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Structural determinants of protein partitioning into ordered membrane domains and lipid rafts

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ABSTRACT

Increasing evidence supports the existence of lateral nanoscopic lipid domains in plasma membranes, known as lipid rafts. These domains preferentially recruit membrane proteins and lipids to facilitate their interactions and thereby regulate transmembrane signaling and cellular homeostasis. The functionality of raft domains is intrinsically dependent on their selectivity for specific membrane components; however, while the physicochemical determinants of raft association for lipids are known, very few systematic studies have focused on the structural aspects that guide raft partitioning of proteins. In this review, we describe biophysical and thermodynamic aspects of raft-mimetic liquid ordered phases, focusing on those most relevant for protein partitioning. Further, we detail the variety of experimental models used to study protein-raft interactions. Finally, we review the existing literature on mechanisms for raft targeting, including lipid post-translational modifications, lipid binding, and transmembrane domain features. We conclude that while protein palmitoylation is a clear raft-targeting signal, few other general structural determinants for raft partitioning have been revealed, suggesting that many discoveries lie ahead in this burgeoning field.

1. Introduction

Biological membranes are relatively impermeable barriers between aqueous compartments, with membrane-spanning proteins representing the central mechanism for transport of materials and signals across the membrane. Such transmembrane proteins comprise approximately 30% of the human genome (Wallin and von Heijne, 1998), underlining their functional ubiquity. An early model of cellular membranes described membrane proteins as freely diffusing in a two-dimensional solvent of bilayer lipids (Singer and Nicolson, 1972). Since then, a plethora of experimental observations have amended this model to provide a more complex picture of membrane protein organization. Most of these measurements have focused on the plasma membrane, both because it is the major site for extracellular signal transduction and because it is the only membrane readily accessible to external labeling and observation. A major takeaway is that very few proteins distribute homogeneously in the plasma membrane. Some – including GPI-anchored proteins (Sharma et al., 2004; Suzuki et al., 2012) and Ras GTPases (Prior and Hancock, 2011; Zhou et al., 2013) – appear to form small, dynamic oligomers. Others show free diffusion on short length/time scales, but remain corralled by a membrane-associated cytoskeleton (Kusumi et al., 2005).

In addition to these, one of the most widely studied mechanisms for organizing the plasma membrane are lipid-driven membrane domains known as lipid rafts. These structures are believed to arise from preferred interactions between saturated lipids, glycosphingolipids, sphingomyelin, and cholesterol that give rise to a sterol-dependent liquid ordered phase (L_o) which can coexist with a liquid disordered (L_d) phase under physiological conditions (Lingwood and Simons, 2010). Proteins and lipids partitioning to this phase would then interact preferentially with each other, thereby spatially confining signaling reactions. Despite a growing body of evidence to support the hypothesis that lipid interactions drive domains in live cells, direct observation remains extremely difficult due to the purported size (tens to hundreds of nanometers) and time (millisecond lifetimes) scales of the putative domains. However, recent developments in isolated plasma membranes have confirmed that liquid–liquid phase coexistence is accessible in biological membranes and that its behavior is consistent with many aspects of the raft hypothesis (Kaiser et al., 2012; Levental and Levental, 2015a,b).

Perhaps the key feature underlying the functionality of lipid rafts is their selectivity for specific proteins. Despite this importance, very few studies have experimentally addressed the

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molecular mechanisms by which this selectivity is mediated. In this review, we will elaborate the characteristics of lipid rafts that may influence protein partitioning, discuss experimental models and techniques for investigation of raft association, and attempt an inclusive overview of the known mechanisms for protein partitioning to ordered membrane domains. Finally, we will address the physicochemical bases behind these results to provide mechanistic, structural insights in the determinants of protein partitioning to lipid rafts.

2. Characteristics of the liquid ordered phase relevant for protein partitioning

The features that bias proteins for preferential partitioning to raft domains are likely those that impart preferential interactions with either the unique lipid composition or physical environment of membrane rafts (Fig. 1A). The L_o phase of synthetic membranes is the most well accepted model for such domains, and the unique properties of this phase have been extensively characterized.

2.1. Structure and composition of the L_o phase

In synthetic systems, and also in more complex cell-derived membranes (Levental et al., 2009), the formation of the L_o phase depends on the unique structural properties of sterols (cholesterol in mammalian membranes) and their interactions with diacyl membrane lipids. In fluid membranes, the rigid, planar ring of cholesterol (and other sterols) inhibits trans-gauche isomerization of lipid acyl chains, enforcing more extended lipid conformations. This acyl chain ordering effect leads to a reduction of lipid molecular area and thickening of the membrane (reviewed in (Rog et al., 2009; Rog and Vattulainen, 2014)). Conversely, cholesterol fluidizes the lipid gel phase (L_β) by intercalating between lipids, with the methylated β-side of the molecule forcing apart closely packed phospholipids. Certain compositions permit the formation of a distinct liquid phase with properties intermediate between the gel and liquid crystalline state, termed the liquid ordered (L_o) phase. The detailed physicochemical interactions that drive the formation of a L_o phase are only partly understood. Interactions between cholesterol and saturated acyl chains have been shown to be energetically favored over interactions with unsaturated acyl chains (Almeida, 2009). Hydrogen bonding between the hydroxyl group of cholesterol and the amide group of sphingolipids might stabilize such preferential interactions, favoring the formation of ordered assemblies (Rog et al., 2009). Other effects, like the umbrella effect induced by the large headgroups of glycolipids could shield hydrophobic cholesterol and thereby contribute to preferential sterol–lipid interactions (Huang and Feigenson, 1999). Finally, stoichiometric 'condensed complexes' of phospholipids and cholesterol have been proposed based on the non-linear reduction of lipid molecular area induced by cholesterol (Radhakrishnan and McConnell, 1999).

In model membranes, the L_o and Lα phases coexist at thermodynamic equilibrium through a large range of lipid compositions and temperatures (Brown and London, 1998; London, 2005). Such behavior can be directly observed by conventional fluorescence microscopy (Korlach et al., 1999; Veatch and Keller, 2003) and atomic force microscopy (Garcia-Saez et al., 2007), or inferred from NMR (Heberle et al., 2013) or FRET (Pathak and London, 2011) data. Despite being reliant on cholesterol for its formation, the L_o phase is believed to be modestly enriched in cholesterol (Feigenson and Buboltz, 2001; Veatch et al., 2006); rather, strong enrichments are expected for saturated lipids and sphingolipids (Niemela et al., 2009; Rog and Vattulainen, 2014).

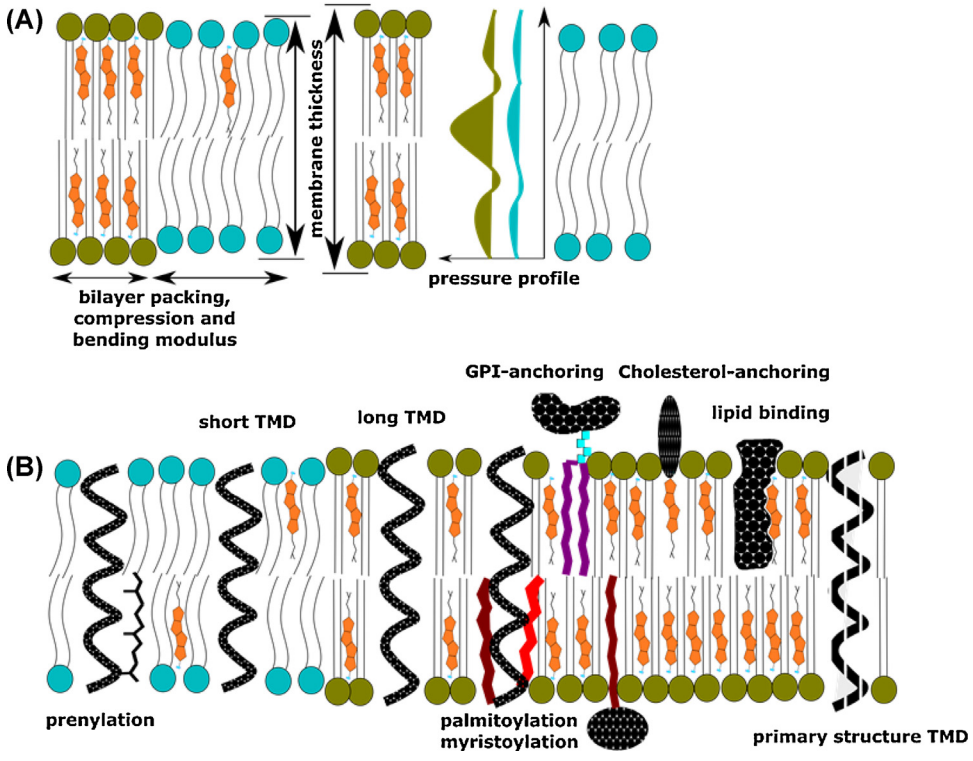

Fig. 1. Biophysical determinants of raft partitioning. (A) Ordered (raft-like) phases in biomimetic and biological membranes are distinguished from disordered (non-raft) by a variety of biophysical characteristics, including their compressibility, bending modulus, hydrophobic thickness, and transbilayer pressure profile. (B) Proteins preferentially interact with one of these phases by a variety of mechanisms, including matching the transmembrane domain length to the thickness of the membrane, post-translational saturated lipid modifications that impart order phase affinity, and specific binding of raft lipids, among others.

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2.2. Diffusion rate

Lipids in ordered phases are more tightly packed, leading to lower diffusivity of lipids and proteins. Differential lateral diffusion between coexisting phases has been measured by NMR (Filippov et al., 2004) and fluorescence correlation spectroscopy (FCS) (Bacia et al., 2004) in model membranes, and confirmed in natural membranes (Levental et al., 2009). These studies converge on diffusion rates approximately 3-5-fold slower in ordered compared to disordered domains. In live cells, such measurements are complicated by a variety of factors, including membrane topology (Adler et al., 2010), membrane traffic, and interaction with cytoskeletal elements (Gowrishankar et al., 2012) and other proteins. Nevertheless, recent super-resolution FCS studies in live cells suggest that sphingolipids possess distinct diffusion behavior from glycerophospholipids, potentially reflective of raft-mediated confinement (Eggeling et al., 2009).

2.3. Membrane thickness

As mentioned above, cholesterol forces the saturated acyl chains of sphingomyelin and phospholipids into a more extended conformation, which leads to an increase of the bilayer thickness. This effect has been confirmed by AFM on supported planar bilayers (Garcia-Saez et al., 2007; Oreopoulos and Yip, 2009), neutron scattering in liposomes (Heberle et al., 2013), and atomistic simulations (Niemela et al., 2007). Moreover, the lipid composition of the bilayer influences membrane thickness, most notable in the effect of longer acyl chains increasing membrane thickness (Lewis and Engelman, 1983). The differences in membrane thickness are compelling in light of previous observations of membrane thickness differences between various subcellular organelles (Mitra et al., 2004). These differences have been proposed to aid segregation of membrane proteins to their intended cellular location, by matching the length of a particular transmembrane segment to the thickness of the appropriate cellular membrane (Sharpe et al., 2010; Diaz-Rohrer et al., 2014 #1576).

2.4. Transmembrane pressure profile

The pressure profile of a membrane can be understood as the depth-dependent distribution of lateral stresses on a probe molecule (e.g., a transmembrane protein) (Cantor, 1999). These pressures, and their gradients through the bilayer normal, can be quite significant and likely affect protein conformations. Although difficult to measure experimentally, these transmembrane pressures have been calculated by computational simulations, and suggest that ordered domains have distinct profiles from non-raft membranes (Niemela et al., 2007, 2009). These differences suggest that changing protein partitioning between coexisting membrane domains may be sufficient to induce a conformation/activity change (Fig. 1A).

2.5. Elastic properties

The bulk mechanical properties of a membrane can be defined by three different types of elasticity: shear elasticity, stretching elasticity and bending resistance (Helfrich, 1973). The latter two are defined by the compressibility (κ_a) and bending modulus (κ_b), respectively. Model membranes and atomistic simulations have shown that the compressibility modulus is greater in ordered membranes because of tighter lipid packing (Needham and Nunn, 1990; Niemela et al., 2009). This effect is due not only to the condensing effect of cholesterol, but also to interfacial hydrogen bonds between sphingomyelin molecules and cholesterol. This greater compressibility modulus can be interpreted to suggest that it would require more work to create a cavity (e.g., for protein insertion) in an ordered/raft domain compared to a non-raft membrane. This effect has indeed been observed for melittin, where a higher compressibility modulus associated with the liquid ordered phase was responsible for excluding the peptide from the bilayer (Allende et al., 2003).

Analogous to the compressibility modulus, the bending modulus of ordered phases is also likely higher than that of the liquid crystalline phase (Evans and Rawicz, 1990; Niemela et al., 2009), i.e., ordered membranes are both more difficult to stretch and to bend than disordered membranes. The effect of cholesterol on increasing bending modulus has also been observed in lipids extracted from red blood cell plasma membranes (Meleard et al., 1997). Thus, it is likely that both protein insertion and protein-generated induction of curvature would require more energy in raft-like ordered domains compared to more disordered ones.

2.6. Caveats of the liquid-ordered model for membrane rafts

It is important to emphasize here the limitations in applying the inferences from experiments on liquid ordered phases in synthetic model systems directly to membrane rafts in live cells. The most important of these are that while the compositions of synthetic membranes are often chosen to be 'biomimetic', they are extremely simplified compared to eukaryotic plasma membranes, which can contain hundreds of different lipid species at various concentrations. Moreover, biological membranes are extremely protein rich, with erythrocyte membranes cross-sectional areas comprised of ~23% transmembrane polypeptide (Dupuy and Engelman, 2008). Most of the physical characterizations above were performed in protein-free membranes, and it is almost certain that biologically relevant polypeptide levels would influence many of these properties, possibly in unexpected ways. In addition to these caveats, ordered domains in model systems are long-lived and often macroscopic, while rafts in plasma membranes of living cells are hard to detect directly, possibly because they are transient and nanoscopic. Moreover, in contrast to synthetic systems, the living membrane is not at thermodynamic equilibrium, with energy consuming processes constantly modifying the shape, composition, and environment of the membrane.

3. Experimental systems to investigate protein partitioning between membrane domains

Because of the difficulties associated with detecting and quantitatively measuring raft properties and compositions directly in cellular membranes, most studies to date have relied on a variety of model membranes and indirect methods to infer protein partitioning to raft domains. In this section, we describe several of the most commonly used experimental paradigms.

3.1. Liposomes

Lipid liposomes have been, and remain, the stalwart membrane model systems due to their ease of handling, tight control over composition and size, and methodological flexibility. They can be produced from purified lipid components or from lipid extracts obtained directly from biological membranes, though it is important to stress that such 'reconstituted' membranes lack the proteins that comprise a major fraction of cellular membranes. The majority of studies of liquid ordered/disordered coexistence have either been performed in microscopic Giant Unilamellar Vesicles (GUVs; >1 μm diameter; formed by electroswelling or gentle hydration), Large Unilamellar Vesicles (LUVs; 100 nm–1 μm diameter; formed by extrusion), or Small Unilamellar Vesicles

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(SUVs; ~30 nm diameter; formed by sonication). Phase separation in GUVs is easily observable by fluorescence microscopy, allowing direct measurement component partitioning between coexisting phases (Kahya et al., 2005; Shogomori et al., 2005; Sezgin et al., 2012a,b, 2015). Further, GUVs can be manipulated after formation to produce highly curved tubules, for example to study the effects of membrane curvature on protein binding (Roux et al., 2005; Tian and Baumgart, 2009; Aimon et al., 2014). Smaller vesicles have been probed by X-ray scattering to determine conformational changes upon membrane binding (Lee et al., 2014), electron microscopy to study membrane tubulation by curvature generating proteins (Shi and Baumgart, 2015) or molecular motors (Roux et al., 2002), and circular dichroism to study the effects of membranes on protein secondary structure (Aoki and Epand, 2012). An important technique for evaluation of partitioning in LUVs is Förster Resonance Energy Transfer (FRET), which measures the molecular proximity (interpreted as co-partitioning) between a protein of interest and a well-characterized marker for a particular membrane domain (Lin and London, 2013).

3.2. Supported planar bilayers (SPB)

Supported planar bilayers are usually prepared on a hydrophilic support like mica, treated glass or silicon, providing an important advantage over free-floating vesicles of being flat therefore easy to analyze by techniques like TIRF and AFM (Oreopoulos and Yip, 2009). They are usually prepared by depositing lipid monolayer films or fusing synthetic lipid liposomes on a planar hydrophilic surface (Kalb et al., 1992; Puu and Gustafson, 1997). SLBs can be imaged microscopically to study the aggregation state of proteins and determine their raft partitioning in a phase-separated membrane (Milhiet et al., 2002; Saslowsky et al., 2002). Another interesting approach is to study the lateral molecular composition of bilayers by Time of Flight Secondary Ion Monitoring (Tof-SIMS) (Kraft et al., 2006; Zheng et al., 2008). Modern instruments can detect sub-microscopic lipid domains, in addition to detailing the molecular composition of those membranes (Vaezian et al., 2010), and this approach has recently been extended to live cells to study both lipid (Frisz et al., 2013) and protein (Wilson et al., 2015) distributions.

3.3. Detergent resistant membranes (DRM)

Detergent resistant membranes were the first, and remain the most common, method to infer raft association in cells. DRMs are produced by extracting live cells with cold, non-ionic detergent (Lingwood and Simons, 2007), but can also be prepared from isolated membranes and synthetic liposomes (Sengupta et al., 2008; Lin and London, 2013). In model membranes, the L_o phase is not extracted under these conditions (Ahmed et al., 1997), implying that un-extracted material from cellular solubilization under the same conditions is reflective of a similar liquid ordered membrane present in live cells. Consistently, DRMs are enriched in stereotypical raft components, including cholesterol, sphingolipids, and GPI-anchored proteins (Brown and Rose, 1992).

3.4. Giant plasma membrane vesicles (GPMVs) and plasma membrane spheres (PMS)

Giant plasma membrane vesicles are large, spherical plasma membrane projections that detach from a variety of cell types after treatment with a cysteine-alkylating chemical (e.g., *N*-ethylmaleimide (NEM) or formaldehyde) in calcium-containing buffer (Scott, 1976; Levental and Levental, 2015a,b). These GPMVs are part of the plasma membrane and therefore contain a representative sampling of the lipids and proteins therein. Because of their large size (up to 10 μm in diameter), they are easily observable by light microscopy (Sezgin et al., 2012a,b; Levental and Levental, 2015a,b). Most importantly, at certain temperatures, GPMVs separate into coexisting liquid ordered and liquid disordered phases (Baumgartet al., 2007; Sezgin et al., 2012a,b; Levental and Levental, 2015a,b). This capacity provides a powerful tool to study protein partitioning to ordered domains in biological membranes—a close proxy for lipid rafts in vivo. GPMVs can be prepared from cells transfected with a plasmid encoding a protein with a fluorescent tag, whose raft partition coefficient can then be directly quantified by fluorescence microscopy (Sengupta et al., 2008; Johnson et al., 2010; Levental et al., 2010a,b; Diaz-Rohrer et al., 2014). Plasma membrane spheres are similar to GPMVs, except that they are prepared without chemical treatments and require cross-linking of glycolipids by CTxB to observe macroscopic domains (Lingwood et al., 2008).

3.5. Live cells

Because of the proposed dynamic and nanoscopic nature of lipid rafts in cellular membranes, it is difficult to directly assess protein raft partitioning in living cells. A common technique is to study low-resolution co-localization of fluorescently labeled proteins with a known raft marker, such as the glycolipid ganglioside GM1 (usually labeled by CTxB) or caveolin. This approach is prone to misinterpretation and is unlikely to provide meaningful data because the putative raft domains are far smaller than the resolution of the light microscope and general staining of the PM is likely to yield artifactual co-localization with PM resident proteins. A variation involves crosslinking the membrane surface with antibodies, which generates large-scale patches on the surface of the cells (Harder et al., 1998). These patches appear to be selective for certain membrane components, and so may reveal inherent raft affinity; however, it is not known how such crosslinking may affect the native partitioning. A number of more advanced microscopic techniques have been applied to study membrane domains in live cells. Examples include hetero-FRET (Engel et al., 2010) and homo-FRET (Varma and Mayor, 1998), FRAP (Meder et al., 2006; Kenworthy, 2007), super-resolution FCS (Eggeling et al., 2009; Sezgin et al., 2012a,b), two-photon microscopy of order-sensitive dyes (Gaus et al., 2003), single particle tracking (Kusumi and Suzuki, 2005), and optical tweezers (Pralle et al., 2000).

3.6. Comparisons and caveats of different membrane models

Obviously, the most relevant information about the cell membrane is gleaned from measurements in live cells. However, interpretation of such experiments is inherently confounded by the complications of live cell membrane topology, composition, dynamics, etc. Thus, most studies have relied on the model systems described above. Unfortunately, there are often disagreements between the different systems, likely driven by distinct caveats associated with each. The major caveats and controversies associated with DRMs have been detailed elsewhere (Lichtenberg et al., 2005; Brown, 2006), so we will only emphasize that results from DRM experiments cannot provide definitive evidence of raft partitioning and must be verified independently. DRMs include many more proteins than enriched ordered phases in model membranes, a difference that remains poorly understood and is discussed in detail in Levental and Levental (2015a,b). For example, GUVs very rarely show protein enrichment in the raft phase. This includes predicted raft proteins, like the linker for activation of T cells (LAT) (Shogomori et al., 2005), which does prefer ordered domains in GPMVs (Levental et al., 2010a,b). This discrepancy between GUVs and GPMVs might be explained by the differences

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in order between their L_o/L_d phases, as the order of the L_d phase of GPMVs (and PMS) is much higher than those of GUVs (Kaiser et al., 2009). The resulting difference between the phases is therefore much lower in the natural compared to the synthetic systems, with this difference affecting probe partitioning between phases (Sezgin et al., 2012a,b, 2015). Although GPMVs are the most biomimetic model membranes, these come with their own set of potential artefacts. The isolation chemicals may reduce palmitoylation of membrane proteins, crosslink and/or otherwise modify proteins non-specifically (Levental et al., 2011), native PM asymmetry is generally lost in GPMVs (Baumgart et al., 2007), there is no assembled cytoskeleton, and GPMVs are at thermodynamic equilibrium. Ultimately, there is as yet no perfect plasma membrane model system, and the strongest results are those that corroborate most closely between synthetic, natural, and in vivo membranes.

4. Structural determinants of protein partitioning to raft domains

As mentioned, protein partitioning to raft domains has mainly been inferred from their association with DRMs. More recently, this field has expanded into quantitative measurements in GPMVs. As pointed out above, the various experimental modalities have their limitations and do not always agree; nevertheless, a few general raft-targeting features can be identified (Table 1 and Fig. 1B).

4.1. Protein lipidation

Of all other factors, lipid conjugation of proteins seems to be the most widespread and consistent factor determining raft partitioning. The various lipid post-translational modifications and specifically their effect on protein partitioning have been extensively reviewed previously (Levental et al., 2010a,b), so below we include only a cursory overview.

4.1.1. Glycophosphatidylinositol (GPI)-anchored proteins.

GPI anchors are synthesized in the endoplasmic reticulum, covalently attached to proteins in the ER lumen, and subsequently delivered via the Golgi network to the exoplasmic leaflet of the plasma membrane. GPI anchors are constituted of a phosphatidylinositol, coupled via a glucosamine, three mannose residues and a phosphoethanolamine group to the C-terminus of the protein via amide bond. While the two acyl chains of the lipid anchor can be unsaturated or saturated, they are most often saturated (Yu et al., 2013). GPI-anchored proteins (GPI-APs) were some of the first to be identified in DRMs, with detergent resistance acquired only after trafficking through the trans-Golgi network, suggesting that it was not protein-intrinsic, but rather a function of membrane environment (Brown and Rose, 1992). Antibody clustering of GPI-APs induces large PM patches that recruit other putative raft associated proteins (Harder et al., 1998). Also, GPI-APs clearly enrich in raft domains of both GPMVs (Levental et al., 2010a,b) and GUVs (Kahya et al., 2005), and associate with ordered LUVs (Benting et al., 1999). It appears that without exogenous clustering, GPI-APs exist in cells as small oligomers (Brameshuber et al., 2010), possibly because of their residence in membrane domains, although alternate explanations rely on active cytoskeletal self-organization and/or actin corrals (Suzuki et al., 2007; Goswami et al., 2008; Gowrishankar et al., 2012). Nevertheless, it is clear that across model systems GPI-anchors direct proteins to raft domains.

Table 1
Overview of protein raft partitioning determinants.

Raft partitioning mechanism	Protein example	Membrane association	Raft partitioning	References
Myristate	HIV-1-Nef	Cytosolic	No DRM association without myristoylation	(Wang et al., 2000)
	MyrAkt	Cytosolic	Only the myristoylated form is associated with DRMs	(Adam et al., 2007)
Palmitate	Src-family tyrosine kinases Fyn and Lck	Cytosolic	Some excluded from L_o phase in GPMVs, whereas doubly palmitoylated Lck showed some ordered phase partitioning	(Pyenta et al., 2001; Baumgart et al., 2007; Johnson et al., 2010)
	$G\alpha_i$	Cytosolic	53% of palmitoylated/myristoylated $G\alpha_i$ was associated with DRMs from a raft mixture	(Brown et al., 2000)
	LAT	Transmembrane	Loss of palmitoylation excluded LAT from DRMs and reduced raft-partitioning in GPMV by 74% ($K_{p,raft}$ 1.7 → 0.44)	(Zhang et al., 1998; Levental et al., 2010a,b)
	HA	Transmembrane	Mutation of the palmitoylation sites reduced DRM association by 58%. Also FRET suggests association with Myr-Pal-YFP and GPI-CFP. No consensus on ordered phase partitioning in GPMVs	(Chen et al., 2005; Scolari et al., 2009; Engel et al., 2010; Nikolaus et al., 2010)
GPI-anchor	Thy-1	Exoplasmic	Thy-1 is associated at ~80% with the raft phase of GPMVs	(Baumgart et al., 2007)
	GFP-GPI	Exoplasmic	GPI-GFP K_p,raft = 1.5–2	(Goswami et al., 2008; Sengupta et al., 2008; Johnson et al., 2010; Zhou et al., 2013)
	Acetylcholine esterase (ACE)	Exoplasmic	In SPB composed of DOPC/brain SM/cholesterol (1:1:1) 41% of GPI-ACE partitioned into the Lo phase	(Garner et al., 2007)
Sterol anchor	Hedgehog (Hh)	Transmembrane	–	(Mao et al., 2009; Shi et al., 2013)
Transmembrane domain primary structure	CD40, CD44, CD154	Transmembrane	CD154 DRM association diminished by 40% when TMD was mutated	(Perschl et al., 1995; Bock and Gulbins, 2003; Benslimane et al., 2012)
	HA	Transmembrane	Mutation in the middle of TMD reduced DRM association from 38 to 2.2%	(Scheiffele et al., 1997; Lin, et al., 1998)
Transmembrane domain length	LAT	Transmembrane	Decreasing TMD length by 6 residues (~1/4 of TMD) decreased $K_{p,raft}$ from ~1.1 to 0.6	(Diaz-Rohrer et al., 2014)
	PFO	Multi-span TM toxin	In GUVs, increasing TMD length by two residues increased $K_{p,raft}$ from 3.48 to 5.44 Reducing TMD length by 2 residues completely eliminated ordered partitioning	(Lin and London, 2013)
Cholesterol binding motif	Gp41	Transmembrane	–	(Schwarzer et al., 2014)

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4.1.2. Palmitoylation

The conjugation of a saturated palmitic acid to a protein cysteine is called *S*-palmitoylation. Originally discovered in viral proteins (Berger and Schmidt, 1984), modern proteomic techniques have identified hundreds of proteins modified by palmitoylation (Kang et al., 2008; Martin and Cravatt, 2009) involved in a slew of functions including signaling, trafficking, recycling, apoptosis, and protein stability (Kummel et al., 2006; Linder and Deschenes, 2007; Song et al., 2013; Diaz-Rohrer et al., 2014; Rossin et al., 2014). Palmitoylation differs from other lipid modifications in two important ways: it is reversible and it modifies many proteins with transmembrane domains. Moreover, there is a wealth of literature showing that palmitoylation targets proteins to DRMs (reviewed in Levental et al. (2010a,b)), and the important and wide-spread role of palmitoylation as a signal for raft association was definitively confirmed in GPMVs (Levental et al., 2010a,b). These observations are intriguingly suggestive of a possible role for palmitoylation in the dynamic regulation of transmembrane protein partitioning to raft domains. Physicochemically, it is facile to suggest an explanation for the effect of palmitoylation on raft partitioning: the saturated acyl chain has high affinity for the more ordered environment of the lipid raft. However, this hypothesis has not been formally demonstrated.

4.1.3. Myristoylation

Another saturated fatty acid modification is the addition of a 14-carbon myristoyl residue to the N-terminal glycine of certain proteins by *N*-myristoyltransferase, or NMT (Farazi et al., 2001). In general, myristoylation occurs co-translationally and remains with the protein throughout its lifetime, mediating attachment of otherwise cytoplasmic proteins to membranes. There is an exception, in that post-translational cleavage of proteins by proteases (e.g., caspases during apoptosis) can uncover an N-terminal glycine, which can become myristoylated (Zha et al., 2000). Myristoyl-dependent DRM association has been shown for several different proteins, including Src, the HIV protein Nef, and annexin A13b (Mukherjee et al., 2003; Djordjevic et al., 2004; Turnay et al., 2005). However, in general, myristoylation is insufficient for raft targeting. Instead, a number of proteins are simultaneously myristoylated and palmitoylated, with this dual acylation efficiently promoting DRM affinity for proteins including Gα subunits, Src-family tyrosine kinases, reggie-1/flotillin-2, BAALC 1-6-8, UL-11 and TXNRD1-v3 (Moffett et al., 2000; Mukherjee et al., 2003; Neumann-Giesen et al., 2004; Wang et al., 2005; Koshizuka et al., 2007; Cebula et al., 2013). In surprising – and as yet unexplained – contrast with these results, myristoylated/palmitoylated constructs often do not partition efficiently into the L_o phase of GPMVs (Baumgart et al., 2007; Sengupta et al., 2008; Johnson et al., 2010).

4.1.4. Prenylation

The isoprenoid modifications – farnesylation and geranylgeranylation – are likely antagonistic for raft partitioning. This is likely due to the resistance of the branched and bulky prenyl group to insert into tightly packed raft-domains (Melkonian et al., 1999). Some proteins (e.g., H-Ras and N-Ras) bear both raft-preferring (palmitate) and raft-avoiding (prenyl) lipidations. It has been proposed that this combination may confer affinity to the interface between raft and non-raft domains (Weise et al., 2009), with a potential role for these proteins as line-active modifiers of domain separation (Trabelsi et al., 2008).

4.1.5. Sterol-conjugation

The only known proteins covalently modified by a cholesterol residue are the Hedgehog (Hh) family. Endoproteolytic cleavage precedes cholesterol addition via ester linkage to the glycine of the C-teminus, while the N-terminus becomes palmitoylated through a peptide bond (Mann and Beachy, 2000). This modification allows Hh proteins to associate with DRMs (Rietveld et al., 1999).

4.2. Protein transmembrane domain (TMD) features

The proteinaceous, hydrophobic, membrane-inserted domains of integral membrane proteins are a critical determinant of raft partitioning (Scheiffele et al., 1997; Lucero and Robbins, 2004; Benslimane et al., 2012). As pointed out above, the lipid ordered environment is not optimally suited for the insertion of transmembrane polypeptides. This is especially true in most synthetic model membrane systems, where the order difference between the raft and non-raft phase is relatively high (Kaiser et al., 2009; Schafer et al., 2011; Sezgin et al., 2015). Thus, it is unsurprising that most proteins are excluded from raft-mimetic domains in GUVs, and even in the more natural GPMVs (Brown, 2006; Sengupta et al., 2008; Levental et al., 2011), though it should be noted that several proteins do show significant L_o phase enrichment in GPMVs (Levental et al., 2010a,b; Diaz-Rohrer et al., 2014). Below we review the scant literature regarding transmembrane domain features that impart raft affinity.

4.2.1. Primary structure.

Most such studies have been performed by mutagenesis of TMDs in single-pass transmembrane proteins, assaying detergent resistance as a proxy for raft association. A consistent finding is that the TMD itself can be an independent determinant of raft partitioning, as TMD chimeras typically follow the partitioning of the TMD, not the host protein (Perschl et al., 1995; Scheiffele et al., 1997; Bock and Gulbins, 2003; Benslimane et al., 2012). Alanine scanning mutations of the TMD of influenza hemagglutinin (HA) revealed that mutations of the exoplasmic half induced the loss of detergent resistance, independent of protein palmitoylation (Scheiffele et al. 1997; Lin et al., 1998). The net charge of the TMD also influenced protein partitioning for Lck constructs in GPMVs (Johnson et al., 2010). The mechanisms behind either of these observations are currently unclear. However, structural features – e.g., the GxxxG oligomerization motif (Russ and Engelman, 2000) – can reside in single-pass transmembrane α-helices, so it is not unthinkable that a raft-partitioning motif remains to be discovered.

4.2.2. Transmembrane domain length.

Liquid ordered phases are usually thicker than the liquid disordered regions. Proteins also have a wide distribution of transmembrane domain lengths (Sharpe et al., 2010). Thus, it stands to reason that to minimize the hydrophobic mismatch between the lipid membrane and the polypeptide, proteins with longer TMDs would preferentially partition into the thicker raft domains. This attractive hypothesis has recently received convincing experimental support in both synthetic and natural membranes. In GPMVs, it was shown that the length of a protein's transmembrane domain was strongly and quantitatively correlated with raft phase partitioning, with this effect being quite general among the four single-pass transmembrane proteins assayed. A linear correlation between the length of TMDs of 11 variants of a model single-pass protein and the raft partitioning coefficient ($K_{p,raft}$) was also established. By decreasing the length of the transmembrane domain from the native 24 amino acids to 18 (~0.9 nm for an α-helix), the raft partition coefficient decreased from 1.1 to ~0.65 (Diaz-Rohrer et al., 2014).

As an aside, alanine mutations in the TMD had little or no effect on ordered phase partitioning, in contrast to the hemagglutinin studies cited above. The most exciting aspect of this study was the observation that raft partitioning had a clear cellular readout, with

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raft preferring constructs localizing to the plasma membrane while non-raft mutants were internalized and degraded via endo/lysosomes (Diaz-Rohrer et al., 2014). This TMD length result in GPMVs mirrors an elegant set of experiments in liposomes, in which Perfringolysin O (a β-barrel pore forming toxin) was clearly shown to partition between domains based on the length of the membrane spanning region (Lin and London, 2013).

4.2.3. Interactions with membrane lipids.

Proteins bind membranes through a wide variety of specific interactions with membrane lipids. It is also possible that some of these interactions with raft-preferring lipids would specifically target these proteins to ordered membrane domains. Recently, a proteome-wide analysis revealed 250 cholesterol-binding proteins, including a variety of membrane-embedded enzymes, channels and receptors (Hulce et al., 2013) that may bind cholesterol via their transmembrane domain. An exciting example of this effect was recently shown for the amyloid precursor protein (APP), whose cholesterol-binding domain involves a GxxxG motif that has been previously implicated as a helix–helix oligomerization motif (Russ and Engelman, 2000; Barrett et al., 2012). The implications of this surprising finding remain to be resolved, but it is possible that cholesterol plays a crucial role in the many GxxxG-mediated protein–protein interactions, perhaps by recruiting proteins to raft domains. Cholesterol binding by GxxxG echoes the more recognized cholesterol recognition amino acid consensus, or CRAC, motif (Li and Papadopoulos, 1998; Baier et al., 2011). CRAC motifs are ubiquitous, largely because they are relatively loosely defined (L/V-X_{1-5}-Y-X_{1-5}-(R/K)), though few have been directly shown to interact with cholesterol (Song et al., 2014). Nevertheless, CRAC motifs in several proteins have been implicated in raft partitioning (Li and Papadopoulos, 1998; Epand, 2006; Schwarzer et al., 2014; Ruysschaert and Lonez, 2015), suggesting that specific cholesterol binding may be one way to 'lubricate' a protein for raft association.

Finally, because of their high concentration in lipid rafts, binding to sphingolipids like sphingomyelin and more complex glycosphingolipids might also influence raft recruitment (Fantini, 2003). Examples of specific binding between transmembrane domains and sphingolipids were recently demonstrated for p. 24 (Contreras et al., 2012) and the EGF receptor (Coskun et al., 2010), although a direct role in raft recruitment has not yet been demonstrated.

5. Conclusion

Although the specific determinants of protein partitioning to lipid rafts have been identified in a few isolated cases, no general mechanisms have yet emerged. In part, this is because too few proteins have been analyzed in detail. Additionally, previous studies have relied on different experimental modalities that may be probing different aspects of membrane domain association. For example, it is possible that DRMs recruit all proteins that bind intact membranes remaining after detergent solubilization, whereas L_o phases in GUVs only select proteins with very high ordered domain affinity and miss those which require specific protein-lipid or protein–protein interactions. For the purpose of evaluating raft affinity, we believe GPMVs are the best available system because they maintain the complexity of biological membranes while yielding direct quantitative partitioning information (Levental and Levental, 2015a,b). Moreover, generating and testing variants is simple, with modern DNA synthesis technologies allowing affordable and rapid production of dozens of sequence variants, which can be synthesized, transfected and assayed on the time frame of days. Important caveats of this system (discussed above) must be considered, but it is our hope that continued detailed analysis of raft partitioning mechanisms for transmembrane proteins will soon yield general insights that can be applied to the entire proteome toward a clear picture of the protein composition of membrane rafts.

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