

Review

Molecular Dynamics and Interactions for Creation of Stimulation-Induced Stabilized Rafts from Small Unstable Steady-State Rafts

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We have evaluated the sizes and lifetimes of rafts in the plasma membrane from the existing literature, with a special attention paid to their intrinsically broad distributions and the limited time and space scales that are covered by the observation methods used for these studies. Distinguishing the rafts in the steady state (reserve rafts) from those after stimulation or unintentional crosslinking of raft molecules (stabilized receptor-cluster rafts) is critically important. In resting cells, the rafts appear small and unstable, and the consensus now is that their sizes are smaller than the optical diffraction limit (250 nm). Upon stimulation, the raft-preferring receptors are clustered, inducing larger, stabilized rafts, probably by coalescing small, unstable rafts or cholesterol-glycosphingolipid complexes in the receptor clusters. This receptor-cluster-induced conversion of raft types may be caused by suppression of alkyl chain isomerization and the lipid lateral diffusion in the cluster, with the aid of exclusion of cholesterol from the bulk domain and the boundary region of the majority of transmembrane proteins. We critically inspected the possible analogy to the boundary lipid concept. Finally, we propose a hypothesis for the coupling of GPI-anchored receptor signals with lipid-anchored signaling molecules in the inner-leaflet raft.

Key words: boundary lipid, cholesterol, crosslinking, GPI-anchored receptor, lifetime, miscibility, receptor-cluster rafts, signal transduction, size, steady-state rafts

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Elucidating the Sizes and Lifetimes of Rafts Is the Key to Understanding How They Work

The cell membrane has a two-dimensional, liquid-like structure, but it is not a simple liquid. Rather, it is a *non-*

ideal liquid mixture of molecules with *variable degrees of mutual miscibilities*, and thus it contains a variety of molecular complexes and domains, which might occupy a large fraction of the membrane surface. These molecular complexes and domains range from small protein clusters with lifetimes on the order of 10 μ s, like transient dimers of rhodopsin (1), to large, micron-sized domains with lifetimes on the order of 1–10 h stabilized by, in addition to the molecular interactions within the membrane, interactions with the cytoskeleton, like desmosomes (2–4). These molecular complexes and domains form and disperse continually, with various lifetimes. Since many key functions of the plasma membrane, such as signal transduction, are thought to be carried out by these temporary, dynamic structures, a keen awareness of their lifetimes and sizes is critical for understanding their functions and regulatory mechanisms. The dynamic characteristics of these temporary structures in the cell membrane may be determined by the delicate balance of the affinities between constituent molecules, their miscibilities with various lipid molecules, and thermal agitation/fluctuation.

Rafts are considered to be representative of dynamic domains and molecular complexes, although the research has not yet reached the stage at which they can be clearly defined. Lai called the rafts 'unidentified floating objects' (5). To advance the argument in this review, we will employ the following working definition of the raft. We propose that a molecular complex should be called a 'raft' (for a 'general' raft without further specifications) if the molecular complex in the membrane consists of at least three molecules that include a molecule with a saturated alkyl chain or a cholesterol molecule that plays a critical role in the creation of the complex itself. Such a definition for the raft, which includes or even alludes to very small structures, may surprise many researchers in the raft field, but it is important to include and to raise all of the possibilities that cannot be excluded from the first principles of physics/chemistry. We exclude two-molecule complexes from our definition of the raft, because these could defeat the whole purpose of establishing a raft concept: this concept is interesting because such a raft could provide the means, based on the lipid interactions between saturated alkyl chains and cholesterol, to flexibly and dynamically (i.e. temporarily) recruit two or more molecules that are to interact with each other in the molecular

complex. In this review, we concentrate on the rafts in the plasma membrane. The major functions of the rafts in the plasma membrane appear to be facilitating signal transduction and trafficking of proteins and lipids. In this review, we focus on the function of rafts in signal transduction. Recent reviews have given excellent descriptions of the rafts in cytoplasmic membranes and those involved in trafficking (6–11).

The sizes and lifetimes of rafts are mostly intermediate between those of the transient rhodopsin dimers and the rather stable desmosomes. This intermediate nature of the raft in time–space scales and the lipid nature of its molecular interactions, upon which raft formation itself is based, make the raft unique among various biomolecular complexes. First, the size and the lifetime of the raft can be varied greatly, by modulating the delicate balance between the molecular interactions (details given later) (12–17). Second, the raft may be sufficiently small and short-lived to allow for rapid long-term diffusion of its (part-time) constituent molecules to quickly reach the site of signal input ('reserve raft' before stimulation ((18,19), see also (20)). Third, the greater, stabilized raft ('receptor-cluster raft' induced by liganding and crosslinking of raft-associating receptor molecules) may have a sufficient size and lifetime to facilitate the incorporation of various key signaling molecules to carry out rather complicated reactions (21–23).

Throughout this review, we emphasize the importance of clearly distinguishing between these two types of rafts—the small, unstable 'reserve rafts' present in resting cells and the 'receptor-cluster rafts' induced by liganding and crosslinking of raft-preferring molecules. The lack of this distinction appears to be creating unnecessary conflicts and confusions in the raft research field (15–17). One also should consider the fact that raft molecules can be crosslinked quite readily, and thus could easily become crosslinked unintentionally. Meanwhile, since the sizes and the lifetimes of both types of rafts are likely to be broadly distributed, one must be keenly aware that various observation methods may detect different subsets in each type of rafts.

Rafts Have Not Been Directly Observed in the Plasma Membranes of the Resting Cells

Almost all of the efforts to visualize rafts in the plasma membrane have attempted to observe the concentration of putative raft molecules in specific regions. Caveolae, raft-related structures (24–26), may be one of greater stabilized rafts stabilized by the assembly of caveolin accompanied by cholesterol and glycosphingolipids (27–29) and can be imaged in intact membranes. Direct observations of rafts in resting cells, however, have turned out to be difficult. Close examination of the reports describing the imaging of rafts in steady-state cells using light and electron microscopy has revealed that, in almost all of the cases, the sample preparation protocol involved one a combination of the following:

- induction of the clustering of raft molecules, using crosslinkers like antibodies and fixatives;
- use of probes that might crosslink raft molecules, such as cholera toxin, a pentavalent reagent, and latex beads conjugated with antibodies (21,22,30–33);
- employment of low temperatures (even cooling to room temperature may result in the artificial growth of domains) (34–38).

The use of low concentrations of formaldehyde, which 'fixes' the amino-containing molecules at their intrinsic locations, enhances the clustering of raft molecules rather than fixing these molecules *in situ*, probably because sphingolipids and cholesterol, which cannot be crosslinked by formaldehyde, are critically involved in raft formation (39). For the observations at the light microscopy level, the use of at least 4% formaldehyde has been recommended (39), whereas for observations at the electron microscopic level, inclusion of (at least) 0.1% glutaraldehyde in the fixation medium appears to be essential (11,39–41).

Therefore, *rafts have only been visualized after crosslinking or ligating raft molecules or at lower temperatures* (22,34,39), with only a few possible exceptions (31,35). This suggests that the raft size in the resting cell is smaller than the optical diffraction limit (≈ 300 nm) and might increase after stabilization by crosslinking raft molecules or lowering the temperature (16,17,20). In steady-state cells under physiological conditions, even if some raft molecules are concentrated in a raft, if the raft size is smaller than the optical diffraction limit, then their assembly in the raft would be blurred and smeared out by averaging over the area determined by optical diffraction (e.g. a 10 \times concentration in a 50-nm raft would appear as only $\approx 30\%$ increase in the fluorescence signal at a diffraction limit of 300 nm).

It is notable that an antibody to a ganglioside GM3 and a bacterial toxin with cholesterol affinity, θ -toxin, have higher affinity when these lipid molecules are in microdomains (42,43). The anti-GM3 antibody exhibits clear staining of cells only when the raft microdomains are induced, again suggesting the absence of large, stable raft domains in the resting cell membrane.

Electron microscopy has much higher spatial resolutions, but the rafts do not exhibit any morphological signature, and thus their visualization has to depend on the labeling of putative raft molecules by antibodies or other probe molecules. Even when the putative raft molecules of interest (antigen molecules) are all localized within rafts, if each individual raft is small, only a few antigen molecules may be present in a single raft. This would make detection of individual rafts by antibody labeling difficult. By observing the distribution of small gold probes (4 nm in diameter) bound to various raft molecules (mostly localized on the inner surface of the membrane like H- and K-Ras), and then

by analyzing a modified pair-distribution function of the probes, Prior et al. (41) indicated that approximately one-third of the inner leaflet of the plasma membrane consists of raft domains 40 nm in diameter that concentrate raft molecules like H-Ras (before its activation). This was an elegant study, and it will become furthermore interesting if fixation reagents, negative staining and natural drying can be avoided in their future studies. These processes might have induced the clustering of raft-philic molecules that could be detected at the electron microscope level (remember that only raft associating molecules, and only in the presence of rafts, could be this sensitive to mild clustering conditions), but not at the optical microscope level.

The Rafts in the Plasma Membrane of the Resting-State Cell May Be Small/Short-Lived and/or Their Constituent Molecules May Rapidly Exchange with Those in the Bulk Domain

As described in the previous section, the bottom line in the literature on the size of the raft in the steady-state cell

appears to be that the raft size is smaller than the optical diffraction limit of ≈ 300 nm in the absence of extracellular stimulation, except for several isolated observations. Although the size variations may be large and the estimated size may depend heavily on the method of observation, most data are consistent with the raft size smaller than 300 nm in diameter.

Indirect estimates of the sizes of lipid raft domains in living cells, employing single particle tracking and photonic force microscopy, suggest even smaller sizes, ranging between 50 and 200 nm in diameter (30–32). The problem with these methods is that since they employ gold or latex particles as probes, it is difficult to totally disregard the possibility of probe-induced crosslinking. In fact, as methods for probe preparation improve, these particle probes less often exhibit the transient confinement zone (TCZ, which is a site or a domain where raft-philic molecules temporarily stop diffusion; see Box 1), which may represent rafts (A. Kusumi and K. Jacobson, unpublished results). Nichols (44) detected GM1 clustering using FRET imaging microscopy, but he found that these complexes did not form discernible domains and were uniformly

Box 1

Difference between the membrane compartments and transient confinement zones (TCZs)

The diffusion rates of membrane molecules in the cell membrane are reduced by a factor of 5–50 compared to those in artificial membranes. The reduction mechanism has puzzled membrane biophysicists for over a quarter of a century. Previously, our group showed that the major mechanism for such reduction is membrane compartmentalization, which induces short-term confinement of all membrane molecules (both proteins and lipids) in a compartment and long-term hop diffusion over the compartments (Figure A). Namely, diffusion in the plasma membrane is slow not because diffusion *per se* is slow, but because molecules are temporarily confined in these compartments and it takes some time to hop from a compartment to an adjacent one. The compartment size ranges between 30 and 250 nm, and the residency time within a compartment varies greatly (1 ms – 1 s), both of which depend on the cell and molecule.

Two technological developments made the observations of hop diffusion and membrane compartmentalization possible. First, observations of single molecules (or small groups of molecules) enabled direct visualization of each individual hop movement from a compartment to an adjacent one. With the conventional methods (like FRAP) to observe the ensemble averaged behavior of many molecules, each hop movement of individual molecules would be totally smeared out by averaging

over all molecules under observation. Second, enhanced time resolution (25 μ s) was necessary as the time resolution must be much shorter (about a factor of 40 or shorter) than the residency time, to detect confinement. As it turned out, the residency time within a compartment is as short as 1–25 ms for phospholipids

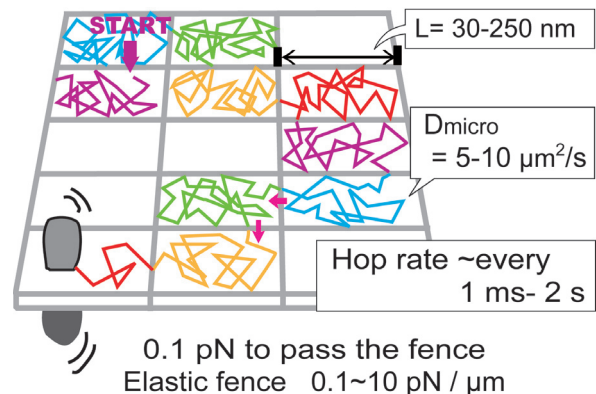


Figure A: The plasma membrane is compartmentalized with regard to translational diffusion of membrane proteins and lipids. These membrane molecules undergo hop diffusion over the compartments. For detection of these compartments and hop diffusion, observations at the level of single molecules at higher time resolutions are required. The average force to get over the compartment boundary is about 0.1 pN as found by optical trap experiments. These experiments also showed that the inter-compartmental barrier is elastic, with an elastic constant in the range of 0.1–10 pN/ μm (108,120).

and 3 ms – 1 s for transmembrane proteins (Murase, Fujiwara, Iwasawa, Ritchie, Suzuki, Umemura, and Kusumi, unpublished observations; these residency times depend on both cells and molecules), and over 40 measured points are required to clearly detect individual compartment, thus the time resolution of 25 μ s may be required for many cases. Since these residency times, particularly those for lipids, are often shorter than the frame time of normal video (33 ms or 40 ms), no wonder such hop diffusion could not be observed previously. In video-rate observations, a membrane molecule undergoing hop diffusion looks as if it were undergoing slow simple Brownian diffusion (apparent simple Brownian diffusion).

The compartment boundaries are likely to be made of the membrane skeleton (the cytoplasmic domains of transmembrane proteins directly collide with the membrane skeleton; 'fence' model) (Figure B1) (107,108) and rows of transmembrane-protein pickets anchored to the membrane skeleton ('picket' model, Figure B2) (56). Therefore, for transmembrane proteins, both the fence and pickets work together to suppress their diffusion in the membrane.

Transient confinement zones (TCZs), a term coined by Jacobson's group (32,109), are the membrane domains where the diffusion of a membrane molecule is substan-

tially slowed. It is defined as a domain where the observed molecule (normally tagged with a colloidal gold particle) stays much longer than expected from the average diffusion coefficient of the molecule (109). Note that this is somewhat an operational definition, because it assumes that the observation is carried out at slow rates, like normal video rate, at which individual membrane compartments are usually smeared out due to time-averaging on (low time resolution of) the camera. Therefore, the observed molecules are undergoing apparent simple Brownian diffusion with a macroscopic diffusion rate determined by the hop movements over many compartments, outside the TCZs.

These TCZs have appeared on the central stage of the raft research when Jacobson's group showed that (i) DRM-philic molecules exhibit TCZs much more often than DRM-phobic molecules (30), and (ii) TCZ is formed in a cholesterol-dependent manner (32).

The membrane compartments (corrals) and TCZs have been confused quite often, but they are two completely different structures and concepts. In the following, we try to clarify the difference between the membrane compartments and TCZs.

The membrane compartments cover the entire cell membrane, except for other membrane structures like

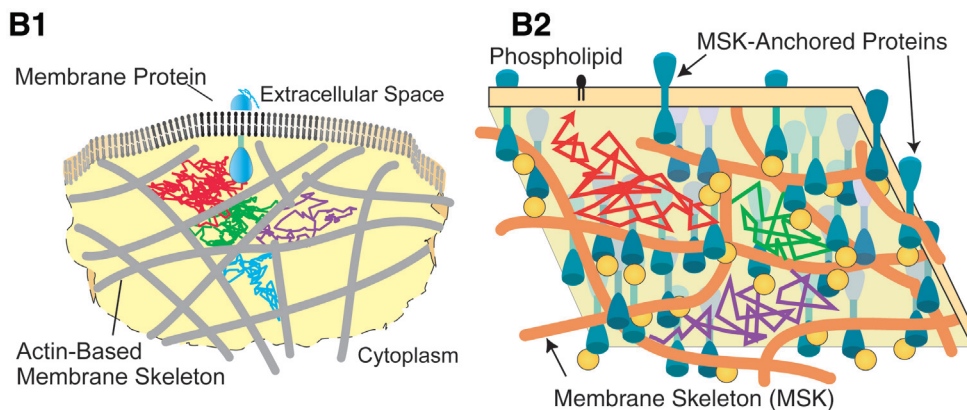


Figure B: The membrane skeleton 'fence' model and the anchored-protein 'picket' model. B1) The membrane skeleton fence model. Transmembrane proteins are sticking out into the cytoplasm and, in this model, the cytoplasmic domain collides with the membrane skeleton, which induces temporary confinement of the transmembrane protein in the membrane skeleton mesh (compartments). Transmembrane proteins could hop from a compartment to an adjacent one when the actin filament temporarily and locally breaks, when the membrane-to-skeleton distance fluctuates to allow the passage of the transmembrane protein, and/or the protein has by chance kinetic energy enough to push through the barrier when it is in the boundary area. B2) Anchored protein picket model, in which various transmembrane proteins anchored to and lined up along the actin-based membrane skeleton mesh act as rows of pickets which temporarily confine phospholipids (even those in the outer leaflet of the membrane) and proteins in the membrane skeleton mesh (compartments), due to the steric hindrance as well as the hydrodynamic friction-like effects of immobilized picket proteins. The compartment boundaries do not have to be totally closed off by picket proteins because the friction-like effects of immobile proteins are propagated for quite a distance. Here it is important to realize that transmembrane proteins that are not immobilized (via anchoring on the membrane skeleton) will not make effective diffusion obstacles. Based on this model, one could predict that the size of the compartment as 'felt' by transmembrane proteins and phospholipids (even those in the outer leaflet) is practically the same, and indeed this turned out to be true.

clathrin-coated pits, caveolae, and cell-to-substrate and cell-to-cell adhesion structures. Contiguity of the compartments with each other is one of the important characteristics of the compartmentalized structure, seen as close apposition of compartments in the trajectories of membrane molecules observed at sufficiently high time resolutions. The fence and picket models are consistent with these observations, because they insist that the compartments are separated by narrow quasi-linear (at around 1- μm level, the persistent length for actin filaments) fences or by the fence posts that keep these fences near the bilayer, and so there is no significant diffusion-distance between adjacent compartments.

In contrast, contiguity of TCZs with each other is not generally seen (although TCZs may be concentrated in a small region), and TCZs are mostly separated from each other, often a micron or farther away. It is not correct to call the inter-TCZ times 'hops', since they can be quite

long, both spatially and temporally. The residency time within the membrane compartment is 1–25 ms on average, whereas that within a TCZ is on average on the order of a second. The diffusion coefficient within the compartment is comparable to those found in artificial membranes (5–10 $\mu\text{m}^2/\text{s}$), whereas the accurate measurements of the diffusion rates of molecules in TCZs have not been carried out.

In addition, all of the molecules examined thus far, including phospholipids, transmembrane proteins, gangliosides, and GPI-anchored proteins, exhibited hop diffusion (15). Oligomerization of membrane molecules greatly reduces the hop rate in general (110). Even after partial depletion of cholesterol, membrane molecules undergo hop diffusion, whereas TCZ behavior is dramatically decreased (12–14). As described in the text, only clustered (by crosslinking or liganding) raft-philic molecules appear to exhibit TCZs at statistically significant time fractions.

spread over the cell surface (except in the clathrin-coated pits) at the resolution of the light microscope, thus showing that the GM1 complexes or domains are smaller than the optical diffraction limit ($\approx 300\text{ nm}$). However, since FRET was detected between pentavalent cholera-toxin molecules, it may be induced by the assembly of crosslinked GM1. If this is the case, then it is interesting because it shows that crosslinked GM1 could form greater complexes. Why FRET could not be seen in the clathrin-coated pits has not been explained: the clustering efficiency of cholera-toxin in the pit may be lower, the accessibility of cholera toxin to GM1 in the pit may be limited, or the concentration of GM1 in the pit may be lower than that in the bulk membrane.

FRET and pulse EPR spin-labeling experiments suggested that the rafts are even smaller and/or short-lived, and/or that the raft molecules rapidly diffuse in and out on a time scale of 100 μs or less (45,46). Recent FRET work on a GPI-anchored protein, folate receptor, revealed that 20–40% of these proteins may be in clusters smaller than pentamers, with the remaining 60–80% existing as monomers (47,48). In line with these observations, McConnell has advanced the concept of the condensation complex of cholesterol and saturated-alkyl-chain lipids, which is conceptually different from the domain made of the liquid-ordered phase (49–55). It would be of interesting if the lifetimes of these complexes could be estimated.

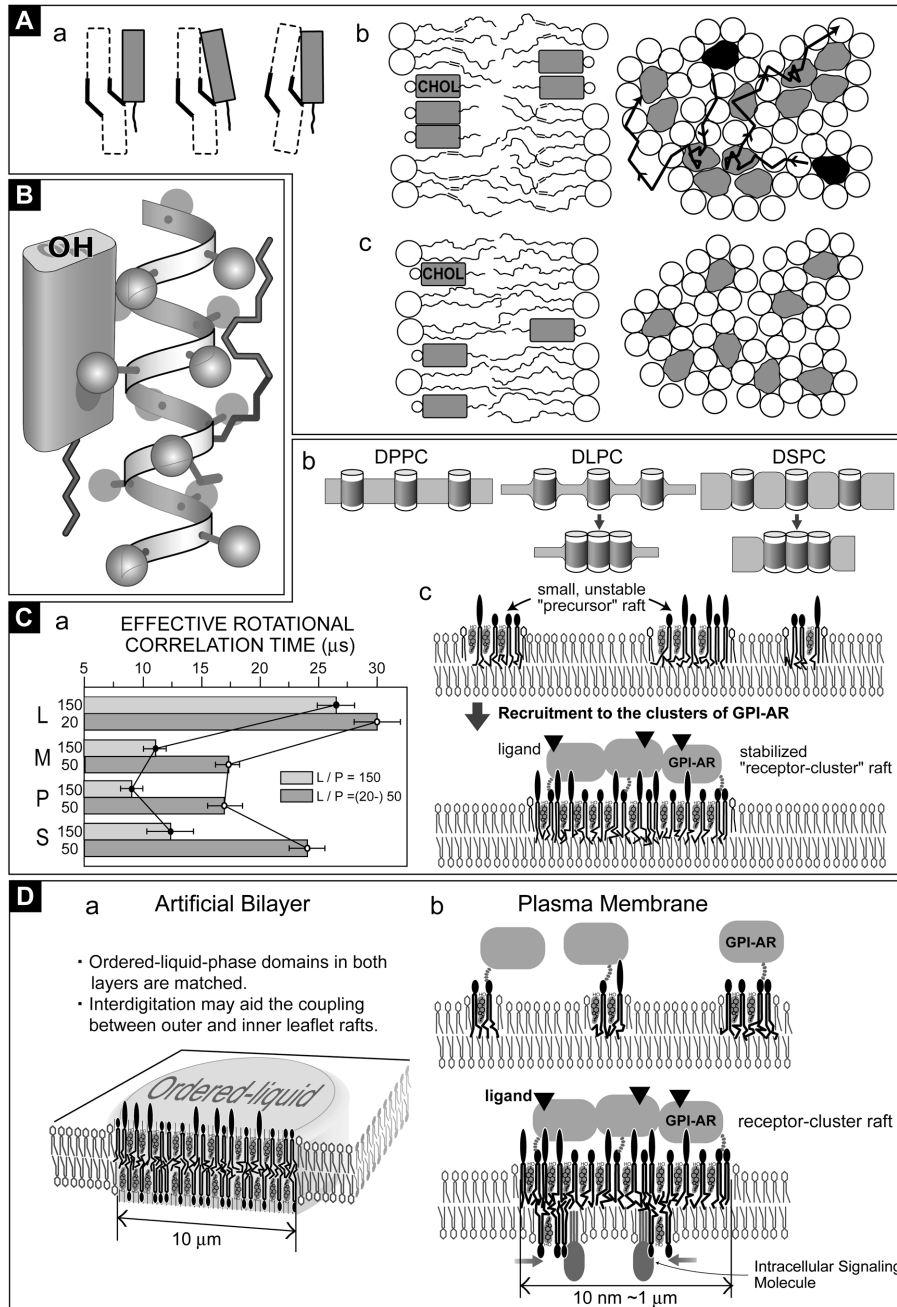
Using single molecule techniques (both single particle tracking at a 25- μs resolution and single fluorescent molecule imaging at video rate), Suzuki et al. found that the raft that monomeric CD59, a typical raft-preferring GPI-anchored protein, is associated with may consist of only several molecules with a lifetime perhaps on the order

of a millisecond or less (12–14). To explain these results, let us begin from the basic structure of the plasma membrane. The plasma membrane is compartmentalized into 30–250-nm compartments (their sizes depend on the cell type) for both lipids (including those located in the outer leaflet of the membrane) and transmembrane proteins. Both undergo short-term confined diffusion in a compartment and a long-term hop movement over many compartments, a phenomenon termed hop diffusion (56; Murase and Kusumi, unpublished observations). The average hop rate is once every 1–25 ms (again depending on the cell type). See Box 1 for details. By repeating such temporary confinement and hop movement, the membrane molecules undergo macroscopic diffusion over many compartments. The compartment boundaries are made of the actin-based membrane skeleton (called the 'fence') as well as various transmembrane proteins anchored to and lined up along the membrane skeleton (called 'pickets'). Rows of anchored transmembrane-protein pickets are responsible for temporarily confining GPI-anchored proteins and phospholipids within the compartments in the outer leaflet of the plasma membrane (see Box 1).

If a GPI-anchored raft protein, CD59, is always associated with large and stable rafts, the raft and CD59 would diffuse together, and one would expect that their hop rate across the compartment boundaries would be much smaller than that for a typical nonraft phospholipid, DOPE. However, in reality, they both hopped at the same rate, once every 25 ms on average. Furthermore, in CHO cells, where the compartment size is much smaller ($\approx 40\text{ nm}$), a GPI-anchored form of major histocompatibility complex (MHC) Class II and DOPE diffused at the same rate (57; Suzuki and Kusumi, unpublished observations). Although

we note that these results are not inconsistent with an extreme possibility that GPI-anchored proteins do not partition into the raft in the resting cells at all, we prefer the idea that the rafts that monomeric GPI-anchored proteins associate with in steady-state cells may be very small and/or short-lived, perhaps on the order of a millisecond or less (because both phospholipids and CD59 hop across the picket line at about the same rate of once every 25ms on average). These are qualitatively similar to the observations described above, in that the rafts in the resting cell membrane are small. In the present review, we call these small, unstable rafts 'reserve rafts', as stated at the beginning of this review.

Anderson & Jacobson (58) proposed a model of a 'lipid shell' surrounding raft-associated molecules. The difference between the lipid shell model and our small/unstable reserve raft model is the lifetime (although the size may also differ by a factor of 3–10 in diameter or 10–100 in the number of molecules). They assume that the lipid shells have long lifetimes, and they argue that this model could explain the fast hop frequency of CD59 across the row of pickets (compartment boundary), because the shells are soft and can change their shape, allowing for passage between the pickets. However, such a property may not readily be compatible with the raft integrity (long lifetime), i.e. if it were very soft, then it would tend to break easily,



reducing its lifetime, and thus the shell concept becomes very similar to the small/unstable reserve raft concept.

Basic Interactions between Membrane Molecules for Understanding Small/Unstable Reserve Rafts and Stimulation-Induced Stabilized Rafts

In the following, we consider why the rafts in the plasma membrane tend to be small and unstable, whereas the ordered-liquid-phase domains in artificial membranes are large, often exceeding 10 μm (58–63). We also consider why and how the raft size can be increased by clustering raft molecules (by ligation or crosslinking), i.e. how the clustering of a single raft molecular species induces the recruitment of other raft components, like cholesterol and sphingolipids, and stabilize the raft structure.

Before considering the mechanism for the conversion of raft types, we summarize the following six important interactions between membrane molecules that influence the creation of the raft as well as its size and lifetime (stability).

A) Immiscibility of cholesterol and unsaturated alkyl chains (Figure 1A). This occurs due to lateral nonconformability of the rigid and bulky tetracyclic sterol structure of cholesterol and the rigid double bond of unsaturated lipids (Figure 1A, a) (64,65). Consider an artificial membrane that consists of only cholesterol and an unsaturated phospholipid 1,2-dioleoylphosphatidylcholine (DOPC). In such a membrane, cholesterol is segregated out of the DOPC domain, due to the conformational nonconformability of the cholesterol's rigid backbone and the rigid *cis* double bond at the position of C9:C10 on the oleoyl chain,

Figure 1: The four basic interactions between membrane molecules for understanding small/unstable reserve rafts and stimulation-induced, stabilized receptor-cluster rafts (two additional interactions in the text). A) Lateral nonconformability between *cis*-unsaturated alkyl chains and cholesterol (a) induces dynamic segregation of cholesterol out of the bulk phase (b), whereas cholesterol mixes with saturated alkyl chains at various ratios and promotes *trans* conformations, which increases alkyl chain order (c). (a) Lateral nonconformability between the rigid structure of cholesterol (rectangles with short tails) and the rigid bend at the C9–C10 *cis* double bond in an unsaturated oleoyl chain (bent rods). This nonconformability creates packing problems when placing cholesterol and unsaturated alkyl chains side-by-side in the membrane. Due to the mismatch in the lengths between the cholesterol and the alkyl chains, the assembly of cholesterol could induce vacant pockets (packing defects) in the central part of the membrane, which causes interdigitation with longer alkyl chains. (b, c) Snapshot drawings of the side view (left) and the top view (right) of L- α -dioleoylphosphatidylcholine (DOPC)-cholesterol (b), and L- α -dipalmitoylphosphatidylcholine (DPPC)-cholesterol (c). In the top view, the open circles represent phospholipid hydrocarbon chains, and the solid structures indicate cholesterol molecules (the cholesterol shape is based on 117). The top view of (b) shows that cholesterol molecules frequently and repeatedly become incorporated in and dissociated from cholesterol clusters. The lifetime of these clusters has been proposed to be 1–100 ns. To simplify the presentation, the movement of only two cholesterol molecules is drawn. See (118,119). B) Cholesterol is excluded from the boundary region of the transmembrane protein due to the lateral nonconformability of the rigid, tetracyclic cholesterol backbone and amino acid side chains protruding from the transmembrane α -helix of the transmembrane protein (surface roughness). Alkyl chains can assume various conformations due to *trans*-gauche isomerization of each methylene segment, thus accommodating the protruding amino-acid side chains. C) Segregation of molecules due to mismatches in hydrophobic lengths. (a) The rotational correlation times of rhodopsin in reconstituted membranes with di-saturated PCs with various chain lengths. L, M, P, and S respectively represent dilauroyl-, dimyristoyl-, dipalmitoyl-, and distearoyl-PCs, which have 12, 14, 16, and 18 carbon atoms in each alkyl chain. In contrast to translational diffusion, rotational diffusion is very sensitive to the size of the diffusing unit, and the formation of dimers or greater oligomers can be sensitively detected. With an increase in the protein concentration, the rotational correlation time sharply increases, suggesting the formation of oligomers. In the case of L, rhodopsin exists in greater oligomeric forms even at low protein-to-lipid mole ratio (1/150). Rhodopsin is most miscible with P, and the rotational correlation time of rhodopsin sharply increases by lengthening or shortening the alkyl chains, suggesting the formation of dimers or larger oligomers. The level of the increase in the rotational correlation time suggests that this oligomerization is transient. (b) Since mixing molecules with different hydrophobic lengths is energetically unfavorable, the molecules that have either longer or shorter hydrophobic lengths than that of the bulk domain tend to be segregated out to form oligomeric complexes. This result also indicates that, in the membrane, monomers and molecular complexes tend to be in a delicate balance, and that very small changes that modulate the molecular interactions could greatly change the oligomer fraction. (c) The long, saturated alkyl chains of glycosphingolipids that are in contact with cholesterol tend to be extended. This creates a hydrophobic length mismatch, which drives segregation of glycosphingolipid-cholesterol complex out of the bulk unsaturated lipid domain. Coupled with the tendency for cholesterol to be segregated away from the bulk domain, this promotes the formation of glycosphingolipid-cholesterol clusters. However, these alone would not be sufficient to form large stable rafts, but form small, unstable 'reserve rafts' (above). When a GPI-anchored receptor (GPI-AR) is clustered upon ligand binding, the reserve rafts may be stabilized in the cluster, forming stabilized 'receptor-cluster rafts' (below). Therefore, the small unstable rafts in the resting-state cells are termed 'reserve rafts' for the formation of receptor-cluster rafts. D) Large ordered-liquid-phase domains found in artificial membranes may be stabilized by interdigitation between the two leaflets, whereas the outer-leaflet stabilized receptor-cluster raft in the plasma membrane could recruit small inner-leaflet rafts beneath them that contain lipid-anchored cytoplasmic signaling molecules. (Left) The 10- μm -level, ordered-liquid-phase domains in the artificial bilayer appear to be made of two matched, ordered-liquid domains in both the outer and inner leaflets of the bilayer, suggesting that they may be stabilized by the interdigitation of lipids between the two layers (left). The formation of such large, ordered-liquid domains in the artificial bilayer may be optimized by adjusting the lipid composition, lowering the temperature (like room temperature), avoiding inclusion of raft-breaking transmembrane proteins and the membrane skeleton that anchors the transmembrane proteins. (Right) In the plasma membrane, such large domain formation is unfavorable, and the plasma membrane is likely to contain many small, unstable reserve rafts consisting of several GPI-AR, sphingolipid and cholesterol molecules (right top). Ligand-induced clustering of GPI-AR creates stabilized, enlarged receptor-cluster rafts, like the one shown in Figure 1C (c, bottom). Such receptor-cluster rafts formed in the outer leaflet could recruit inner-leaflet rafts that contain lipid-anchored signaling molecules, possibly transiently, probably due to interdigitation interactions between the outer- and inner-leaflet rafts.

and forms cholesterol-enriched (clustered) domains (Figure 1A(c)). However, these cholesterol-enriched domains are made up of only a few cholesterol molecules and they last only 1–100 ns, i.e. they are forming and dispersing continuously (64,65). Such cholesterol-cluster domains may be stabilized if saturated-chain lipids are included in the membrane, because they tend to mix with cholesterol at certain molar ratios to form molecular complexes (Figures 1A (b)) (5,64). Some alkyl chains of sphingomyelins have a *cis* double bond at a C15:C16 position (66), but this will not have a conformational conflict with the ring structure of cholesterol, further contributing to good miscibilities of cholesterol and sphingolipids.

B) Exclusion of cholesterol from the boundary regions of transmembrane proteins (Figure 1B). Cholesterol is excluded from the boundary regions around transmembrane proteins in the reconstituted membranes of sarcoplasmic reticulum ATPase, phospholipids, and cholesterol (67). Band 3 in the erythrocyte membrane is also immiscible with cholesterol in reconstituted membranes (Kusumi and Tsuji, unpublished observations). Consistent with these observations, a large majority of transmembrane proteins exhibit very low levels of partitioning into the DRM fraction. We propose that these results are due to lateral *nonconformability* of the rigid, bulky sterol ring of the cholesterol with the rough surface of the transmembrane domain of the majority of transmembrane proteins (Figure 1B). This surface roughness is due to the amino-acid side chains protruding from the transmembrane α -helix. The surface of the transmembrane domain may be rough, but the alkyl chains must have sufficient flexibility to accommodate the protruding amino-acid side chains (Figure 1B). Beyond a layer or two of the alkyl chains from the transmembrane α -helix, the effects of the protruding amino-acid side chains would be smoothed out, and cholesterol could be incorporated there.

The surface roughness of the transmembrane domain is probably the reason the majority of transmembrane proteins are excluded from the cholesterol-rich raft domains. Therefore, we would predict that the transmembrane receptors that take advantage of rafts for their signaling, such as the Fc ϵ receptor or the T-cell receptor, would have relatively smooth surfaces on their transmembrane α -helices to facilitate their incorporation within the raft.

These results further suggest that most transmembrane proteins may act as *raft breakers*, by driving cholesterol away from their boundary regions (see below). Another possibility to be considered is that the membrane may contain two types of domains: one containing cholesterol and other raft molecules, and the other transmembrane proteins. How large each individual domain grows remains unknown, but we envisage that these domains remain small (see **3** in the next section).

C) Segregation of molecules due to the mismatch in hydrophobic length (Figure 1C). In reconstituted mem-

branes composed of bovine rhodopsin, a G-protein-coupled receptor, in di-saturated phosphatidylcholines (PC) with various chain lengths (from C12 up to C18, i.e. dilauroyl-PC, dimyristoyl-PC, dipalmitoyl-PC, and distearoyl-PC), rhodopsin is monomerically dispersed in C16 dipalmitoyl-PC, whereas the dimer (and greater oligomer) fraction sharply increases as the chain length either increases or decreases (Figure 1C (a, b)). Since mixing molecules with different hydrophobic lengths is energetically unfavorable, the molecules that have either a hydrophobic length longer or shorter than that of the bulk domain tend to be segregated out to form oligomeric complexes (Figures 1C (b)) (1,68,69). An argument was advanced in which the monomers and oligomers (including dimers) are in such a delicate balance that only very small changes in the molecular interactions could greatly change the oligomer fraction (1).

The second chains in glycosphingolipids tend to be slightly longer than those in phospholipids and tend to be saturated (66). When they are in contact with cholesterol, the *trans* conformation in the alkyl chains is preferred and the alkyl chain order increases (Figures 1A (b)), making the long saturated chains reach deeper into the membrane. This would create mismatches in the hydrophobic length, enhancing the tendency to segregate the glycosphingolipids (or glycosphingolipid-cholesterol complex) out of the bulk domain (Figures 1C (c, top)), and this tendency would be facilitated by the presence of clustered GPI-anchored receptor molecules (Figures 1C (c, bottom)). We stress the importance of the ordering effect of cholesterol on the saturated alkyl chains in this segregation because, in the presence of cholesterol, since the alkyl chains next to the rigid sterol ring of cholesterol tend to assume the *trans* conformation, the conformational freedom of the alkyl chains down beyond C12 would not be able to fully absorb the extra length of the glycosphingolipid alkyl chains.

D) Stabilization of the large ordered-liquid-phase domains found in artificial membranes by interbilayer coupling (Figure 1D). Among the large ordered-liquid-phase domains created due to phase separation in artificial membranes, those reported thus far appear to be either made of two matched, ordered-liquid domains in both the outer and inner leaflets of the bilayer (59,60,70) or backed by the supported layer of lipids fixed on the solid material or by the solid material itself (61). Generally, fluorescence microscopy observations of the bilayers containing these large domains have reported the presence of two clearly separated domains, ordered- and disordered-liquid-phase domains, and not the presence of regions where the disordered-phase domain in one leaflet overlaps with the ordered-phase domain in the other leaflet. These results suggest that for large ordered- and disordered-liquid-phase domains to coexist stably they have to be stabilized in some ways: in supported membranes, the ordered-liquid-phase domains may be stabilized by the solid nature of the support, and those in the bilayer may be stabilized by

matching the ordered domains in both layers and enhancing the coupling of the two layers through interdigitated lipids (Figure 1D (a, b)) (7,71,72).

E) Equally high miscibility of transmembrane proteins with saturated and unsaturated alkyl chains in the disordered-liquid domain (Figure 1B). In reconstituted membranes of bovine rhodopsin, rhodopsin was equally well-dispersed in the membranes of dipalmitoyl-PC (a saturated PC) or DOPC in the disordered liquid phase (1). Therefore, transmembrane proteins tend to be miscible with both saturated and unsaturated chains if they are in the disordered liquid domains.

F) Possible involvement of the molecular dipole moment or charge in partitioning into the raft or the ordered-liquid-phase domain. This argument has been advanced by Vaz and coworkers (73). Supporting this model, Dietrich et al. (60) indeed found that NBD (no charge)-DPPE and TexasRed (both + and – charges with no net charge)-DPPE preferentially partition into the ordered and disordered liquid-phase domains, respectively, in artificial membranes with liquid–liquid phase separation. Fluorescein (negative charge depending on pH)-DPPE partitioned equally well in both domains. Further elucidation regarding the involvement of the molecule's dipole moment or the charge in raft partitioning is clearly required.

Why Can't the Large Ordered-Liquid Domains Found in Artificial Membranes Be Detected in the Plasma Membrane?

If the raft domains in the plasma membrane are in some way related to the liquid-ordered-phase domains observed in artificial membranes, then why are they so small, to the point of being undetectable (in the resting state), compared with those seen in artificial membranes? Many studies have shown that the size of these domains often exceeds 10 μm in artificial membranes (58–63). It is often assumed that since the plasma membrane contains such a variety of molecular species, they tend to work like detergents towards each other, relaxing domain boundaries, with concomitant loss of the line tension around the domain. These effects would make the domain size very small. However, admitting such effects, we would like to be more specific here. Based on the interactions between membrane molecules described in the previous section, here we further consider why large, stable ordered-liquid-phase domains cannot be found in the plasma membrane.

1 The coexistence of liquid-ordered and disordered phases observed in artificial membranes only occurs in narrow lipid composition ranges, which are particularly restricted at 37 °C (70). (However, see also the results of de Almeida et al. (74). Based on the microscopic observation methods, they argue that this coexistence takes place in

much broader lipid composition ranges. When one talks about phases, we feel more comfortable with more macroscopic observations and definitions). Two main interactions probably drive the creation of cholesterol/saturated-lipid domains. First, cholesterol tends to be segregated out of the unsaturated–lipid phase (Interaction A, Figure 1A). Second, such a cholesterol-enriched domain (its lifetime may be very short without further stabilization, as described in section A above) may be stabilized by high concentrations of long, saturated lipids, such as glycosphingolipids, sphingomyelin, and phospholipids with long saturated alkyl chains, i.e. raft-preferring lipids. Plasma membranes generally contain 30–50 mol% cholesterol, but only very specific types of cell membranes, such as the apical membrane of several epithelial cells (75), have sufficient concentrations (over 50 mol% (70)) of raft-preferring alkyl lipids that satisfy the lipid composition for the formation of the liquid-ordered phase. However, since all of the cells investigated thus far appear to have GPI-anchored receptor molecules and raft-based signaling and trafficking mechanisms (7), the raft must form without liquid–liquid phase separation.

One could argue that the majority of raft-preferring alkyl lipids exist in the outer leaflet of the cell membrane, which would double the amount of these lipids in the outer leaflet, making liquid–liquid phase separation in the outer leaflet possible (76). If this is the case, the problem would be which lipids are responsible for the formation of rafts in the inner leaflet. (Since many signaling proteins located in the ordered liquid phase in and on the inner leaflet of the plasma membrane require cholesterol for their functions (8,11,19,77,78), we believe the presence of lipid rafts in the inner leaflet of the membrane.) Furthermore, even if large ordered-liquid-phase domains are formed in the outer leaflet, they may soon become fragmented if these domains are not backed by the rafts in the inner leaflet (Interaction D, Figure 1D). Therefore, if the rafts are made of the liquid-ordered phase, such a phase must exist in both layers of the bilayer, but this may not be able to be achieved with the lipid compositions of the majority of the cell membranes, again suggesting that the existence of large liquid-ordered domains is unlikely.

One should also note that the majority of published studies employing artificial lipid membranes have been carried out at lower temperatures (e.g. room temperature), where the two distinct domains are more readily observable (60,61,79,80). Since the lipid composition in the cell membrane is optimized for the function at physiological temperatures (81,82), lowering the observation temperature is likely to induce the artifactual formation of various membrane domains and phase separations that lack direct relevance to physiological events occurring in the cell membrane at 37 °C. Lowering the temperature would also induce domains with solid-phase characteristics that are different from those of the ordered liquid phase. This, in turn, drives many membrane

molecules away from the solid phase, thus inducing clustering due to their locally increased concentrations.

2 As described above, the plasma membrane is compartmentalized by various transmembrane proteins (pickets) anchored to and lined up along the membrane skeleton mesh (12–14,56). The picket proteins are aligned along the membrane skeleton 3–10 nm away from each other (Figure 2; assuming that the diameter of a transmembrane domain is 0.7–4 nm). Furthermore, cholesterol tends to be excluded from the boundary regions of transmembrane proteins (Interaction B, Figure 1B). Therefore, it follows that, on the membrane skeleton, 1.5–5 nm diameter zones that exclude cholesterol are lined up 2–9 nm away from each other (Figure 2; assuming that the diameter of the area that an alkyl chain occupies is 0.4–0.5 nm). These rows of cholesterol-excluding areas would act as barriers that block the growth of the raft domains. This would limit the raft size to less than the compartment size (30–250 nm depending on the cell type), which is smaller than the optical diffraction limit.

3 The mutual exclusion of cholesterol and transmembrane proteins may induce cholesterol-enriched domains and protein-enriched domains in the membrane. However, we envisage that these would only form nanodomains, consisting of several to several tens of molecules. The cholesterol content in the plasma membrane is 30–50 mol% on average; if cholesterol is excluded from the protein-rich domains, then the cholesterol mole fraction in cholesterol-enriched domain would easily be increased to over 50 mol%. However, the maximal mole fractions of cholesterol in PC and PE membranes are 51 and 66 mol% (83). Hence, such cholesterol-enriched domains could only exist as very small domains, and therefore the transmembrane-protein-enriched domains would also stay small.

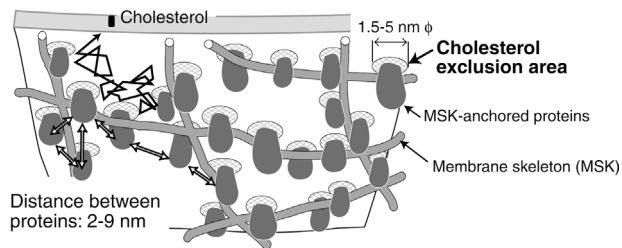


Figure 2: The plasma membrane is compartmentalized, thus inhibiting the growth of steady-state rafts by lining up cholesterol-excluding zones along the membrane skeleton.

Various transmembrane picket proteins are anchored to and lined up along the membrane skeleton mesh, 3–10 nm away from each other. Since cholesterol tends to be excluded from the boundary region of a transmembrane protein (Figure 1B), on the membrane skeleton, zones of 1.5–5 nm in diameter that exclude cholesterol are lined up 2–9 nm away from each other. These rows of cholesterol-excluding areas would act as barriers that block the growth of raft domains over 30–250 nm (depending on the cell type).

4 Many phospholipids have one saturated and one unsaturated alkyl chains (1 and 2 positions, respectively), and thereby are miscible with both raft-philic and raft-phobic molecules, although they tend to be excluded from the low-density, detergent-resistant membrane fractions (DRM) (84,85). These 1-saturated, 2-unsaturated phospholipids are expected to 'solubilize' the raft-preferring molecules into the bulk membrane, thus inhibiting the growth of the domains of raft-preferring molecules (however, see de Almeida et al. (74)).

The relative importance of these effects is hard to determine, but collectively they would block the growth of the raft in the steady-state cell membrane, and only allow for the formation of small, unstable rafts, i.e. the local, transient assembly of raft-philic molecules all over the membrane. However, we need to stress the delicate balance between the concentration and the dispersion of these raft molecules. Only a slight suppression of thermal movements, for example, by the local concentration of raft molecules by the ligand-induced clustering of raft-preferring receptor molecules (such as GPI-anchored receptors) or by lowering the temperature, could induce large rafts, although they may not be as large as 10 μm .

These considerations indicate that the dynamic behaviors of raft constituent molecules in resting-state cells may bear some similarities with those in the ordered-liquid phase in artificial membranes (86). However, the rafts in the steady-state cells would not represent the ordered-liquid phase in the majority of the cells ((55), however see (72)).

Clustering of raft molecules by ligand binding or crosslinking induces stabilized rafts, 'receptor-cluster rafts'

Abundant evidence exists for the formation of greater, stabilized rafts by the clustering of raft-philic molecules (21–23,34,38,61). In fact, the results of unintentional crosslinking described in the earlier section, 'Rafts have not been directly observed...', would provide good examples for such clustering-induced stabilized rafts. Crosslinking of Thy1, another GPI-anchored protein, causes cholesterol and glycosphingolipids to become concentrated, leading to the formation of greater, stabilized rafts (Thy1-cluster rafts) with lifetimes longer than several min (Figures 1C, (c, bottom right) (87). In neutrophils, after chemotactic stimulation, large ordered membrane microdomains were formed (17). In T cells, the flotilla at the front of the cell is marked by the ganglioside GM3 (and possibly CD44), whereas the flotilla at the rear contains GM1 (and possibly PIP₂, (16)). However, it is important to realize that the constituent lipid molecules of such "receptor-cluster rafts" may still be able to exchange with those in the bulk disordered-liquid domain, just like the lipids moving back and forth between the ordered- and disordered-liquid-phase domains in artificial membranes (71).

How Can Raft Molecule Clustering Induce Stabilized Rafts? A Lesson from the Boundary-Lipid Research

How can the clustering of GPI-anchored proteins or glycosphingolipids (induced by ligand, lectins, pathogens or other reagents) induce greater, stabilized rafts? What makes the clustered raft molecules different from the monomeric raft molecules? As an example, consider the hypothetical oligomerization of 10 GPI-anchored receptor (GPI-AR) molecules, which induces the close assembly of 10–20 saturated alkyl chains. Note that since the protein domain is much larger (say 3–5 nm in diameter) than the two alkyl chain anchors (say 0.6×1.2 nm), there is always enough space for other lipids to stay among the anchoring chains in a GPI-AR cluster.

We suggest that such a loose saturated-alkyl-chain cluster would recruit cholesterol, glycosphingolipids and raft-preferring molecules into the cluster, due to the following interactions.

First, cholesterol mixes well with saturated chains while it tends to be excluded from the bulk, disordered liquid domains because of its immiscibility with unsaturated alkyl chains (Interaction A, Figure 1A). If given a choice, cholesterol would then move to the region where the saturated chains are concentrated.

Second, since the long alkyl chains of sphingolipids might also be excluded from the bulk, disordered-liquid domain due to mismatches in their hydrophobic lengths, particularly when the long saturated chains are in contact with cholesterol (Interaction C, Figures 1C (c), they tend to assemble in the clusters of GPI-AR-cholesterol, where the hydrophobic chains are longer and the membrane is thicker.

Third, importantly, since the GPI-AR oligomerization prevents their saturated chains from diffusing away from the cluster and blocks the changes in their relative positions, it reduces the diffusion of other molecules in the area of the GPI-AR oligomer, thus restricting the movements of the recruited cholesterol and glycosphingolipids.

Fourth, since the cholesterol-saturated alkyl chain interaction enhances the *trans* conformation and thus higher orders of saturated alkyl chains, which are predominant in the GPI-AR region, this further reduces the thermal agitation in this region and prolongs the residency time of cholesterol, glycosphingolipids, and sphingomyelin in this GPI-AR cluster region.

Each of these processes would involve a tiny free energy gain, but collectively these gains may be sufficient to maintain high concentrations of cholesterol and sphingolipids in the GPI-AR oligomer area. Further, we again would like to stress the delicate balance between the concentra-

tion and the dispersion of these raft molecules: the oligomerization of GPI-AR only slightly suppresses thermal diffusion in the oligomer area, but the small reduction in the thermal motion could greatly enhance the ability of GPI-AR to collect other raft molecules to form large and stable rafts.

This argument is analogous to that for the effect of transmembrane protein clustering on lipid movement found in boundary lipid research. When transmembrane proteins are monomeric, the lipids at the boundary (i.e. the lipids that are in direct contact with the transmembrane protein) exchange with the lipid in the bulk domain at a rate of 10^7s^{-1} , or once every $0.1 \mu\text{s}$ on average (88,89). Note that if the transmembrane protein is anchored to the membrane skeleton, its anchoring effect on the surrounding lipids is greatly enhanced and distantly propagates due to the hydrodynamic friction-like effects (56,90,91). Such rapid lipid exchange is often overlooked by raft researchers. Upon transmembrane protein oligomerization, the lipids become sandwiched between two transmembrane proteins (including the lipids trapped between boundary lipids) or trapped in the protein-rich domain, and the rate of their exchanges with lipids in the bulk domain drops dramatically (1,92,93). Both the geometric and packing effects of the clustered proteins would contribute to the prolonged residency of lipids in the clustered protein area. GPI-AR clustering would similarly suppress the movement of molecules in the cluster region, thus prolonging their stay in the cluster domain.

The essential behavior of boundary lipids that raft researchers must understand can be summarized as follows. In the instances that the alkyl chain enters the boundary region, the *trans*-gauche isomerization in the alkyl chain is strongly restricted (thus free volume in this area is substantially smaller than that in the bulk region) (94–97), but the chain leaves the boundary region within $0.1 \mu\text{s}$ on average (a new lipid comes into the boundary region, and the isomerization of its alkyl chains instantaneously stops; therefore free volume there is always less than that in the bulk region). Prolonged residencies of lipids in the boundary region of a transmembrane protein only takes place when the transmembrane proteins are clustered or form protein-enriched domains. For further details of the boundary lipid behaviors, see Box 2. An analogy can be made to GPI-AR: cholesterol and glycosphingolipids would not remain in the boundary region of GPI-AR unless GPI-AR clustering is induced, leading to the prolonged, but temporary, trapping of other raft lipids and cholesterol in the cluster domain.

The receptor-cluster rafts are the rafts that are directly involved in biological functions. The considerations given in this section suggest that the receptor-cluster rafts are on the verge of forming even in resting state cells, and with only small modulations of the delicate balance of molecular interactions, such as clustering of GPI-AR,

receptor-cluster rafts are formed. Therefore, the small, unstable rafts found before stimulation are those ready for forming greater rafts, and this is why these molecular complexes are called 'reserve rafts' in this review.

Enhanced Awareness of the Characteristic Time Scale of the Observation Method Is Required in Raft Research: A Lesson from the History of the Boundary-Lipid Research

Perhaps, the best lesson one could learn from boundary lipid research (see Box 2) is to always consider the time

scales of the raft-related events as well as those of the methods used for the observation. Since various raft events take place on different time scales, the use of different methods would easily lead to apparently different conclusions, just like the cases of EPR and NMR for boundary lipid dynamics (see Box 2).

In the following, we give two examples that highlight the need for the clear understanding of the time scale that is covered by the observation method, before believing in the presence of stable rafts. The ordered liquid domain may be detected by a spectroscopic method, like EPR spin labeling of lipids (86), but how long does the raft have to exist to be

Box 2

The danger of making simplistic analogies to boundary lipids in the effort to understand rafts

Many raft researchers seem to start thinking of rafts on the basis of the boundary lipid concept. This could be useful, as discussed in the text. However, the boundary lipid concept is rather complicated, and simple-minded analogies could introduce further complications into the raft field. For example, very few researchers seem to be aware that (i) almost all of the lipids in the boundary region exchange with those in the bulk domain within 0.1 μs on average, and (ii) cholesterol may not partition into the boundary region of most of transmembrane proteins (Interaction B, Figure 1B).

Furthermore, the boundary lipid literature is confusing because the period during which the researchers in the field lacked an awareness of the time-scale issue lasted over 10 years. EPR spin labeling, which is sensitive to *events shorter than 10 ns*, detected *apparent immobilization or ordering* of lipids on the surface of transmembrane proteins (94,95). Conventional EPR using nitroxide spin probes is sensitive to the rotational diffusion of the probe, due to the anisotropy of magnetic interactions of 10^8 s^{-1} , and therefore it is very sensitive to reorientational events occurring in the time scale of 1 ps – 10 ns. (However, note that EPR T_1 (spin-lattice relaxation time) methods, like pulse saturation recovery EPR (46,64), saturation transfer EPR (1) and ELDOR (111) are sensitive to time scales up to 1 ms). In contrast, NMR spectroscopy of deuterated lipids, which is sensitive to reorientational motion in *time scales shorter than 10 μs* , revealed *apparent disordering of lipids* when transmembrane proteins were incorporated in reconstituted membranes (112–114).

It took almost 10 years to resolve this apparent contradiction. It turned out that the boundary lipids exchange with the lipids in the bulk domain on the order of 100 ns

(0.1 μs) (88,89). When a lipid partitions into the boundary region next to a transmembrane protein, the alkyl chain motion becomes slower and looked immobilized on the EPR time scale (10 ns), and thus the EPR order parameter of the lipid was greatly increased from that in the bulk (little alkyl chain segmental movement during 10 ns (115)). However, deuterium NMR is sensitive to events up to 10 μs , during which over 1000–3000 lipid molecules (100 exchanges during this time for 10–30 lipid sites) enter and exit the boundary domain. Since the lipid-to-protein molar ratio used in these experiments is commonly on the order of 50–200, good mixing of lipids between the boundary and the bulk regions takes place. The orientation of the alkyl chain may be different each time (and in each place in the boundary domain) a new lipid comes in, and may be spread over large angles due to the rough surface of the transmembrane domain (Interaction B, Figure 1B). Since such lipid exchange and binding at different angles in the boundary region are integrated over the NMR time scale of 10 μs , the alkyl chain order in a membrane observed on an NMR time scale would decrease, as more proteins are incorporated in the reconstituted membrane (many of these experiments were carried out using reconstituted membranes with various lipid-to-protein ratios).

The critical problem was that the majority of researchers assumed that both the EPR and NMR methods were measuring the ensemble average of the order parameter, and were not aware that they were taking the time averaging first (before averaging over all molecules under observation), and that the time scales over which the average was taken are different between these methods (10 ns and 10 μs for EPR and NMR, respectively). This problem, coupled with sloppy reconstituted membrane preparations in some investigations and with the measurements at nonphysiological, low temperatures, which were then (and are now) not uncommon, created enormous confusion in the field. Basically, all of the researchers in the boundary lipid field knew and talked about membrane dynamics, but

most still missed this specific point of the characteristic time scale for each method (116).

Therefore, if the boundary lipid analogy is to be made, it has to include the following:

- fast exchanges of the lipids between the boundary and the bulk domains on the order of 100 ns;
- long residency time of lipids in the boundary region only when transmembrane proteins form protein-enriched domains (see text);
- exclusion of cholesterol from the boundary region of most of the transmembrane proteins (Figure 1B);
- enhancement of *gauche* conformations in the alkyl chain and freezing of alkyl-chain segmental movement in the boundary region during the alkyl-chains' stay in the boundary region (Figure 1B);
- enhancement of *trans* conformation in the saturated alkyl chain located next to cholesterol (Figure 1A) (64);
- mutual exclusion of cholesterol and unsaturated lipids (Figure 1A);
- short distance of propagation of transmembrane proteins' ordering effect (about 2–3 lipids away or 1–2 nm from the surface of the transmembrane protein) unless the transmembrane protein is anchored (see text).

detected as such? The answer: about 10 ns. The constituent molecules may be incorporated in such a raft for 40 ns, and spend the next 10 ns in the bulk domain, and then exist for 40 ns again in a raft, and so on (neglecting the fact that the lipid spin probes in the ordered-liquid domain may not be separated from those in the boundary region in the EPR spectroscopy). Since the time scale for conventional EPR is limited up to 10 ns (see Box 2 for details), one could not tell from these data alone whether the ordered-liquid domain lasted for 40 ns or 40 min. Whether or not a raft with 10-ns lifetime is biologically significant is a separate issue.

Assume that FRET between two raft molecules was detected after an image acquisition period of 10 s. However, does this mean that the raft exists over 10 s? Not always. FRET is sensitive to the energy transfer that takes place during the excited state lifetime of the donor molecule, which is generally about 3 ns. If we again assume that rafts are forming and dispersing every 10 and 40 ns (or 10 and 40 μ s or 10 and 40 ms), one cannot differentiate this from the model where 20% and 80% of the donor raft molecules stay forever in (small) stable rafts and in the bulk domain, respectively.

We raise the possibility that the spectrum of the small, transient raft domains are broadly spread in time-space scales, i.e. the sizes and the lifetimes of these rafts may be widely distributed. This may partially explain why the observed sizes and lifetimes of the rafts vary greatly, depending on the employed method. Since different technologies have sensitivities to different time-space scales, each method can pick up only limited sets of rafts in the broad spectrum of the rafts in the time-space scales occurring in the membrane.

One would expect that small rafts with short lifetimes tend to form more often, whereas large rafts with long

lifetimes are likely to be created less frequently. However, one should note that *biologically important events tend to be coupled to rare, but large, fluctuations* (98,99). Therefore, to make the raft work, the balance should be shifted so that greater and more stable rafts could be formed more frequently. We propose that this is exactly what the ligand does to its GPI-AR or raft-preferring transmembrane receptor molecules: by oligomerizing or clustering raft-philic receptor molecules, probably by causing conformational changes in their extracellular domains, such a shift toward the generation of greater, stabilized rafts is induced.

Coupling of GPI-AR Located in the Outer Leaflet, with Lipid-Anchored Signaling Molecules in the Inner Leaflet

GPI-AR molecules span only halfway through the membrane (i.e. they do not reach the cytoplasm), yet they have to transmit the signal from the outside world to the inside of the cell. Interestingly, they are often coupled with lipid-anchored signaling molecules in the inner leaflet of the cell membrane, like some of the Src-family kinases (100), which makes it even harder to couple the GPI-AR with the intracellular signaling network. Since these inner-leaflet signaling molecules are likely to be associated with rafts, many researchers believe that this coupling may take place via rafts, which we call the 'raft hypothesis'. In fact, one of the most important functions of the raft in the cell membrane may be to help with the signal transduction of GPI-AR in the plasma membrane. However, how the raft can achieve such coupling remains unknown.

As described above, the ligation of GPI-AR induces the formation of large, stable rafts (receptor-cluster rafts) in the outer leaflet. Therefore, the problem is how such enlarged, stabilized rafts in the outer leaflet can recruit

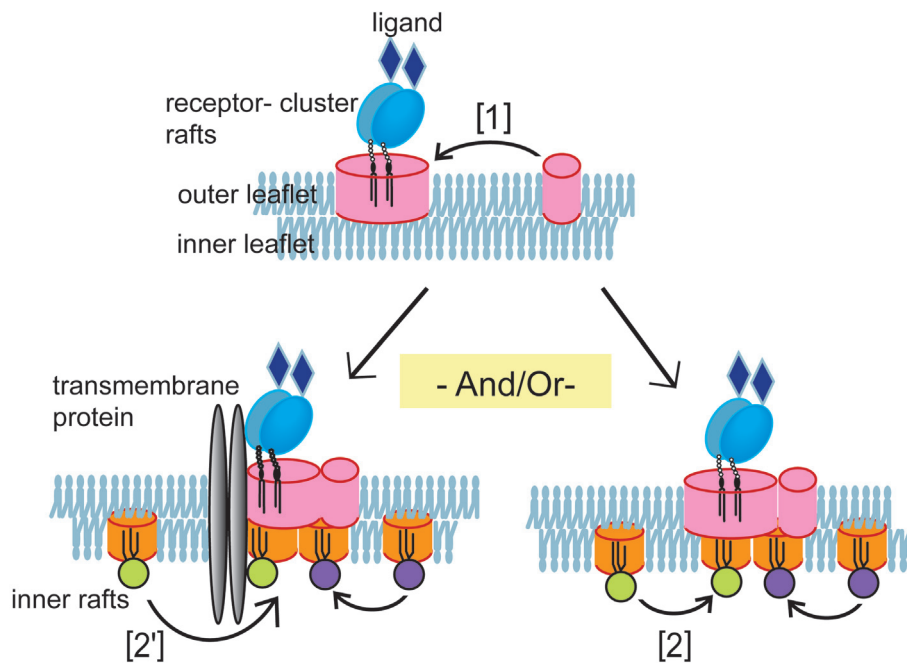


Figure 3: Working hypothesis for the coupling of the outer-leaflet raft with the inner-leaflet, lipid-anchored signaling molecules.

First, ligand binding induces GPI-AR clustering, creating an outer-leaflet stabilized, receptor-cluster raft [1]. The second step may involve the coupling of outer- and inner-leaflet rafts [2] and/or transmembrane proteins with some raft affinity [2']. The raft coupling (the former process 2) may involve diffusional collision of the inner-leaflet raft, which contains lipid-anchored signaling molecules, with the outer-leaflet raft, followed by transient entrapment, probably due to an interaction based on lipid interdigitation. Mediation by transmembrane proteins (the latter process 2') would be initiated by the recruitment of a transmembrane protein with some affinity to the outer-leaflet raft and/or clustered GPI-AR. This affinity would certainly be enhanced by the clustering of GPI-AR and the formation of the stabilized raft. This could lead to recruitment and concentration of the inner-leaflet rafts, which contain signaling molecules that also have some affinity to the transmembrane protein, beneath the outer-leaflet raft. Note that these two processes are not exclusive, but are likely to work synergistically. Also note that the former process [2] would not have any selectivity in the downstream signaling events. In the latter model [2'], the raft coupling aids in the mediation by the transmembrane protein. Although the residency time of inner-leaflet rafts beneath the outer-leaflet receptor-cluster raft may be limited, the inner-leaflet rafts may be somewhat concentrated, which may induce the interactions among the signaling raft molecules on those inner-leaflet rafts, triggering the subsequent intracellular signaling responses.

lipid-anchored signaling molecules beneath them in the inner leaflet. We envisage two possibilities (Figure 3).

First, small, unstable inner-leaflet rafts containing signaling molecules may become associated with an outer-leaflet-stabilized raft perhaps by interactions of interdigitated lipids (Figure 1D (a)), and become somewhat concentrated there. This may induce the interaction of two or more raft molecules in the inner leaflet, leading to intracellular signaling events. The problem with this model is that it lacks specificity in the selection of the downstream signaling pathways, and even ganglioside crosslinking in the outer leaflet could induce signaling, although this has already been observed (23,101).

Second, upon the formation of the stabilized raft in the outer-leaflet, certain raft-philic transmembrane proteins may be recruited there. If this occurs, then the signaling molecules located in the inner leaflet, with affinities for both the cytoplasmic domain of the transmembrane protein and the raft, will be more selectively assembled (although the duration of their stay beneath the outer-

leaflet receptor-cluster raft may be limited). Therefore, it would be interesting to search for transmembrane proteins that mediate (or help to mediate) GPI-AR signal transduction to intracellular signaling molecules. Note that even in such cases where transmembrane proteins are involved, the raft makes an important contribution by assisting in the recruitment of the transmembrane proteins and the inner-leaflet signaling molecules (that have some raft affinity). In the case of a GPI-anchored receptor, $GFR\alpha-1$, which transduces the signal for the glial-cell-line-derived neurotrophic factor (GDNF), a transmembrane protein Ret is known to act as a coreceptor, and to play a key role in signal transduction (102–104).

An important feature of these models is that the interbilayer coupling by the rafts, the recruitment of cytoplasmic lipid-anchored molecules to the coupled rafts, and the molecular interactions there may all occur dynamically and transiently. For the induction of such short-term events, the weak interactions of the outer- and inner-leaflet rafts, mediated by interdigitated lipids, may play key roles.

How the cytoplasmic signaling molecules are recruited and concentrated in receptor clusters is a key problem, which has appeared repeatedly in the studies of many signal transduction pathways in the plasma membrane. Kinases or the receptor kinase domains often play key roles in the signal transduction after receptor clustering, but for some receptors, like many GPI-ARs, the mechanisms controlling the concentration of the kinases in their clusters are totally unknown. The raft hypothesis, in which the raft helps to recruit kinases by lipid-based interactions, may turn out to be the solution for this critical issue (100,105,106). Such a raft mechanism may actually be a general strategy used in the plasma membrane as a critical step in many signal transduction pathways.

Concluding Remarks

We would like to emphasize the importance of always considering the time and space scale of the raft in question. In particular, the observation methods for rafts in live cells all have their specifically sensitive time scales. Arguments promoted without regard to the time–space sensitivities and the limitations of the method being used are not only useless but will turn out to be harmful by increasing confusion in the literature.

The raft has been visualized in most cases after ligating or (unintentionally) crosslinking raft molecules or at lower temperatures. It is critical in raft research to distinguish the small, unstable reserve rafts in the plasma membrane in the steady-state (resting) cells from the induced, greater, stabilized receptor-cluster rafts after stimulation or crosslinking of raft components. Further knowledge of the interactions and miscibilities of membrane constituent molecules will be necessary to understand how the raft size and lifetime are determined, and how the clustering of GPI-AR leads to the formation of stabilized rafts.

One of the key questions regarding GPI-AR signaling is how the signal received by GPI-AR is relayed to the inner-leaflet, raft-philic, lipid-anchored proteins, like some of the Src-family kinases. This signal transfer may be carried out or aided by the coupling of the outer-leaflet, receptor-cluster raft with a small inner-leaflet raft containing lipid-anchored signaling molecules. Such coupling is also likely to take place dynamically and temporarily, and perhaps is mediated by interdigitation of lipids between the two (outer-leaflet and inner-leaflet) raft domains. Further studies must pay special attention to the time scales of the interactions between the outer- and inner-leaflet rafts.

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