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Lipid rafts and plasma membrane microorganization: insights from Ras

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The spatial organization of plasma membrane components in discrete microdomains is thought to be a key factor in the generation of distinct signal outputs. A detailed characterization of plasma membrane microdomains, including descriptions of their size, dynamics and abundance, has proved to be a taxing problem for cell biologists and biophysicists. The use of novel techniques is providing exciting new insights into the challenging problem of plasma membrane microstructure and has allowed the visualization of domains with the characteristics expected of lipid rafts – microdomains of the plasma membrane enriched in cholesterol and sphingolipids. This review focuses on some of these recent advances and uses Ras signaling as a paradigm for understanding inner plasma membrane organization and the role of lipid rafts in cellular function.

The plasma membrane (PM) of eukaryotic cells is composed of hundreds of different lipid species. Increasing evidence suggests a role for these lipid species in the organization of the PM into microdomains and, in particular, a role for cholesterol and sphingolipids in the generation of lipid raft domains [1,2]. The particular properties of lipids in these domains give rise to a liquid–ordered membrane phase, segregated from the bulk disordered phase of the membrane. Consequently, lipid rafts are suggested to act as lipid-based cholesterol-sensitive sorting platforms that can recruit specific components such as acylated proteins anchored to the cytoplasmic surface of the PM [3,4]. As demonstrated by elegant *in vitro* reconstitution experiments [5,6], this sorting mechanism might depend on the specific biophysical properties of particular lipid species. Lipid raft-associated sorting has been proposed to underlie several cellular processes including signal transduction, protein sorting in polarized cells, endocytosis and cell adhesion [7,8]. Lipid rafts might also play an important role in lower eukaryotes, such as yeast, suggesting an evolutionarily conserved role in cell function [9].

Until recently, the study of lipid rafts in cellular membranes has relied on several relatively crude techniques, all of which have limitations. Nonionic detergents are widely used to isolate low-density detergent-resistant domains [10–12]; a specific subset of cellular proteins copurify with these domains, but the validity of assigning these proteins as lipid raft constituents has been criticized on the grounds of specificity and relevance to the *in vivo* situation [13]. Cholesterol depletion is extensively used as a way to specifically disrupt raft domains but can potentially also influence other properties of the membrane [14–16]. Ideally, lipid rafts should be visualized by microscopic techniques under conditions that do not perturb membrane structure. Ultrastructural techniques that allow visualization of specialized PM domains, such as caveolae and clathrin-coated pits, are generally unable to define areas corresponding to lipid rafts, which appear to be indistinguishable from the bulk PM based on morphology alone [17,18]. Light microscopy also fails to yield a consensus on the size and dynamics of lipid rafts, despite a wide range of sophisticated techniques [19–22]. This has led to questions regarding the importance, and even the existence, of lipid rafts in biological membranes [13]. Yet, the lipid raft model provides a plausible and elegant explanation for numerous phenomena that cannot be readily explained by other models. For example, the spatial segregation of signaling components in lipid rafts can readily account for intriguing observations from many experimental systems in which cholesterol and protein acylation play important roles in regulating signal output [8,23,24].

This review focuses on one particular aspect of PM organization – the microdomain organization of the inner, cytoplasmic leaflet of the PM. How several recent studies, using different techniques, have provided key insights into the organization of the PM and the role of raft domains in signaling, is discussed. By focusing on a particular set of proteins, the Ras family of GTPases, the review also demonstrates that some consensus has emerged on the role and properties of lipid rafts and other microdomains involved in signaling pathways.

Analysis of PM microdomains: Ras as a paradigm

The ideal method for studying surface microdomains would yield information on their size, abundance and dynamics, including mobility and residence time of constituent proteins and lipids, to provide a comprehensive framework for understanding how the microdomain functions in time and space. At present, no one technique can provide all this information. Some of the methods used to study the microdomains in the surface of the outer leaflet

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of the PM and a summary of the conclusions from these studies are listed in Table 1. Although the organization of the inner surface of the PM has received less attention, several complementary techniques are now starting to provide new insights into microdomains in the cytoplasmic leaflet. Fluorescence recovery after photobleaching (FRAP) and spatial analysis using electron microscopy are of significance here because they have been applied to the specific area of Ras signaling. In particular, interest has centered on two isoforms of Ras, for which abundant functional data are available.

H-Ras and K-Ras are PM-associated proteins, which are ubiquitously expressed in mammalian cells. These highly homologous proteins interact *in vitro* with the same set of effectors but generate distinct signaling outputs *in vivo* [24]. K-Ras is a more potent activator of Raf-1 than H-Ras, but is a less efficient activator of phosphoinositide 3-kinase [25]. The molecular mechanisms underlying these differences are of considerable biomedical importance, because activating mutations in different Ras isoforms are associated with specific tumor types [24]. H- and K-Ras have identical effector-binding sites. Therefore, biological differences are most probably imparted by the C-termini of the proteins that mediate PM association and differ considerably in amino acid sequence (Figure 1). Both Ras isoforms are farnesylated, but the complete membrane anchor of H-Ras also includes two palmitoyl groups, whereas that of K-Ras includes a polybasic domain of six contiguous lysines. Immediately N-terminal of these well-characterized lipid anchors there are additional differences in the amino acid sequences of Ras isoforms; this region is called the linker domain of the hypervariable region and is located between the anchor and the conserved catalytic domains (Figure 1).

A hypothesis to explain how the Ras isoforms generate distinct signal outputs *in vivo* is that they are localized to distinct subdomains of the PM that regulate their interaction with distinct sets of activators and effectors. Considerable evidence now supports this hypothesis [26–30]. First, Raf activation by H-Ras, but not K-Ras, is sensitive to decreases in PM cholesterol levels, consistent with a role for lipid rafts in H-Ras signaling. Second, cell fractionation experiments show that the PM anchors of H-Ras and K-Ras, when attached to an inert marker protein, such as green fluorescent protein (GFP) – GFP-tH and GFP-tK – (Figure 1) target to distinct surface domains [28]. However, a relatively simple model in which H-Ras and K-Ras reside in raft and nonraft domains, respectively, is ruled out by experiments showing a much more complex behavior of full length H-Ras. Association of H-Ras with low-density membrane fractions is regulated by its GTP-bound state and is also dependent upon the hypervariable-region sequences upstream of the lipid anchor [28,31]. The general conclusion from these biochemical studies is that H-Ras exhibits regulated association with

Table 1. Techniques for studying lipid rafts and other surface microdomains

Technique	Comments	Refs
Flotation of detergent-resistant membranes	Crude technique with many possible artefacts; detergent to lipid ratio important; domains might be artificially generated Selects for limited subset of cellular proteins Weakly interacting proteins might be lost	[40,48]
	Proteomic analysis reveals complex pattern of proteins from different organelles; quantitative analysis of cholesterol-sensitive proteins reveals subset of putative *bona fide* lipid-raft proteins Analysis of isolated membranes cannot be used to determine domain size *in vivo*	[11]
Photonic force microscopy	Specialized technique measuring local viscous drag of single proteins using laser trap; provides information on diffusion rate, size and dynamics of domains Slower diffusion of raft proteins compared with nonraft proteins; cholesterol depletion causes faster diffusion of raft but not nonraft proteins Estimated diameter of cholesterol-sensitive raft microdomains 52 nm; lifetime of minutes	[20]
FRET	Indicates close proximity (<10 nm) of surface markers Conclusions vary regarding evidence for raft domains, size etc. in the outer leaflet of plasma membrane but favor very small rafts Evidence for cholesterol-dependent clustering of acylated probe in the inner leaflet of the plasma membrane	[21,22,39,40,49]
Fluorescence recovery after photobleaching (FRAP)	Measures the diffusion of populations of molecules Evidence for cholesterol-sensitive diffusion of palmitoylated proteins and GPI-anchored proteins	[29,34]
Single particle tracking	High-resolution tracking of GPI-anchored proteins and nonraft lipids in the plasma membrane in real time Evidence for confinement zones restricting diffusion of putative raft and nonraft proteins but similar diffusion rates; conclusion – rafts are small very transient domains that can be stabilized to form larger long-lasting structures	[47,50]

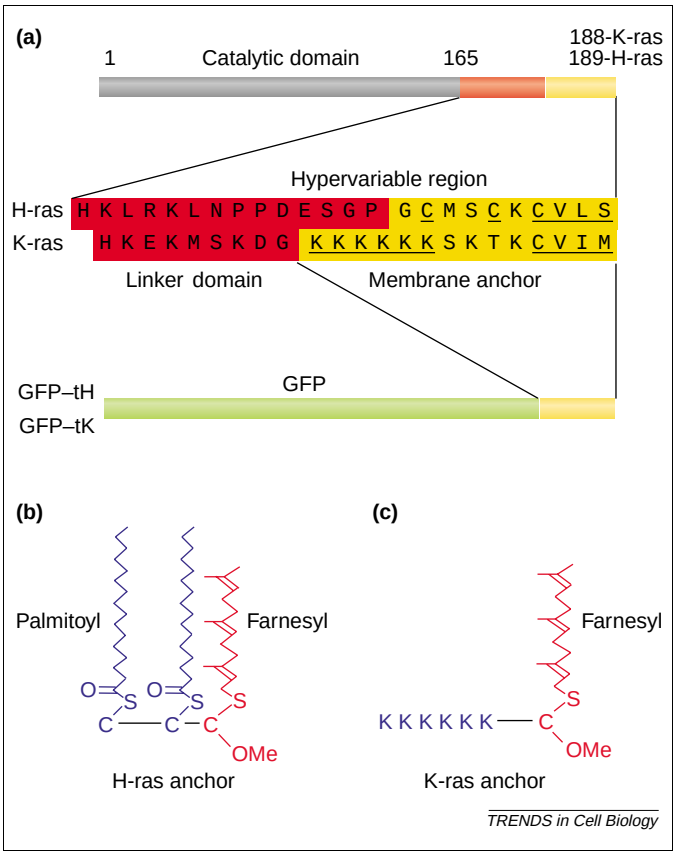

Figure 1. Ras isoforms show differences in C-terminal domains and membrane anchoring. **(a)** The 165 amino acids at the N-termini of H and K Ras (grey) are highly conserved but the 24–25 amino acids at the C-termini of the hypervariable region diverge significantly. The hypervariable region encodes for sequences that direct attachment of the C-terminal anchor (yellow). The protein sequences that direct attachment of the minimal C-terminal anchors of H- and K-Ras, when appended to GFP, target GFP-tH and GFP-tK to lipid rafts and nonraft microdomains, respectively. The adjacent protein sequence of the linker domain (orange) and the N-terminal domains of H-Ras modulate the microdomain interactions of the minimal C-terminal anchor. **(b)** In H-Ras, the C-terminal CAAX motif undergoes farnesylation, proteolytic removal of the three C-terminal amino acids and methylation of the now C-terminal cysteine residue. This is followed by palmitoylation of two upstream cysteines, underlined in (a), to generate the mature H-Ras anchor. **(c)** The K-Ras CAAX motif undergoes the same processing steps as that of H-Ras but the anchor is completed by a polybasic domain, underlined in (a), to generate the mature K-Ras anchor.

the raft domains in the inner leaflet, depending on its GTP/GDP-bound state. Can this conclusion be sustained by techniques that directly visualize Ras–membrane interactions?

Analysis of H-Ras and K-Ras using FRAP

FRAP has been used for many years to study the mobility of fluorescent molecules in living cells. The more recent development of GFP and its derivatives to label proteins of interest [32] has made FRAP an even more useful tool for cell biologists. Fluorescent molecules are irreversibly bleached in a small area by a focused laser beam and the recovery of fluorescence is followed [33]. Two important characteristics can be determined by FRAP – the average mobility of the fluorophore expressed as the diffusion coefficient (D) and the mobile fraction (R_f) estimated from the fractional recovery of fluorescence in a defined time. FRAP also distinguishes between fluorescent proteins that show a stable interaction with the membrane (fluorescence recovery by lateral diffusion), and those that undergo exchange with a cytosolic pool (recovery by exchange). If FRAP occurs by lateral diffusion, the time fluorescence recovery (τ) is proportional to the area illuminated by the beam, whereas if FRAP occurs by dynamic exchange between membrane-bound and cytosolic pools, τ is independent of the beam size.

A recent FRAP analysis showed that GFP-tagged wild-type (WT) H- and K-Ras in their GDP-bound state, or RasG12V, constitutively active GTP-bound Ras generated by a glycine to valine substitution at amino acid 12, associate stably with the PM and had similar rates of lateral diffusion, which were somewhat slower than a freely diffusing lipid probe [29]. On cholesterol depletion, the lateral diffusion of WT-H-Ras increased twofold, whereas that of activated H-RasG12V, WT-K-Ras and activated K-RasG12V was largely unaffected [29]. These results suggest that only WT-H-Ras significantly interacts with cholesterol-dependent surface domains. The FRAP data are therefore entirely consistent with the conclusions from earlier fractionation studies that GDP-bound H-Ras but not activated H-RasG12V or K-Ras interact with lipid rafts [28].

Further important insights were obtained by varying the expression levels of the different constructs. The lateral mobility of K-RasG12V and H-RasG12V increased with increase in their expression levels. The interpretation favored by the authors is that the lateral mobility of activated Ras proteins is retarded by their reversible interactions with saturable binding sites on the PM; when all these sites are occupied at high levels of expression, the mobility of H-RasG12V and K-RasG12V increases. Interestingly, the concentration dependence of D for K-RasG12V and H-RasG12V was different, suggesting that the saturable sites with which K-RasG12V and H-RasG12V interact are not identical [29].

Using similar techniques, the dynamics of raft proteins of the outer leaflet have also been studied [34]. In this study, the authors compared the lateral diffusion of WT influenza hemagglutinin protein (HA), which localizes to lipid rafts through its transmembrane domain, with both an HA mutant that does not associate with lipid rafts and a GPI-anchored form of HA. WT and GPI-anchored HA diffused more slowly than the nonraft HA mutant; however, all three forms showed similar diffusion rates after cholesterol depletion. Next, the authors used antibody crosslinking to patch one type of HA protein and remeasured the lateral diffusion of a coexpressed HA protein. Assuming that the patches are relatively immobile, if the coexpressed protein stably interacts with the patch, this will reduce its R_f, whereas if the interaction is transient then it will predominantly reduce its D. Crosslinking of GPI-HA caused a reduction in the D of WT-HA, whereas crosslinking of WT-HA caused a reduction in the R_f of GPI-HA. Therefore, the interesting conclusions are that WT-HA and GPI-HA interact with the same set of raft domains but the dynamics of their interactions are different and are regulated by the extent of raft clustering.

Box 1. Spatial analysis using electron microcopy

Immunogold labeling of two-dimensional plasma membrane sheets generates point patterns that can be analyzed in a completely unbiased and quantitative manner using spatial statistics (Ripley's K-function) [30,36,37]. This method evaluates all inter-particle distances within the area of interest, calculates the mean number of particles within a range of radii of each particle (e.g. $r = 5–150$ nm) and normalizes the data on the density of the pattern [36]. The K-function, $L(r)$ [51,52] is calculated from the following formula and plotted as $L(r) - r$ against r [30,36,37] (Eqn I).

$$L(r) = \sqrt{\frac{A\sum_{i=1}^{N}\sum_{j=1,i\neq j}^{N} k(i,j)}{\pi N^2}}$$

where N = number of points, A = study area
d(i,j) = the distance between the i^{th} and j^{th} points.
If $d(i,j) \leq r$ then $k(i,j) = 1$, if $d(i,j) > r$ then $k(i,j) = 0$
Border corrections apply to k(i,j) for points close to the edge of the study area.

The null hypothesis is that the point pattern exhibits complete spatial randomness, in which case $L(r) - r$ is zero for all values of r. A positive deviation of $L(r) - r$ from zero and outside of the 99% confidence interval, therefore, indicates that the pattern is not random, but is clustered. The value of r at which the deviation of $L(r) - r$ is maximum indicates the radius of clusters (Figure 2).

Ultrastructural analysis of Ras microdomains

Visualization and quantitative analysis of Ras microdomains at the ultrastructural level would be a considerable step forward in understanding the organization of the PM. In contrast to light microscopy, electron microscopy (EM) can only provide a snap shot of the cell at one particular moment. However, the resolution of EM is far higher than that of light microscopy. In addition, particulate markers can provide the means to gain quantitative insights into domain organization at a molecular level, because these can be analyzed objectively using statistical methods. With the availability of different Ras proteins as molecular markers, which based on FRAP and biochemical data suggest localize to different PM microdomains, is it possible to map these domains of the cell surface by EM?

One method that has directly attempted this is spatial analysis using EM, which relies on the generation of two-dimensional (2D) sheets of PM from cultured cells expressing Ras proteins [28,30,35–37]. By adhering an EM grid to the surface of the cell, the entire dorsal PM can be removed as one continuous sheet. This PM sheet is fixed within seconds of cellular disruption. The PM sheet remains adhered to the grid during the labeling procedure, with cytoplasmic antigens exposed and therefore accessible to labeling. Glutaraldehyde fixation ensures almost immediate immobilization of any proteins and prevents any potential problems associated with crosslinking by multivalent antibodies. This is an important consideration because, without glutaraldehyde fixation, multivalent antibodies can redistribute surface lipid-anchored proteins [18,38]. In addition, there is no detectable mixing of proteins between the inner and outer leaflets of the PM sheets. For example, GPI-anchored GFP (exposed on the outer surface of the cell) could not be labeled with anti-GFP antibodies after preparation of PM sheets when only the cytoplasmic surface of the PM is exposed [30]. Immunogold-labeled PM sheets are analyzed by converting the image into a set of (x,y) coordinates that describes the 2D array of gold particles [30,36,37]. This pattern can be analyzed in a completely unbiased and quantitative manner using spatial statistics (Ripley's K-function) (Figure 2 and Box 1).

Using this technique, the distribution of GFP, anchored to the PM with the targeting domain of H-Ras (GFP-tH), was analyzed in BHK cells labeled with anti-GFP-antibody–gold complexes. The gold labeling showed significant clustering in domains with a diameter of 44 nm (Figure 2) [30]. Mathematical modeling of the

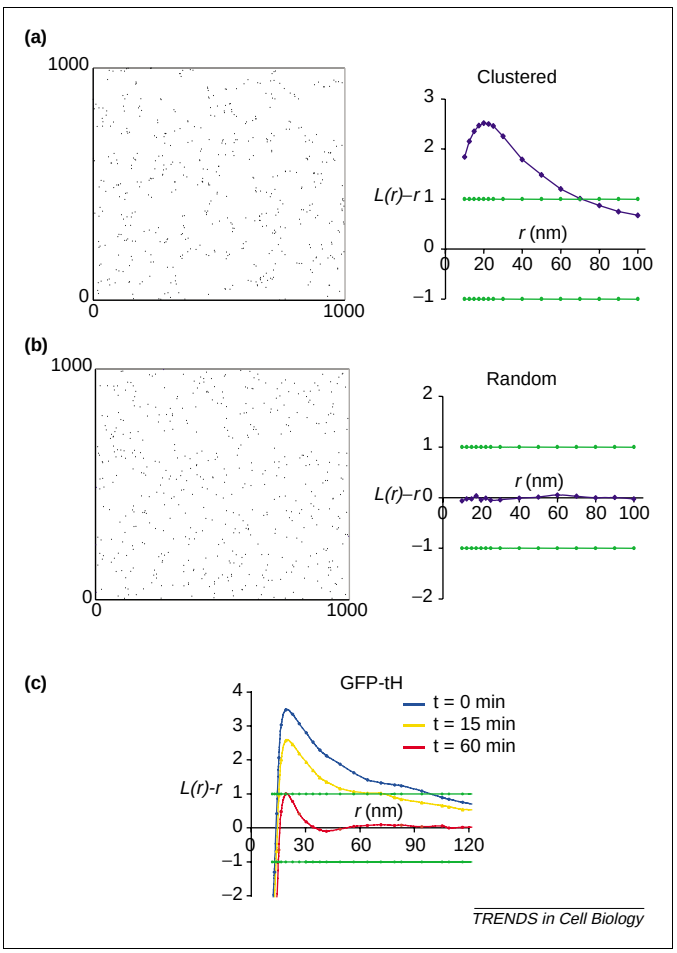

Figure 2. Spatial analysis of immunogold-labeling patterns on plasma membrane 'sheets'. **(a)** Pattern of points corresponding to a realization of a mathematical model of lipid rafts [30]. The rafts, with a mean radius of 22 nm, occupy 35% of the study area (1 μm × 1 μm). 95% of the gold particles label the raft domains and the remaining 5% of the particles are randomly distributed. All features of the gold patterns that are commonly observed on lawns labeled for a raft marker are evident in the modeled pattern including spaces, small clusters and apparent strings of particles [30]. The K-function shows maximal deviation out of the 99% confidence interval for complete spatial randomness (green lines) at a radius of 22 nm, showing that the pattern is significantly clustered. **(b)** A random-point pattern. Note that it is difficult to visually define the pattern as random because small clusters will occur by chance in a true random pattern, where the probability of a particle being at any location in the study area is equal. The K-function clearly shows that the pattern is random because the $L(r) - r$ curve tracks along the x-axis. **(c)** The effect of cholesterol depletion on the distribution of GFP-tH in plasma membrane sheets prepared from BHK cells [30]. There is a time-dependent loss of clustering when cells are incubated with methyl-β-cyclodextrin. The density of labeling for GFP-tH, with anti-GFP antibody coupled to 5-nm gold, was similar in all three experimental conditions (830 gold particles/μm^2). {Panel (c) reproduced from Ref. [30], by copyright permission of The Rockefeller University Press.}

data estimated that the GFP-tH-labeled domains occupied 35% of the PM surface. These values fit well with some theoretical estimates of the size and abundance of lipid rafts in the outer leaflet of the PM [8]; however, is the EM analysis really visualizing lipid rafts? A key experiment suggests that it is. The GFP-tH clusters were completely disrupted upon cholesterol depletion. Concomitantly, there was no change in total density of PM labeling, but the labeling pattern became random (Figure 2) [30]. This result provides evidence for cholesterol-dependent spatial segregation of a lipid-anchored protein. The random distribution of the GFP-tH raft marker after cholesterol depletion is also an important control to show that preparation, fixation and gold labeling of the PM sheet do not induce clustering of the labeled protein *per se*. It also confirms that dimerization of GFP does not artefactually produce a clustered distribution of the probe. Dimerization was a problem for fluorescence resonance energy transfer (FRET) studies of raft markers labeled with yellow and cyan fluorescent proteins (YFP and CFP) [22], although a more recent study did not detect significant dimerization of GFP [39].

The cholesterol-dependent clustering of the dually palmitoylated protein GFP-tH argues for a role of lipid rafts in the organization of the inner-leaflet of the PM. However, although cholesterol depletion has been used extensively as a criterion for a lipid-raft-mediated process, the specificity of this method has been questioned [40]. Undoubtedly, many processes are affected by acute cholesterol depletion. For example, cholesterol depletion can reduce endocytosis and cause a general decrease in the lateral diffusion of membrane proteins [14–16]. Interestingly the reduced mobility of class 1 MHC molecules in cholesterol-depleted fibroblasts and lymphoblasts might in part reflect an effect on the actin cytoskeleton, because lateral mobility is restored by treatment with cytochalasin D, an actin-disrupting agent [16]. A useful aspect of using Ras proteins to probe the structure of the inner leaflet of the PM is that the closely related isoforms H- and K-Ras can be used as reciprocal controls. For example, GFP-tH is unclustered by cholesterol depletion, whereas GFP-tK targeted by the K-Ras membrane anchor (Figure 1) remains clustered when BHK cells are acutely depleted of cholesterol [30]. GFP-tK and GFP-tH differ solely in their membrane anchoring (Figure 1), and only the distribution of the palmitoylated GFP-tH exhibits a cholesterol-dependent change; this is strong evidence for a lipid-based sorting mechanism. In addition, the effect of cholesterol cannot be explained by changes in endocytosis because the labeling density of GFP-tH is the same with or without cholesterol depletion [30], and GFP-tH exhibits minimal constitutive internalization [41]. Thus, overall the available data support the existence of lipid raft domains in the inner surface of the PM. Considerations regarding the possible dimensions of these domains are presented in Box 2 and indicate an encouraging level of agreement with recent studies on the domains of the outer leaflet of the PM using advanced FRET, which predict a maximum cluster size of four GPI-anchored proteins [39].

The extension of the EM approach to double-labeling with different antibodies conjugated to two- and four-nanometer gold particles, which can be analyzed for

Box 2. How many proteins in a raft?

The data from different techniques vary in their estimate of the size of lipid raft domains. Generally, the methods agree that rafts are below the resolution of light microscopy with diameters <100 nm. Studies of the local diffusion of single membrane proteins using a laser-trap and single-particle tracking produced an estimate of domains with a diameter of 52 nm [20]. Such a domain could contain 3500 lipid molecules. At the highest density of membrane protein packing, the domain could contain a maximum of 50–60 membrane proteins. More recent estimates, using advanced FRET to study GPI-anchored proteins, suggest that lipid rafts contain only a few GPI-anchored proteins and might have a diameter that is no larger than 10 nm [39]. How do these estimates compare with electron-microscopy-based spatial analysis of a dually palmitoylated probe in the inner leaflet of the plasma membrane [30]?

Spatial analysis suggests that the radius of the gold clusters is in the range of 20–22 nm for the raft marker GFP-tH (Figure 2c) [30]. However, taking into account the length of the antibody and radius of the gold particle [53], this cluster size could represent labeling of GFP proteins in a raft with a radius anywhere between 7.5 and 22 nm (Figure I). The resolution of the electron microscopical approach can be increased and the spatial uncertainty decreased by using smaller gold particles on shorter spacers.

The K-function analysis also allows the estimation of the mean number of labeled molecules that are clustered in a microdomain (M_c).

If $L_m = \sup_r|L(r) - r|$ for a pattern with a density of λ

and ε = antibody–gold labeling efficiency towards individual molecules

then $M_c = (1 + (L_m + r)^2\ \pi\ \lambda)/\varepsilon$.

Thus, from the data shown in Figure 2c, the estimated mean number of GFP-tH molecules per lipid raft is $\sim$3.35–6.7 when $\varepsilon = 1$ to $\varepsilon = 0.5$, respectively, that is – if the labeling efficiency is in the range of 100 to 50% (ε is high, but experiments are in progress to measure it directly). The results of electron microscopy looking at rafts in the inner leaflet of the plasma membrane, therefore, appear to be in good agreement with the recent FRET methods looking at lipid raft domains in the outer leaflet of the plasma membrane [39].

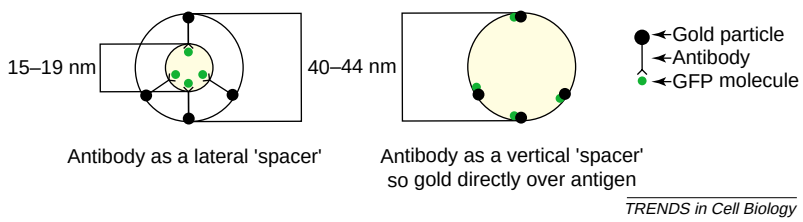

Figure I.

colocalization using a bivariate spatial analysis, allows further conclusions to be made [30]. In particular, the results provide independent support for the hypothesis, based on fractionation [28] and FRAP studies [29], that H-Ras has dynamic interactions with both raft domains (defined by their sensitivity to cholesterol depletion) and distinct nonraft domains (unaffected by cholesterol depletion). WT-H-Ras showed strong colocalization with the raft marker GFP-tH in serum-starved BHK cells, which decreased when cells were treated with serum to stimulate GTP loading of Ras. Constitutively active H-RasG12V showed negligible colocalization with GFP-tH, irrespective of serum treatment [30]. Thus, GTP loading results in a reduced association of H-Ras with lipid rafts.

H-RasG12V and K-RasG12V are both clustered on the PM but in microdomains that are smaller than the estimated size of lipid rafts and are unaffected by cholesterol depletion [30]. The mapping data obtained using EM also concur with the FRAP analysis [29] in showing that the microdomains occupied by H-RasG12V and K-RasG12V are non-overlapping [30]. Determining the nature of these domains undoubtedly will be of great interest in understanding Ras signaling. Interestingly, one player involved in the formation and/or structure of H-RasG12V microdomain appears to be galectin-1, a protein that interacts preferentially with GTP-bound H-Ras [42]. Downregulation of galectin-1 expression reduces, and galectin-1 overexpression increases, clustering of activated H-Ras (J.F. Hancock and R.G. Parton; unpublished) [30]. Ectopic expression of galectin-1 transforms fibroblasts and stabilizes the GTP-bound form of H-Ras [43]. Taken together, these data suggest that a positive feedback cycle might exist, whereby GTP-loaded H-Ras recruits galectin-1 to the PM from a cytosolic pool [42], where it scaffolds H-Ras–GTP in a nonraft microdomain. By contrast, the clustering of K-RasG12V does not require galectin-1, although intriguingly it is sensitive to the length of the prenoid chain attached to the C-terminus [30]. If K-Ras is farnesylated, then it is clustered, but, if it is geranylgeranylated, it does not form clusters. Further work will hopefully elucidate the role of the lipid anchor and polybasic domain interactions in K-Ras association with these domains.

FRET to study microdomains in the inner leaflet of the PM

FRET is a powerful technique to analyze intermolecular interactions in living cells. Energy transfer from an excited donor fluorophore molecule to an acceptor fluorophore results in quenching of the donor channel signal and an increase in the acceptor signal. Because FRET only occurs efficiently between molecules in very close proximity (typically 3–6 nm, efficiency being inversely proportional to the 6^{th} power of the intermolecular distance), it is an excellent technique to study clustering and coassociation of fluorescent markers. This approach was used to investigate the existence of lipid rafts in the inner leaflet of the PM. Analogous to the C-terminal anchor of H-Ras, the N-terminal lipid anchor of the Lyn kinase comprises adjacent myristoyl and palmitoyl groups. The Lyn anchor targets proteins to lipid rafts, at least as defined biochemically [44,45]. CFP and YFP, engineered to abolish inherent dimerization and targeted to the PM by the Lyn anchor, exhibited cholesterol-dependent FRET, consistent with the probes coclustering in lipid rafts [45]. Extrapolating these data and related observations on geranyl-geranylated CFP-YFP FRET pairs into a detailed model of surface organization is not possible at present [46]; however, this FRET study does provide further evidence for the existence of raft domains and illustrates how FRET can be used to probe the organization of the inner surface of the cell.

Concluding remarks

The data from several independent techniques suggest a model in which K-Ras operates in cholesterol-insensitive nonraft domains but H-Ras exists in a dynamic association with raft and nonraft surface domains. Through the use of the techniques described above, we now have a preliminary understanding of the size and abundance of some of these domains and the dynamics of molecules that associate with them. Although the existence of lipid raft domains remains elusive, it is reassuring to see that diverse techniques provide some agreeing data on the abundance and size of lipid rafts in the inner and outer leaflets of the PM (Box 2). We have also gained insights into the mechanisms involved in association with rafts and other domains. Lipid anchors can play a role in localizing molecules to distinct microdomains but this is only part of the story; it is increasingly evident that neighboring protein sequences, and the protein conformation (as in GTP- and GDP-bound forms of H-Ras), can regulate the association of molecules with surface microdomains. The biophysical basis underlying this regulated microdomain localization remains unknown. Dynamic microdomain localization undoubtedly has important implications for understanding how signaling complexes are assembled and disassembled in response to particular stimuli; some components of these signaling complexes might reside permanently in rafts but others could have extremely transient interactions that would be very hard to show using biochemical techniques. In the case of Ras, the detailed characterization of the association of specific effectors and activators to these domains in time and space is now required. This should be facilitated by the use of dynamic methods, such as FRET, together with high-resolution single-particle tracking [47]. In addition, it remains to be seen whether other lipid-anchored small GTPases will show similar regulation of microdomain localization in response to their GDP- or GTP-bound state. The Ras system provides a basic framework for understanding microdomains in the inner leaflet of the PM but how these microdomains are linked to the raft domains of the outer leaflet is now an important question. The challenge ahead is to integrate the information from diverse techniques into quantitative models of how the two leaflets of the PM are coordinated and organized into dynamic signaling domains.

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References

1 Brown, R.E. (1998) Sphingolipid organization in biomembranes: what physical studies of model membranes reveal. *J. Cell Sci.* 111, 1–9
2 Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* 387, 569–572
3 Morris, R.J. *et al.* (2003) Rafts, little caves and large potholes: how lipid structure interacts with membrane proteins to create functionally diverse membrane environments In *Membrane Dynamics and Domains* (Quinn, P.J., ed.), Kluwer Academic/Plenum Publishers
4 Melkonian, K.A. *et al.* (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. *J. Biol. Chem.* 274, 3910–3917
5 Brown, D.A. (2001) Seeing is believing: visualization of rafts in model membranes. *Proc. Natl. Acad. Sci. U. S. A.* 98, 10517–10518
6 Dietrich, C. *et al.* (2001) Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc. Natl. Acad. Sci. U. S. A.* 98, 10642–10647
7 Brown, D.A. and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* 14, 111–136
8 Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1, 31–39
9 Bagnat, M. and Simons, K. (2002) Lipid rafts in protein sorting and cell polarity in budding yeast *Saccharomyces cerevisiae*. *Biol. Chem.* 383, 1475–1480
10 Brown, D.A. and Rose, J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68, 533–544
11 Foster, L.J. *et al.* (2003) Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. U. S. A.* 100, 5813–5818
12 Schuck, S. *et al.* (2003) Resistance of cell membranes to different detergents. *Proc. Natl. Acad. Sci. U. S. A.* 100, 5795–5800
13 Munro, S. (2003) Lipid rafts. Elusive or illusive? *Cell* 115, 377–388
14 Rodal, S.K. *et al.* (1999) Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell* 10, 961–974
15 Subtil, A. *et al.* (1999) Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6775–6780
16 Kwik, J. *et al.* (2003) Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc. Natl. Acad. Sci. U. S. A.* 100, 13964–13969
17 Fra, A.M. *et al.* (1994) Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem.* 269, 30745–30748
18 Mayor, S. *et al.* (1994) Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* 264, 1948–1951
19 Varma, R. and Mayor, S. (1998) GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* 394, 798–801
20 Pralle, A. *et al.* (2000) Sphingolipid–cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol.* 148, 997–1008
21 Kenworthy, A.K. and Edidin, M. (1998) Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of
22 Zacharias, D.A. *et al.* (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296, 913–916
23 Van Laethem, F. and Leo, O. (2002) Membrane lipid rafts: new targets for immunoregulation. *Curr. Mol. Med.* 2, 557–570
24 Hancock, J.F. (2003) Ras proteins: different signals from different locations. *Nat. Rev. Mol. Cell Biol.* 4, 373–384
25 Yan, J. *et al.* (1998) Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J. Biol. Chem.* 273, 24052–24056
26 Matallanas, D. *et al.* (2003) Differences on the inhibitory specificities of H-Ras, K-Ras, and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization. *J. Biol. Chem.* 278, 4572–4581
27 Roy, S. *et al.* (1999) Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. *Nat. Cell Biol.* 1, 98–105
28 Prior, I.A. *et al.* (2001) GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. *Nat. Cell Biol.* 3, 368–375
29 Niv, H. *et al.* (2002) Activated K-Ras and H-Ras display different interactions with saturable nonraft sites at the surface of live cells. *J. Cell Biol.* 157, 865–872
30 Prior, I.A. *et al.* (2003) Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J. Cell Biol.* 160, 165–170
31 Jaumot, M. *et al.* (2002) The linker domain of the Ha-Ras hypervariable region regulates interactions with exchange factors, Raf-1 and phosphoinositide 3-kinase. *J. Biol. Chem.* 277, 272–278
32 Tsien, R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* 67, 509–544
33 Reits, E.A. and Neefjes, J.J. (2001) From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat. Cell Biol.* 3, E145–E147
34 Shvartsman, D.E. *et al.* (2003) Differently anchored influenza hemagglutinin mutants display distinct interaction dynamics with mutual rafts. *J. Cell Biol.* 163, 879–888
35 Parton, R.G. and Hancock, J.F. (2001) Caveolin and Ras function. *Methods Enzymol.* 333, 172–183
36 Prior, I.A. *et al.* (2003) Observing cell surface signaling domains using electron microscopy. *Sci STKE* 2003, PL9
37 Wyse, B.D. *et al.* (2003) Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. *J. Biol. Chem.* 278, 23738–23746
38 Parton, R.G. *et al.* (1994) Regulated internalization of caveolae. *J. Cell Biol.* 127, 1199–1215
39 Sharma, P. *et al.* Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* (in press).
40 Edidin, M. (2003) The state of lipid rafts: from model membranes to cells. *Annu. Rev. Biophys. Biomol. Struct.* 32, 257–283
41 Roy, S. *et al.* (2002) H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. *Mol. Cell. Biol.* 22, 5128–5140
42 Paz, A. *et al.* (2001) Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation. *Oncogene* 20, 7486–7493
43 Elad-Sfadia, G. *et al.* (2002) Galectin-1 augments Ras activation and diverts Ras signals to Raf-1 at the expense of phosphoinositide 3-kinase. *J. Biol. Chem.* 277, 37169–37175
44 Field, K.A. *et al.* (1995) Fc epsilon RI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. *Proc. Natl. Acad. Sci. U. S. A.* 92, 9201–9205
45 Zacharias, D.A. (2002) Sticky caveats in an otherwise glowing report: oligomerizing fluorescent proteins and their use in cell biology. *Sci STKE* 2002, PE23
46 Kenworthy, A. (2002) Peering inside lipid rafts and caveolae. *Trends Biochem. Sci.* 27, 435–437
47 Subczynski, W.K. and Kusumi, A. (2003) Dynamics of raft molecules in the cell and artificial membranes: approaches by pulse EPR spin labeling and single molecule optical microscopy. *Biochim. Biophys. Acta* 1610, 231–243
48 Heerklotz, H. (2002) Triton promotes domain formation in lipid raft mixtures. *Biophys. J.* 83, 2693–2701
49 Kenworthy, A.K. *et al.* (2000) High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. *Mol. Biol. Cell* 11, 1645–1655
50 Dietrich, C. *et al.* (2002) Relationship of lipid rafts to transient confinement zones detected by single particle tracking. *Biophys. J.* 82, 274–284
51 Ripley, B.D. (1977) Modelling spatial patterns. *J. R. Stat. Soc.* B39, 172–192
52 Besag, J.E. (1977) Contribution to the discussion of Dr. Ripley's paper. *J. R. Stat. Soc.* B39, 193–195
53 Amit, A.G. *et al.* (1986) Three-dimensional structure of an antigen–antibody complex at 2.8 A resolution. *Science* 233, 747–753