty principle: We can change and/or induce heterogeneity in membranes simply by trying to observe it. Initially, this required moving away from detergent extraction as a means to infer native organization. In a first step, detergent-free, chemical cross-linking of GPI-anchored proteins at the plasma membrane suggested that the intrinsic heterogeneity by rafts was present in nanoscale complexes below the optical resolution limit set by the diffraction of light (*19*). This nanometer-size scale was later supported by viscous drag measures of antibody-bound raft proteins (*21*) and electron microscopic observation of immunogold-labeled raft antigens (*20*). Indeed, recent near-field scanning optical microscopy has confirmed a nanoscale bias in the distribution of raft-associating proteins in fixed cells (*22*). Less perturbing measures of spatial and temporal dynamics in living cells have also provided correlating data. For example, single-particle tracking of colloidal gold–labeled GPI-anchored receptors reveals "stimulation-induced temporary arrest of lateral diffusion," or STALL, in short-lived (~0.5-s) 50-nm areas as a bioactive feature of receptor function (*23*). Parallel advances in microscopy and spectroscopy have revealed similar heterogeneity

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Max Planck Institute for Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany.

*To whom correspondence should be addressed. E-mail: simons@mpi-cbg.de

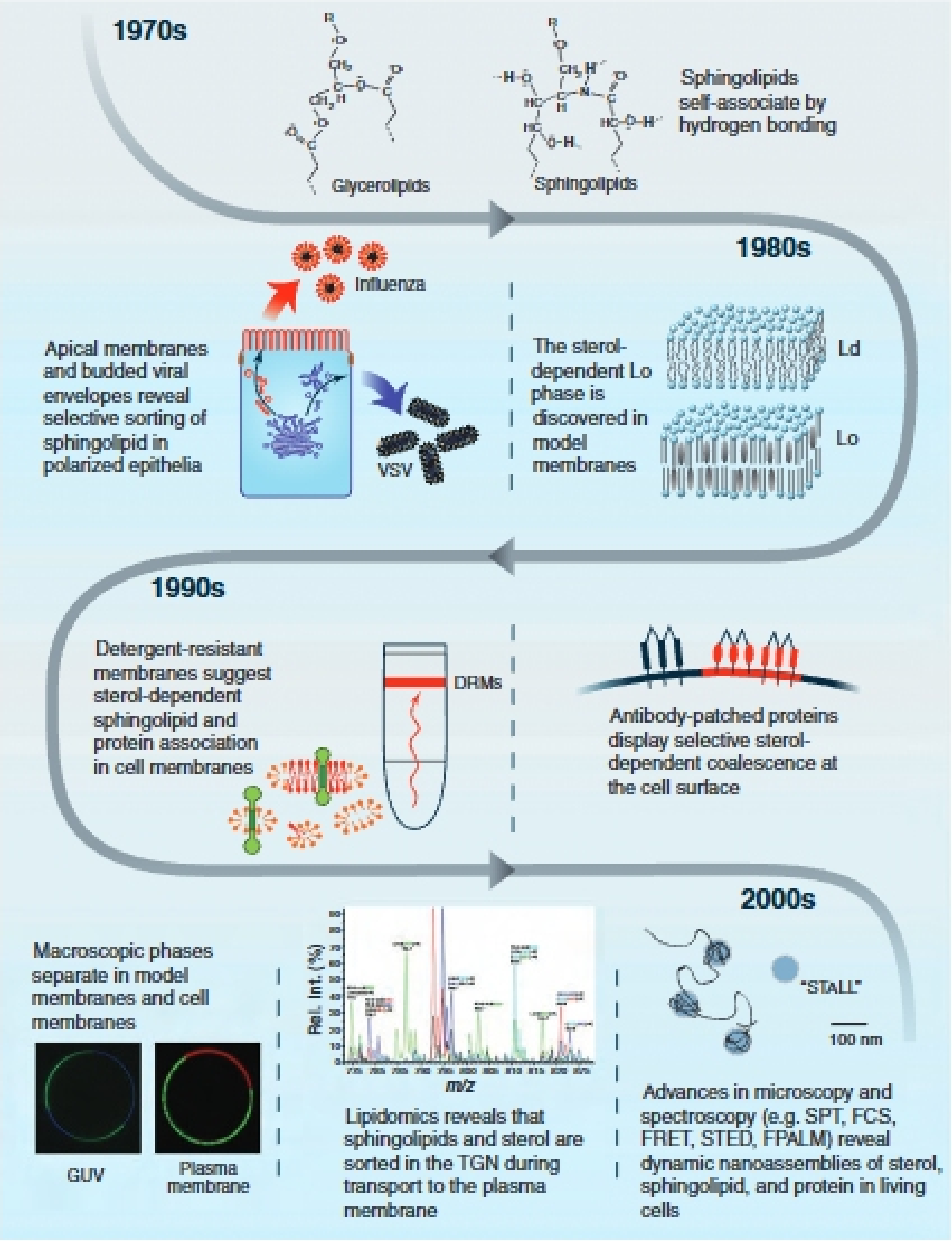

Fig. 1. Evolution of the raft concept for subcompartmentalization in cell membranes. A bold H indicates hydrogen bonding. VSV, vesicular stomatitis virus; DRMs, detergent-resistant membranes; GUV, giant unilamellar vesicle; *m/z*, mass/charge ratio; SPT, single-particle tracking; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; STED, stimulated emission depletion; FPALM, fluorescence photoactivation localization microscopy.

for raft molecules in uncross-linked, "resting" conditions. For GPI-anchored proteins, variable waist fluorescence correlation spectroscopy points to <120-nm assemblages that fluctuate on a subsecond time scale (*24*). High spatial and temporal resolution fluorescence resonance energy transfer (*25*) has generated a more conservative size estimate with GPI-anchored receptors residing in more temporally stable clusters of ~10 nm. Assembly formation is always cholesterol-dependent, and, in some cases, an actin requirement has also been seen (*23*, *25*). However, other techniques have indicated that nanoheterogeneity is actin-independent (*26*). The situation for TM proteins is not yet clear. However, fluorescence photoactivation localization microscopy has revealed a dynamically clustered nanoscale distribution of hemagglutinin (*27*), a TM protein previously described as raft-associating (*21*). The role of the association between cholesterol and sphingolipids in assembly formation has been analyzed recently by stimulated emission depletion microscopy. This study revealed that, unlike glycerophospholipids, plasma-membrane sphingolipids display transient cholesterol-dependent confinement in areas of <20 nm (*28*). In this case, differences in diffusion were attributed to differential hydrogen-bonding capacities of glycerol- versus sphingosine-based lipids. However, spin-labeled lipid probes at the cell surface have also revealed heterogeneity in membrane order on an electron spin resonance time scale (*29*).

Different techniques are yielding a range of values for different molecular constituents in diverse cell types. However, these methods all point to the existence of small, dynamic and selective cholesterol-related heterogeneity in the plasma membranes of living cells. Recent data point to critical behavior as a potential physical basis for the existence of fluctuating nanoscale assemblies in plasma membranes (*30*).

Functionalization of Nanoscale Heterogeneity

Antibody cross-linking at the cell surface causes raft proteins and lipids to co-patch and exclude non-raft proteins (*31*). This selectivity in patching is cholesterol-dependent and can be transmitted across the plasma-membrane leaflets (*32*). The nonrandom coalescence behavior observed in these artifactual cross-linking studies suggests how raft organizing potential may be functionalized to larger, more physiologically relevant temporal and spatial scales (Fig. 2B). A contention of the lipid raft hypothesis is that dynamic nanoscale heterogeneity can be stabilized to coalesce into larger raft domains by specific lipid-lipid, protein-lipid, and protein-protein interactions (*20*). In this sense, cell membranes would possess an underlying sphingolipid/cholesterol-based connectivity that can be activated to cluster membrane bioactivity with little energy cost. Indeed, multimerization promotes the sorting of GPI-anchored proteins into sphingolipid/cholesterol-enriched carriers during clathrin-independent endocytosis (*33*). Along similar lines, clustering of cell surface Gb_3 or GM1 (both GSLs) by their respective ligands Shiga toxin and cholera toxin induces energy-independent tubular invaginations of sphingolipid-biased membrane composition (*34*, *35*). Similar behavior has also been reported during the multivalent binding of SV40 virus to its GM1 receptor (*35*). Invagination from the plasma membrane was dependent on having longer receptor hydrocarbon chains, which are common to sphingolipid, and suggests that the effect is mediated by line tension arising between membrane domains of different compositional order (*35*). Coalescence of dynamic heterogeneity also occurs during signaling, for example, during the formation of B cell receptor (BCR) or T cell receptor (TCR) foci. Antigen binding induces the dynamic association of BCR to its signaling effector Lyn kinase and leads to the formation of an immune synapse. The interaction is dependent on the nature of Lyn lipid anchorage, with membrane order–disrupting bulky hydrocarbon chains preventing association with the BCR (*36*). During TCR activation, raft components of this receptor complex (e.g., GPI- anchored proteins) become selectively immobilized in nanoscale clusters (*37*), seeding the accumulation of cholesterol, sphingomyelin, and saturated and long-chain phosphatidyl choline into the synapse, effectively sorting proteins according to their affinity for raft domains (*38*). Rafts in this "activated" or coalesced condition constitute a more ordered assemblage: a fluid membrane environment in which proteins can be modulated specifically (*39*), yet that exists separately from the surrounding membrane rich in unsaturated glycerophospholipid. Raft activation is often stabilized or nucleated by scaffolding elements such as cortical actin (*40*) and may become dominating when the mole fraction of sphingolipids and cholesterol increases, as is the case in the apical membrane of epithelial cells (*41*).

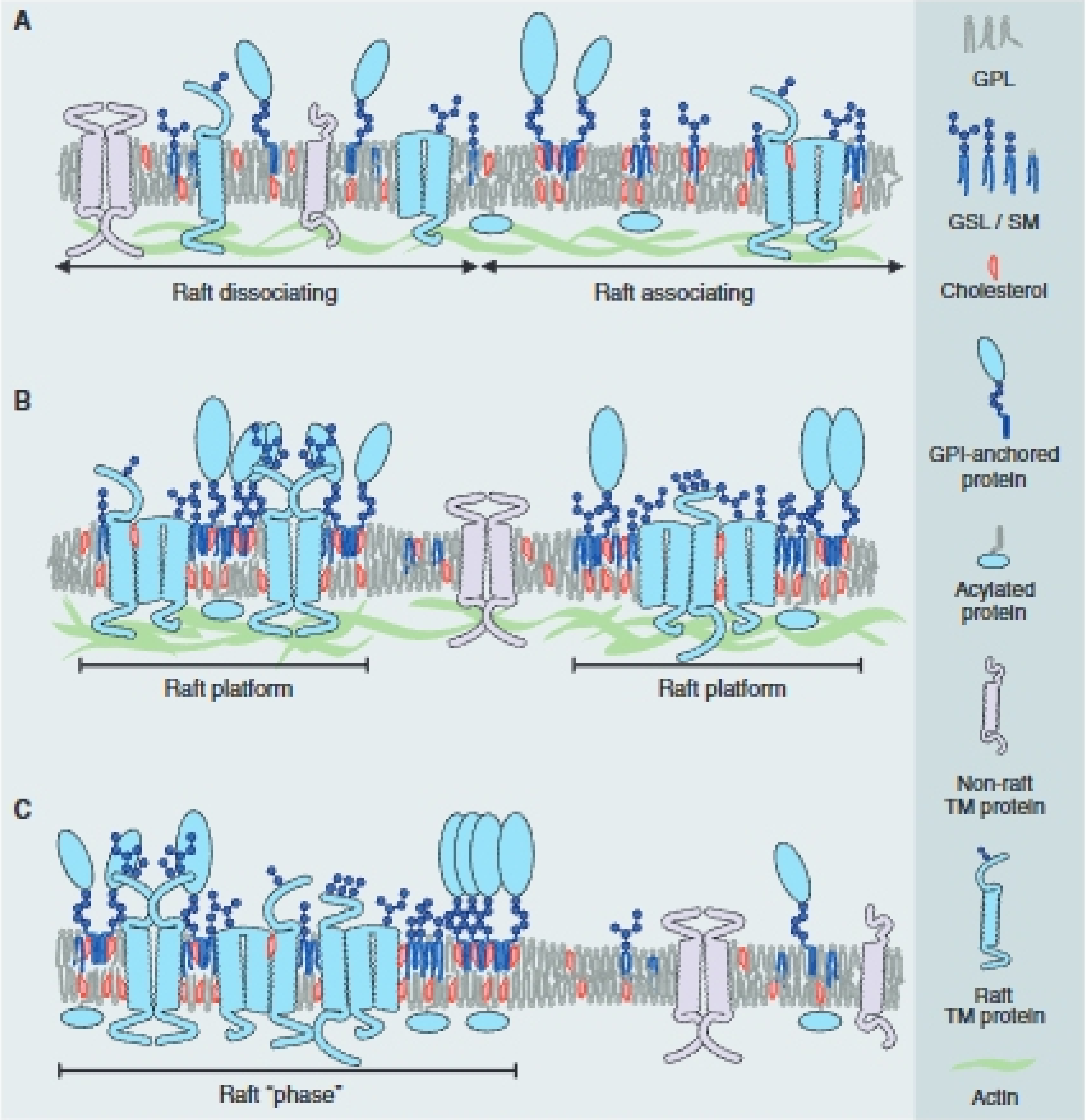

Fig. 2. Hierarchy of raft-based heterogeneity in cell membranes. **(A)** Fluctuating nanoscale assemblies of sterol- and sphingolipid-related biases in lateral composition. This sphingolipid/sterol assemblage potential can be accessed and/or modulated by GPI-anchored proteins, certain TM proteins, acylated cytosolic effectors, and cortical actin. Gray proteins do not possess the chemical or physical specificity to associate with this membrane connectivity and are considered non-raft. GPL, glycerophospholipid; SM, sphingomyelin. **(B)** Nanoscale heterogeneity is functionalized to larger levels by lipid- and/or protein-mediated activation events (e.g., multivalent ligand binding, synapse formation, protein oligomerization) that trigger the coalescence of membrane order–forming lipids with their accompanying selective chemical and physical specificities for protein. This level of lateral sorting can also be buttressed by cortical actin. **(C)** The membrane basis for heterogeneity as revealed by the activation of raft phase coalescence at equilibrium in plasma-membrane spheres. Separated from the influence of cortical actin and in the absence of membrane traffic, multivalent clustering of raft lipids can amplify the functional level to a microscopic membrane phase. Membrane constituents are laterally sorted according to preferences for membrane order and chemical interactions.

Phase Separation in Cell Membranes

Despite their selective co-patching with raft markers at the cell surface, raft TM proteins are depleted from the tightly packed Lo phase when reconstituted in model systems (*42*, *43*). Thus, the Lo phase as it exists in simple model membranes is unlikely to be identical to raft-based heterogeneity in plasma membranes that selectively includes TM proteins (*44*, *45*). Giant plasma-membrane vesicles isolated by a chemical membrane blebbing procedure can be cooled to phase separate into Lo- and Ld-like phases (*46*), and here also, raft TM proteins are typically excluded from the ordered membrane phase (*47*). Remarkably, this phase coexistence indicates that after chemical modification of protein (e.g., formaldehyde cross-linking, thiol treatment), the capacity for physical or lipid-based liquid-liquid phase separation can be manifested by the plasma membrane, despite its compositional complexity. Now the question is, how might phase length–scale separation take place in plasma membranes at physiological temperatures?

Some insight has come from a cell-swelling procedure to separate plasma-membrane spheres from the influence of cytoskeletal, endocytic, or exocytic processes in a cell line enriched in the raft ganglioside GM1. Pentavalent clustering by cholera toxin resulted in sterol-dependent coalescence of a micron-scale raft "phase" at 37°C, selectively reorganizing the lateral distribution of proteins and lipids according to their predicted affinity for raft domains (*44*). In this case, selective incorporation of TM proteins was achieved at a lipid-ordering level far below that observed in model-membrane Lo phases (*45*). Thus, whereas preferential lipid-lipid associations do under-