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REVIEW

SUBJECT COLLECTION: LIPID BIOLOGY

The role of lipid rafts in vesicle formation

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ABSTRACT

The formation of membrane vesicles is a common feature in all eukaryotes. Lipid rafts are the best-studied example of membrane domains for both eukaryotes and prokaryotes, and their existence also is suggested in *Archaea* membranes. Lipid rafts are involved in the formation of transport vesicles, endocytic vesicles, exocytic vesicles, synaptic vesicles and extracellular vesicles, as well as enveloped viruses. Two mechanisms of how rafts are involved in vesicle formation have been proposed: first, that raft proteins and/or lipids located in lipid rafts associate with coat proteins that form a budding vesicle, and second, vesicle budding is triggered by enzymatic generation of cone-shaped ceramides and inverted cone-shaped lyso-phospholipids. In both cases, induction of curvature is also facilitated by the relaxation of tension in the raft domain. In this Review, we discuss the role of raft-derived vesicles in several intracellular trafficking pathways. We also highlight their role in different pathways of endocytosis, and in the formation of intraluminal vesicles (ILVs) through budding inwards from the multivesicular body (MVB) membrane, because rafts inside MVB membranes are likely to be involved in loading RNA into ILVs. Finally, we discuss the association of glycoproteins with rafts via the glycocalyx.

KEY WORDS: Endocytic vesicles, Enveloped virus, Exosomes, Extracellular vesicles, Membrane, Rafts, Synaptic vesicles, Transport vesicles

Introduction

The formation of membrane vesicles is a common feature across bacteria, *Archaea* and eukaryota (Rothman, 2002; Makarova et al., 2010; Toyofuku et al., 2019). However, the mechanism of vesicle formation and involvement of molecular factors vary between them. Eukaryotic cells contain different types of vesicles enclosed by a membrane, including cell organelles, such as vacuoles and lysosomes (although they are not typically considered vesicles, they are actually giant vesicles), as well as vesicles that mediate transport within the cell, secretory vesicles and extracellular vesicles (O'Brien et al., 2020). Membrane lipid rafts, whose role in the vesicle formation is discussed in this article, are domains found in both eukaryotes and prokaryotes (Bramkamp and Lopez, 2015); however, studies on model membranes suggest they also exist in *Archaea* membranes (Chong et al., 2022). In this Review, we present the current knowledge on the interactions of molecules within rafts and the cellular roles of rafts, as well as methods to study them. We will then focus on a range of vesicles, including transport vesicles, synaptic vesicles (SVs), intracellular vesicles and extracellular vesicles (EVs), and discuss how rafts contribute to their formation, as well the role of rafts in the formation of enveloped viruses.

Composition and formation of membrane lipid rafts

The lipid rafts hypothesis, the idea that there are specific lipid microdomains with the selective incorporation of certain protein, was proposed in 1997 (Simons and Ikonen, 1997), and at a later conference on the topic, the following definition of membrane rafts was suggested: "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" (Pike, 2006). The following steps in raft formation can be considered (Sezgin et al., 2017a; Trybus et al., 2022 preprint): relatively unstable nanoscale raft precursors within the membrane, namely, mostly oligomeric protein–lipid complexes, could associate into larger ($\sim\geq$20 nm in diameter), more stable ($\tau_{1/2}$>1 s), and possibly detergent-resistant nanodomains, also known as membrane/lipid 'rafts' and more precisely, resting-state rafts; further clustering of the latter leads to formation of micrometer scale 'raft platforms', for example, immunological synapses. The presence of rafts was confirmed in animal cells (Furukawa et al., 2022) and in yeast (Hurst and Fratti, 2020), whereas in plant cells, a biochemical counterpart of membrane rafts was found (Yu et al., 2020) in which sterol and sphingomyelin are replaced by phytosterol and glycosyl inositol phosphorylceramide, respectively. Initially, the presence of rafts was postulated only in the plasma membrane. However, over time, similar ordered domains were also found in the intracellular membranes of organelles, such as the Golgi, endoplasmic reticulum (ER) and early endosomes (Wang et al., 2020b). It is important to note that not all membrane domains are lipid rafts; membrane lipids can additionally form, for instance, non-liquid ordered domains, a two-dimensional crystalline phase known as a gel phase or a solid-ordered phase (Wang et al., 2020b).

A number of studies have addressed the question of what percentage area is occupied by rafts in membranes. Depending on cell type, temperature and activation status, the area of domains in the liquid-ordered phase in membranes can vary significantly, and these domains have been found both in the inner and outer leaflet of the membrane (Owen et al., 2012; Levental et al., 2020). Using single-particle microscopy, the raft surface was estimated to cover 13% of the entire membrane surface (Schutz et al., 2000), whereas fluorescence lifetime imaging microscopy (FLIM) indicates that the ordered lipid fraction can occupy up to 76% of the membrane surface (Owen et al., 2012). The extracellular leaflet predominantly includes sphingolipids with saturated hydrocarbon chains and cholesterol molecules located in between, whereas the cytoplasmic leaflet contains phospholipids with both saturated and unsaturated fatty acids (Levental et al., 2020; Wang et al., 2020b). Rafts are platforms for various cellular processes, regulating the activity of membrane-associated molecules (Lingwood and Simons, 2010). For instance, rafts have been shown to be involved in membrane trafficking (El-Sayed and Harashima, 2013; Hanzal-Bayer and Hancock, 2007), cell signaling (Gupta and DeFranco, 2003; Dinic

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et al., 2015; Lyu et al., 2019), cell metabolism and migration (Gomez-Mouton et al., 2001; Denz et al., 2017), and pathogen invasion and disease (Van der Goot et al., 2004; Kawabata et al., 2009; Raghu et al., 2010); some of these topics are discussed below.

Interactions within rafts

Here, we discuss the physicochemical principles underlying raft formation and stabilization, that result from interactions between lipid molecules and between lipid and protein molecules.

Lipid–lipid interactions

The van der Waals interactions between saturated fatty acid chains of sphingolipids and cholesterol (Fig. 1B) play a crucial role in the formation of lipid rafts (Hirano et al., 2022). The interaction between cholesterol and sphingolipids is also stabilized by hydrogen bonds formed between the amide group in sphingolipids and the 3-hydroxyl of cholesterol (Ramstedt and Slotte, 2002). In addition, hydroxy and amide groups at the hydrophilic–hydrophobic interface of sphingolipids can donate and accept hydrogen bonds with neighboring sphingolipids (Hirano et al., 2022). However, although these interactions are sufficient for the formation of a lipid domain in a liquid-ordered state, which is equivalent to raft-like regions in model lipid membranes, under physiological conditions, lipid raft formation also results from interactions between the lipids and proteins that constitute the biological membrane (Sezgin et al., 2017a; Levental et al., 2020).

Although initially membrane rafts were considered to contain mainly cholesterol, sphingomyelin and glycosylphosphatidylinositol (GPI)-anchored proteins (Simons and Ikonen, 1997), there is growing evidence that the majority of glycosphingolipids are enriched in these lipid domains; therefore, rafts can also be defined as a glycolipid-enriched microdomain (Furukawa et al., 2022). A dynamic reorganization of cholesterol, sphingomyelin and negatively charged monosialoganglioside GM1, commonly associated with rafts, provides direct evidence for an attractive interaction among these raft components into a cluster (Lozano et al., 2016).

Lipid–protein interactions

Based on analyses of the protein composition of detergent-resistant membranes (DRMs), a database of proteins that preferentially localize to the raft region was established (Shah et al., 2015). These proteins either localize to rafts through their transmembrane domain, or are peripherally associated via protein–protein or protein–lipid interactions, or are linked to them through post-translational modifications, such as addition of a GPI anchor, palmitoylation or myristoylation (Fig. 1) (Levental and Lyman, 2023).

Some proteins, such as caveolin, can bind to cholesterol directly (Levental and Lyman, 2023), which promotes their location at rafts, whereas others such as p24 (also known as TMED2; part of the COPI coat of membrane-bound transport vesicles) can directly interact with N-stearoyl sphingolipids through a motif within its transmembrane domain (Contreras et al., 2012). Proteins that are anchored in the membrane by lipids localize in accordance with the tendencies of their anchoring lipids. Thus, saturated lipid anchors, such as GPI or palmitoyl groups, enable proteins to be incorporated into the raft regions of the membrane, whereas unsaturated or branched anchors preclude the association of proteins with lipid rafts (Levental et al., 2010). Furthermore, non-raft transmembrane glycoproteins outside of the raft region, for example, polysialylated neural cell adhesion molecule 1 (NCAM1), can be linked to the raft region through direct interactions between their oligosaccharide chain (e.g. a long polysialic acid chain) and lipids in liquid-ordered state within raft domain (Sapoń et al., 2019) or through indirect interactions with raft glycosphingolipids via the glycocalyx (Fig. 1).

The importance of the protein transmembrane domain for targeting to a lipid raft has also been noted (Sych et al., 2022; Levental and Lyman, 2023), including the role of protein motifs for specific lipid binding. The effect of lipid rafts on intracellular protein trafficking and their localization using the linker for activation of T-cells (LAT) protein has been studied (Diaz-Rohrer et al., 2014). By systematic mutation of the transmembrane domain (TMD) and juxtamembrane domains of LAT, a panel of mutants with different affinities for lipid rafts has been created, pointing to the importance of palmitoylation, the length of the transmembrane domain and the protein amino acid sequence for association with rafts. Moreover, plasma membrane localization of LAT was found to be strongly dependent on raft phase partitioning across all the mutants, suggesting that the association with rafts is necessary and sufficient for LAT sorting to the plasma membrane (Diaz-Rohrer et al., 2014). Another study found that the cytoplasmic tail and the cholesterol recognition amino acid consensus (CRAC) membrane domains of the gp41 protein of HIV have intrinsic properties that promote its partitioning to lipid rafts (Schwarzer et al., 2014). The CRAC domain is also required for gp41 protein oligomerization at the plasma membrane, suggesting that oligomers are preferentially formed and stabilized in the raft region, thus enabling virus assembly at the plasma membrane of infected cell. A correlation of transmembrane protein partitioning to raft domains with the combined factors of TMD surface area, palmitoylation and length has been demonstrated (Lorent et al., 2017).

Another type of interaction important in modulating membrane organization is that between actin filaments and membrane. Research results indicate the possibility of coupling actomyosin to the intracellular leaflet of the membrane (Fig. 1), leading to membrane segregation into corrals and the formation of a stabilizing domain, even with relatively low connectivity to the membrane (Levental et al., 2020). In addition, active processes arising from actomyosin can induce the transient accumulation of actin-binding membrane proteins and lipids (Koster et al., 2016), and actin cortex remodeling in cells might control dynamic rearrangements of lipids and other molecules inside domains without directly binding to actin filaments (Vogel et al., 2017). Although initially phosphatidylserine (PS) (with a single negative charge) was thought to be the major lipid responsible for adaptor protein binding to membranes (Sezgin et al., 2017a), further studies have revealed that these proteins preferentially bind to lipids with double or triple negative charges [phosphatidylinositol bisphosphate (PIP2) and phosphatidylinositol trisphosphate (PIP3)] compared with PS, phosphatidylinositol (PI) or monophosphorylated phosphoinositide (PIP) (Senju et al., 2017). From these, PI(4,5)P2 is the most abundant at the plasma membrane, and directly regulates the activities and subcellular localizations of numerous adaptor proteins (both actin- and membrane PIP2-binding proteins), such as cofilin and profilin proteins (Senju and Lappalainen, 2019). PIP2 with saturated fatty acyl chains localizes preferentially in lipid rafts (Edwards-Hicks et al., 2023).

Relationship between rafts and membrane stiffness and bending

Membranes have several features, such as a high elastic modulus (compressibility modulus), as a result of the small stretch in bilayers, a variable viscosity depending on membrane composition, a low shear modulus due to the fluid nature of the lipid bilayer and a bending stiffness (bending rigidity, resistance to bending) that is

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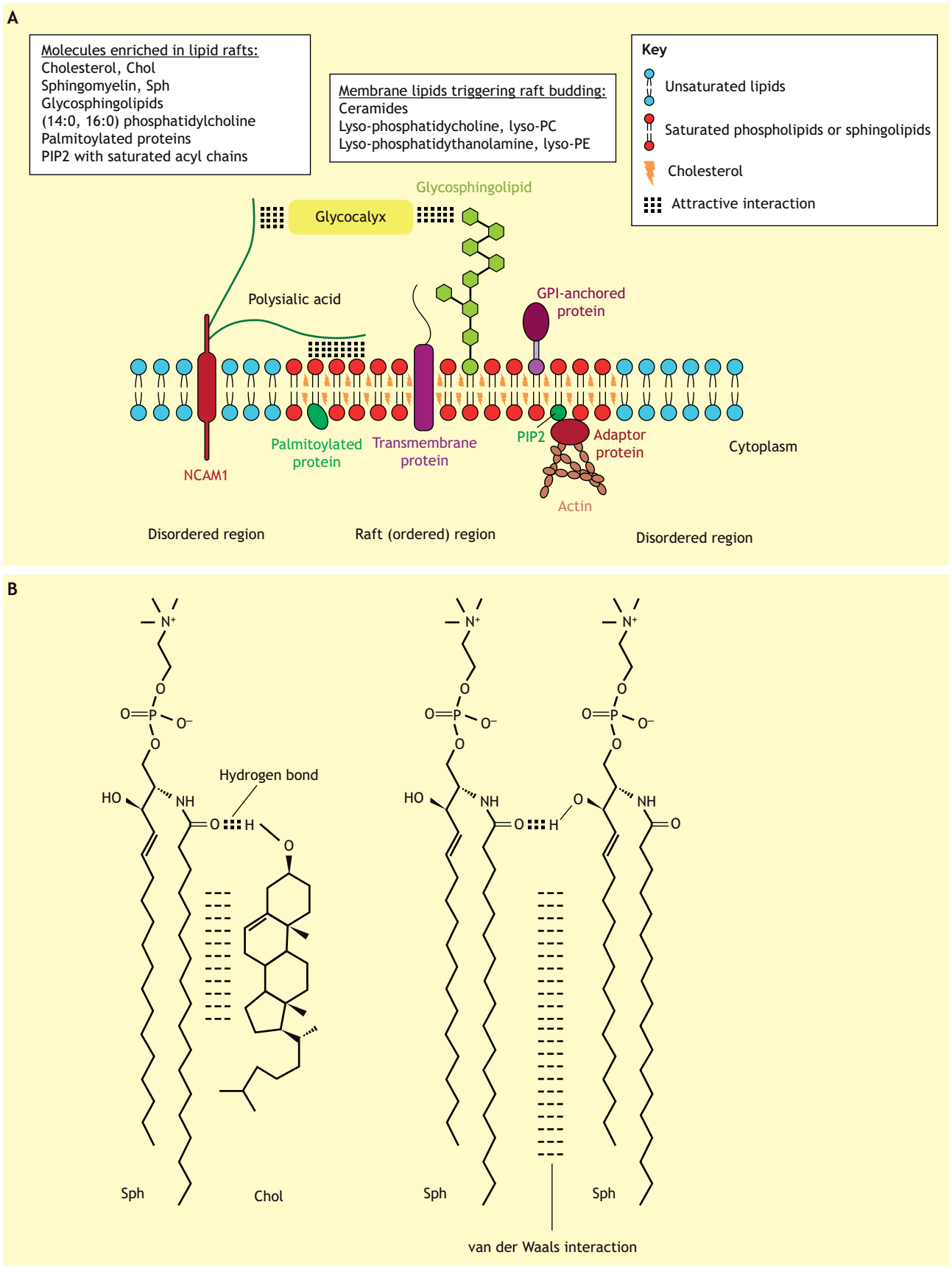

Fig. 1. See next page for legend.

Fig. 1. Plasma membrane with raft domain. (A) Lipid rafts are microdomains in the plasma membrane, characterized by more densely packed lipids in a liquid-ordered state and enriched with cholesterol and glycolipids, which have the ability to selectively interact with certain proteins. For example, palmitoylated proteins, transmembrane proteins with a cholesterol-binding motif and GPI-anchored proteins localize to rafts. Rafts can also interact with cytoskeletal actin through an adaptor protein (e.g. cofilin or profilin) that possess PIP2-binding domains and actin-binding domains. Raft domains can concentrate certain molecules, forming signaling platforms. Transmembrane glycoprotein outside of the raft region (for example polysialylated NCAM1) can be linked to raft region through interactions of their oligosaccharide chain (for example polysialic acid) with the lipid raft domain, or through indirect interactions with raft glycosphingolipids via the glycocalyx. (B) Interactions between cholesterol and sphingolipids, and between neighboring sphingolipids.

influenced by cholesterol, membrane proteins and cytoskeletal elements (Himbert and Rheinstädter, 2022). Cholesterol, a major component of rafts, locally increases the bending rigidity of both unsaturated and saturated lipid membranes, owing to changes in molecular packing (Doole et al., 2022); therefore, rigid membranes require higher energetic inputs during membrane vesiculation. Cholesterol not only effectively stiffens lipid bilayer membranes, but also induces membrane fusion when either the vesicle or the planar membrane is cholesterol rich (Chng et al., 2022).

Observations of rafts by microscopy

The small size of rafts (up to 200 nm) renders them unobservable using standard methods of optical microscopy owing to the diffraction limit of light microscopy. However, recently developed high-resolution techniques have made it possible to observe membranes at a higher resolution. Lipid rafts and actin-induced membrane compartmentalization have been studied by single-molecule imaging techniques and Förster resonance energy transfer (FRET) microscopy (Suzuki and Kusumi, 2023). For instance, stimulated emission depletion (STED) microscopy has been used to detect single lipid molecules within nanometer-sized areas of the plasma membrane in living cells; this allowed to study of individual spots of the membrane 70 times below the diffraction barrier, and demonstrated that sphingolipids and GPI-anchored proteins, unlike phosphoglycerolipids, are temporarily (~10–20 ms) trapped in 20 nm diameter cholesterol-mediated molecular complexes (Eggeling et al., 2009), thus suggesting that lipid rafts persist over this time frame in the plasma membrane of studied living PtK2 cells. FRET microscopy can be exploited to study interactions between rafts and biomolecules (Rao and Mayor, 2005), for example, to study interaction between RNA molecules (Janas et al., 2006; 2019; 2021) or polysialic acid molecules (Sapoń et al., 2019) with liquid-ordered lipid raft domains. It has been suggested that miRNAs act as critical regulators of lipid raft structure by targeting raft-associated proteins (caveolins and flotillins) (Varshney et al., 2016). FRET spectroscopy has also been applied to detect interaction between rafts, membranes and biomolecules, for example, to determine the differences in affinity of RNA molecules containing RAFT motifs to exosome-like lipid vesicles, to vesicles containing raft-like lipid domains and to exosomes (Mańka et al., 2021), or raft-mediated interactions between exosomes (Sapoń et al., 2020b). Colocalization of ordered lipid domains with B cell receptor clusters has been directly visualized using super-resolution fluorescence localization microscopy, (Stone et al., 2017). The performance of the polarity-sensitive membrane dyes has also been tested in live cells and single virus particles (Sezgin et al., 2017b); these dyes can detect raft regions because more tightly packed (ordered) lipid raft regions accommodate a lower number of water molecules and are therefore relatively less polar than a disordered region. To enable the direct investigation of fast dynamics of fluorescent proteins and lipids (including cholesterol) in plasma membranes of live cells, fluorescence correlation spectroscopy (FCS) has been combined with super-resolution STED microscopy (Sezgin et al., 2019); this allowed the accurate characterization of molecular processes in living cells, including lipid raft formation. The concentration of probe molecules in the vicinity of B cell receptor clusters has been measured at high spatial resolution using single-molecule fluorescence localization microscopy, thus showing that phase separation (into raft and non-raft nano-domains) also occurs in intact cell membranes in living cells (Shelby et al., 2021 preprint). In conclusion, observations of rafts by microscopy have supported the lipid-raft concept and revealed molecular mechanisms related to lipid raft formation, dynamics and interactions.

Role of rafts in the formation of membrane vesicles

Lipid rafts present both in the plasma membrane and the membranes of intracellular organelles appear to be involved in the entire lifetime of a vesicle (Levental et al., 2020). Indeed, rafts have been found to be involved in the formation of vesicles and their loading with cargoes, as well as with their anchoring and fusion with a target membrane (Wang et al., 2020b). With regard to vesicle budding, two mechanisms of raft involvement have been proposed. First, proteins and lipids located in these cholesterol-enriched microdomains might interact with coat proteins, leading to a small invagination and subsequent formation of a budding vesicle (Fig. 2) (Bonifacino and Glick, 2004). Another mechanism for the sorting of lipids into the raft region and subsequent vesicle budding has been suggested to be determined by the lipid shape (Janas et al., 2015) (see Fig. 5 below). Here, the action of sphingomyelinase (producing cone-shaped ceramide) and phospholipases (producing inverted cone-shaped lyso-phospholipids) triggers budding from the raft region (Trajkovic et al., 2008; Janas et al., 2015). In addition, cholesterol might relax tension in liquid-ordered domains (despite their increased stiffness) by lowering the energetic barriers of membrane bending and fission, thus explaining why lipid rafts could be preferential sites for membrane budding and endocytosis (Anderson et al., 2021). Below we discuss the role of lipid rafts in formation of endocytic vesicles, transport vesicles, exocytic vesicles, SVs, EVs and enveloped viruses.

Rafts and endocytic vesicles

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the main entry route for most cell surface receptors and their ligands (Baschieri et al., 2020). CME is the predominant form of endocytosis, whereby cargo such as epidermal growth factor (EGF) and transferrin, are internalized from the cell surface by a vesicle coated with clathrin (Stella and Gregory, 2018). This process begins with the recruitment of clathrin, adaptor protein and other cytosolic proteins to the sites containing cargo proteins, resulting in a small invagination that will grow into a clathrin-coated pit (CCP). Once of sufficient size, CCPs undergo scission from the plasma membrane to form the internalized vesicle. Interestingly, metastasis of colon cancer cells is dependent on glycosphingolipid in the plasma membrane, with the mechanisms responsible for this possibly related to changes in the lifetime of CCPs and signaling induced upon crosslinking of glycosphingolipids (Skotland et al., 2020). When membrane tension (the force needed to deform a membrane) is high, a dramatic increase in CCP lifetime is observed, likely reflecting the

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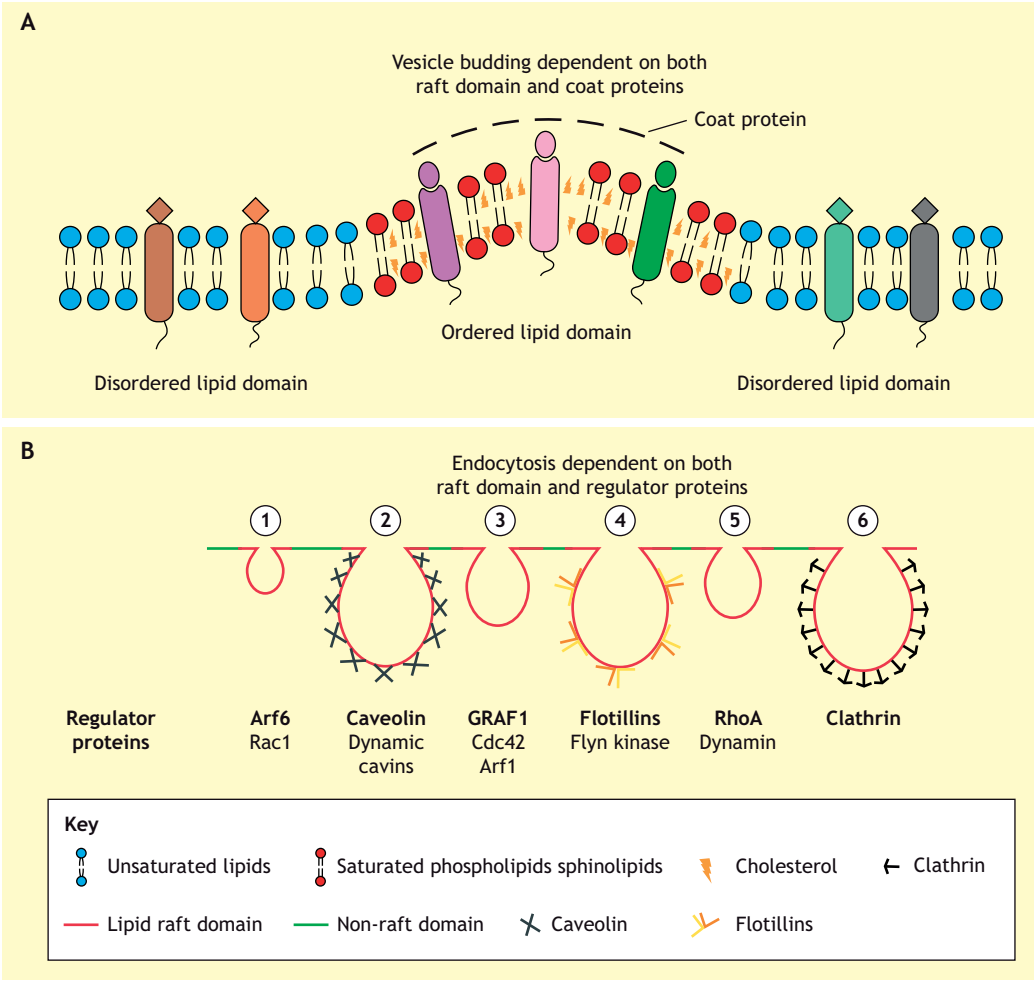

Fig. 2. Role of lipid rafts in vesicle budding. (A) Coat proteins associate with raft proteins or raft lipids, which leads to budding and vesicle formation; induction of curvature is also facilitated by relaxation of tension of the raft domain. Newly formed vesicles are also enriched in the lipids of the raft they originate from. (B) Role of rafts in different types of endocytosis. Rafts (red line; 1–6) associate with endocytic factors to form endocytic vesicles. Rafts are involved in caveolin-mediated endocytosis and flotillin-dependent endocytosis, as well as endocytosis dependent on small GTPases, such as Arf-6, GRAF-1 and RhoA. Clathrin-mediated endocytosis can be both raft dependent or raft independent (not shown); the latter might involve invagination from a non-lipid-raft domain.

difficulties of the clathrin coat to transition from a flat to a curved topology (Baschieri et al., 2020). Therefore, a high membrane tension can perturb the budding ability of CCPs, resulting in shallow invaginations or flat clathrin lattices. Results suggest that the ability of a sterol to pack tightly with other lipids to form a liquid-ordered domain is likely to be critical for a flat clathrin lattice to be able bend into a CCP (Rohrbough and Broadie, 2005; Stella and Gregory, 2018). Therefore, clathrin-dependent endocytosis can be raft dependent; however, there are reports of clathrin-dependent but raft-independent endocytosis, for instance, that of histamine H1 receptors (Hishinuma et al., 2010) or cholera toxin (Shogomori and Futerman, 2001) (Fig. 2B). In another example, the paracrine protein S100B in the glioma microenvironment is internalized by mesenchymal stem cells via clathrin- and lipid raft-mediated endocytosis (Zhang et al., 2021).

Clathrin-independent and raft-dependent endocytosis

Rafts are involved in (1) caveolin-mediated endocytosis and (2) flotillin-dependent endocytosis, as well as (3) endocytosis dependent on small GTPases, such as Arf6, GRAF-1 (also known as ARHGAP26) and RhoA. One of the molecules involved in caveolin-mediated endocytosis is the integral membrane protein caveolin-1, which, when palmitoylated, binds to cholesterol in the lipid rafts region, thereby triggering the formation of caveola (invaginations of the cell membrane) (Murata et al., 1995; Matthaeus et al., 2022). In flotillin-dependent endocytosis, flotillins bind the cytosolic leaflet of the plasma membrane and endomembranes, where they form a complex through hetero-oligomerization of flotillin-1 and flotillin-2, and drive invagination of the membrane preceding pinching off a vesicle (Greenlee et al., 2021). Endocytosis dependent on small GTPases includes GRAF-1-dependent endocytosis (Sabharanjak et al., 2002), Arf6-dependent endocytosis, which is mediated by the ADP-ribosylation factor 6 (Arf6) and Rac1, a member of the Rho family of GTPases (Vidal-Quadras et al., 2011; Li and Guo, 2022), and endocytosis dependent on the small GTPase RhoA (Lamaze et al., 2001; Gundu et al., 2022).

Rafts and transport or exocytic vesicles

Transport vesicles (also called ‘membrane trafficking vesicles’) bud from the donor membrane, are transported through the cytosol and eventually fuse with the acceptor membrane (Fig. 3A), resulting in

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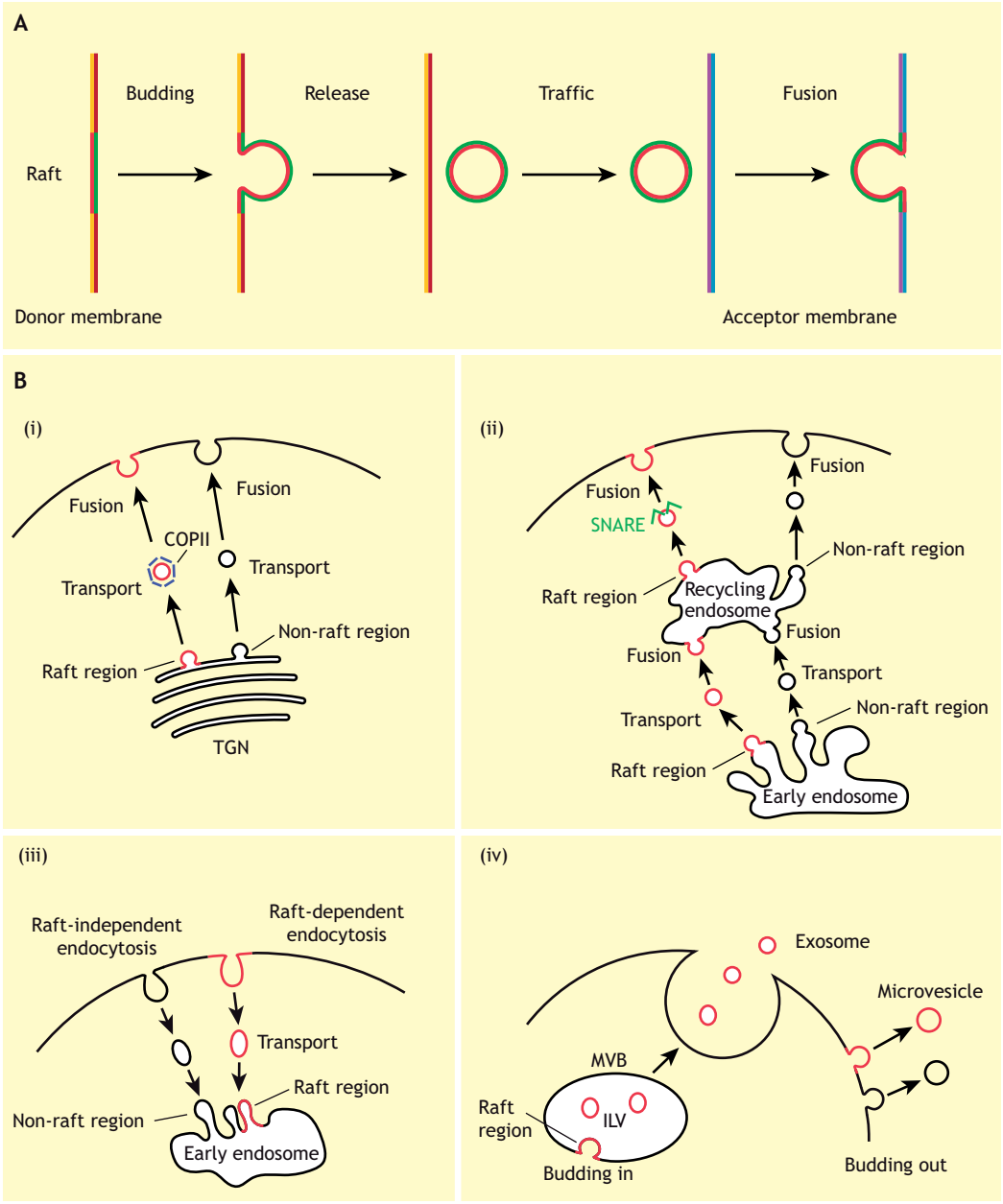

Fig. 3. Role of rafts in vesicular transport. (A) Asymmetrical membranes in transport vesicles. The transport vesicle is formed in the source membrane, cleaves from it and is transported through the cytosol to reach the target membrane to which it fuses. The transport vesicle can have a different composition to the donor membrane and target asymmetrical membranes. The transport vesicle itself can also have an individual asymmetric membrane composition. (B) Trafficking routes inside the cell. Rafts (red line) participate in several intracellular trafficking routes: (i) plasma membrane trafficking of components derived from trans-Golgi network (TGN) followed by their exocytosis; (ii) protein segregation both in early endosomes and recycling endosomes, as well as vesicle formation from these organelles and their transport; (iii) endocytic pathways at the plasma membrane; (iv) the formation of intraluminal vesicles (ILVs) through inward budding into the lumen of a multivesicular body (MVB), followed by their release into extracellular space (as exosomes). Budding of microvesicles from the plasma membrane can be raft dependent (red line) or raft independent.

the coordinated and regulated trafficking of cargoes, either from one organelle to another or for their secretion. Vesicles frequently have a different lipid and protein composition compared with that of the original membrane (because of e.g. involvement of raft domains in the budding process), and their cargo might differ from the content of the compartment they derive from (due to, for example, active loading process). In the case of vesicles that originate from asymmetrical membranes (Fig. 3A), both the outer and inner leaflet of the lipid membrane might have a composition that is different from their source membrane (Van Meer and Sprong, 2004).

Vesicle transport inside the cell can be classified into anterograde transport of lipids and proteins synthesized in the ER, transferred to the Golgi by vesicles coated with COPII (named from coat protein complex II), and then transported further to the other organelles or cell membrane (Taylor et al., 2022). Conversely, retrograde transport involves coat protein complex I (COPI), which forms vesicles for cargo transport from the Golgi to the ER, as well as vesicles for intra-Golgi transport. This type of vesicle is unlikely to include rafts regions given that COPI-coated vesicles have reduced levels of cholesterol and most sphingolipid

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species, except for N-stearoyl sphingolipids (Brügger et al., 2000). Recruitment of COPI onto membranes is mediated by the GTP-binding protein Arf1, which binds to membrane and influences cargo selection, vesicle scission and vesicle uncoating (Taylor et al., 2022).

The role of membrane domains in the formation of transport vesicles involved in anterograde transport in the cell was noted early on (Simons and Van Meer, 1988). These cholesterol-rich domains sort molecules between organelles by recruiting components, either through accumulation from other membrane regions or through specific binding from the water phase, for the controlled transfer from one compartment to an acceptor compartment via raft-enriched vesicles (Klemm et al., 2009) (Fig. 3A). There are several means by which rafts affect vesicle formation – first, cholesterol (in cholesterol-rich rafts) could relax tension in liquid-ordered domains, thus inducing membrane budding and endocytosis (Anderson et al., 2021); second, rafts could recruit coat proteins, which induce invaginations (Levental et al., 2020); and third, rafts could recruit enzymes that generate lipids with specific shapes, thereby facilitating bending (Janas et al., 2015). Raft-mediated vesicular transport also includes the recycling of certain components from the endosomal system in the early endosomes (EEs) and recycling endosomes (REs) back to the PM (Diaz-Rohrer et al., 2014); in the case of glycosphingolipids, the sorting mechanism comprises a C14 motif in the fatty acid of the ceramide and lipid rafts (Schmieder et al., 2022). Transport routes that modulate cellular cholesterol distribution appear to trigger a change both in cholesterol content of endomembranes and in the location and functioning of SNARE proteins (Enrich et al., 2015), which mediate raft-dependent membrane fusion steps of transport vesicles with their destination membranes in the secretory pathway (Warner et al., 2022) (discussed further below). Exocytosis has been found to be inhibited upon cholesterol depletion (Chamberlain et al., 2001); these studies led to the discovery that interactions between lipid rafts and SNARE proteins are important for exocytosis (Chamberlain et al., 2001; Salaün et al., 2005).

Rafts present in the membrane of the trans-Golgi network (TGN), containing cholesterol and sphingomyelin, give rise to vesicles that are then transported to the cell membrane and exocytosed (Laurenzana et al., 2015) (Fig. 3B). In this context, rafts constitute platforms for preferential actin polymerization from a subset of Golgi-derived membrane vesicles (Rozelle et al., 2000). Specifically, synthesis of the phospholipid PI(4,5)P2 and tyrosine phosphorylation of actin induce polymerization of actin surrounding membrane vesicles, leading to the formation of the characteristic actin comet tails that propel the Golgi-derived vesicles within the cytoplasm (Rozelle et al., 2000).

Raft-mediated vesicle exocytosis can occur constitutively, such as upon the fusion of vesicles derived from the TGN with the plasma membrane, or can be precisely regulated, as is the case for SVs and EVs (discussed below). Here, the role of lipid rafts in can be both passive, such as acting as a spatial coordinator of exocytic factors, and direct, by affecting membrane fusion itself, as reviewed elsewhere (Salaün et al., 2004; Meldolesi, 2022).

Rafts and SVs

Membrane rafts are involved in several facets of SV function (Fig. 4). Their importance in the regulation of the SV cycle, in SV budding from sorting endosome, or SV fusion with plasma membrane, is well established, as reviewed elsewhere (Rohrbough and Broadie, 2005; Pfrieger, 2003; Morgan et al., 2013). Raft cholesterol has been shown to associate with SV membrane proteins

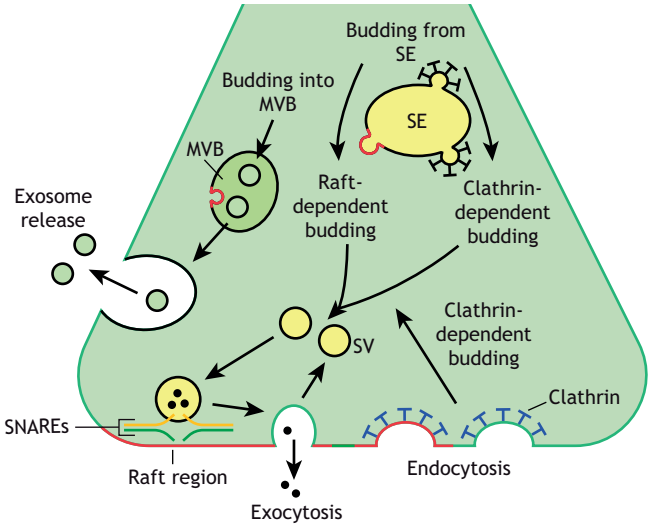

Fig. 4. Rafts at the synapse and their role in the SV cycle. Rafts have been found in both the synaptic vesicle (SV) membrane and the neuron presynaptic membrane, where they promote the localization of SNAREs, suggesting that SV fusion takes place in the raft region of the presynaptic membrane. Some SV components remain in close proximity (probably within raft domains) throughout their entire lifecycle. Rafts in the membrane of sorting endosomes (SEs) allow for the sorting of synaptic membrane proteins into different SVs that repopulate the synaptic vesicle pool. SVs can bud from SEs either through a pathway that involves clathrin and the adaptor complexes AP-1 and AP-3, or from a lipid raft-region in SE membrane; induction of curvature is owing to either clathrin coat formation and/or the relaxation of tension of the raft domain. Multivesicular bodies (MVBs) located in the presynaptic terminal can fuse with the presynaptic membrane, thus releasing exosomes. At the neuromuscular junction, exosomes are released from the presynaptic terminal at the periactive zone.

involved in the SV cycle, such as synaptophysins (Thiele et al., 2000), synaptotagmin and synaptobrevin (Takamori et al., 2006; Puchkov and Haucke, 2013). Synaptobrevin is the receptor for SNAREs and drives SV fusion with the presynaptic membrane via interactions with its cognate SNARE partners (Cousin, 2021). Synaptophysin is involved in the efficient retrieval of synaptobrevin during SV endocytosis, whereas synaptotagmin is a Ca^{2+} sensor for neurotransmitter release (Cousin, 2021). Both SVs and presynaptic membranes contain proteins of the so-called SNARE complex, comprising synaptobrevin, syntaxin and SNAP25, which enable their anchoring to and fusion with membranes (Cousin, 2021). The depletion of cholesterol from the plasma membrane causes the dispersion of rafts and their associated SNARE complexes, pointing to the importance of rafts for the proper location of SNARE proteins (Lang et al., 2001). The lipid raft region of SV membrane markedly weakens the association of the extravesicular domain of synaptobrevin with the SV membrane, which induces a detachment of this domain from it, thus facilitating the interaction of the extracellular domain (while being still connected to the transmembrane domain of synaptobrevin) with its cognate SNARE partners (located in the presynaptic membrane) and SNARE complex assembly (Wang et al., 2020a). Studies in *Drosophila melanogaster* have also confirmed a role of lipid rafts in mediating vesicle fusion in neuronal synapses in embryonic and larval, as well as adult neuronal and non-neuronal tissues (Rohrbough et al., 2004).

It is worth mentioning that rafts also affect the selective accumulation of Ca^{2+} channels in presynaptic membranes, as

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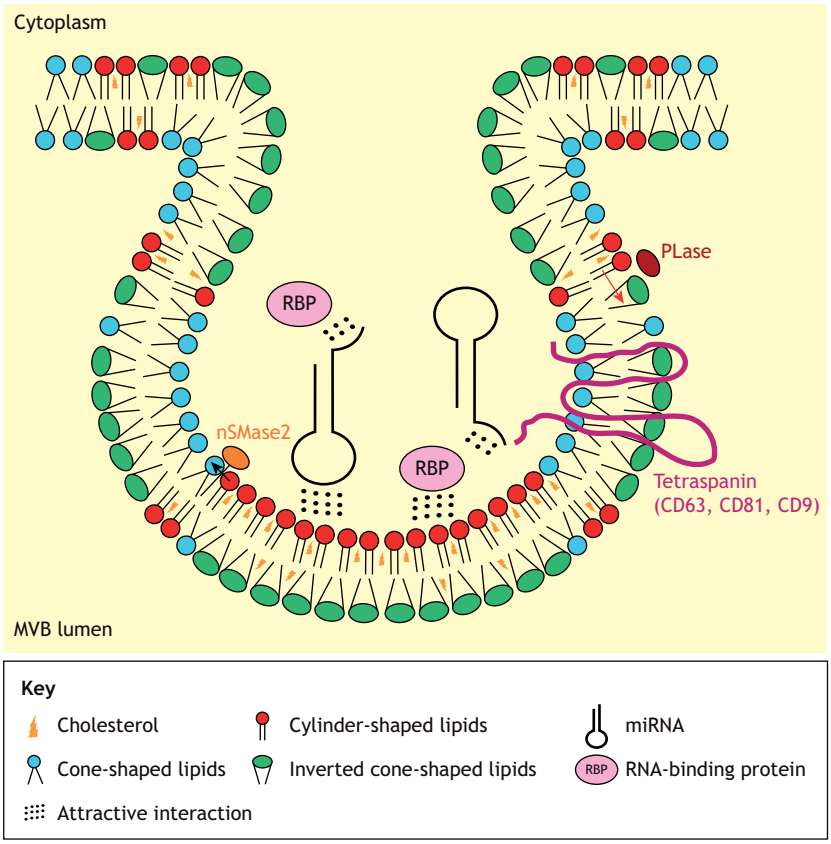

Fig. 5. Lipid-shape-dependent ILV budding-in from an MVB membrane. Budding of a raft region into the interior side of the MVB is preceded by the formation of a raft domain in the cytoplasmic leaflet of the MVB membrane (not shown, see Fig. 1). Internal budding of the raft region of the MVB membrane is triggered by the action of neutral sphingomyelinase 2 (nSMase 2), which binds to the cytoplasmic leaflet of the MVB membrane and catalyzes the breakdown of sphingomyelin (red head group) into phosphocholine and ceramide (a cone-shaped lipid, shown with a blue head group), and phospholipase (PLase), which catalyzes the breakdown of saturated phospholipid (a cylinder-shaped lipid, shown with a red head group) into lysophospholipid (inverted cone-shaped lipid, shown with a green head group). Induction of curvature is also facilitated by glycosphingolipids (in the shape of an inverted cone) in the inner layer of the membrane and by relaxed tension of the raft domain. These can lead to the spontaneous process of budding-in from the MVB membrane, and the formation of an ILV within MVB. Loading the cargo (RNA transported by an RNA-binding protein, RBP) into ILVs involves interaction of the inward-budding raft domain with the RNA–RBP complex through either direct RNA interaction with the raft region (through a RAFT motif within the RNA sequence) or/and indirect interaction via RBPs. When ILVs are released from the cell, they become exosomes.

voltage-gated P/Q-type ($Ca_v2.1$) Ca^{2+} channels, responsible for Ca^{2+} influx in nerve terminals, have been found in the raft fraction, which allows their interaction with proteins of the SNARE complex (Taverna et al., 2004). Thus, rafts both in the SV membrane and in presynaptic membranes are important in organizing membrane sites specialized for SV exocytosis.

Another role of rafts within the SV cycle is in the function of nerve-ending endosomes. There are two endosome populations in the presynaptic terminal involved in raft-dependent vesicle formation – multivesicular bodies (MVBs) and sorting endosomes (Fig. 4). Two mechanisms of SV budding from the endosomal membrane in the presynaptic terminal have been suggested, one based on clathrin-adaptor sorting (Morgan et al., 2013) and the second dependent on raft sorting (Hoopman et al., 2010). The adaptor complexes AP-1 and AP-3 together with SV v-SNAREs are involved in clathrin adaptor sorting of membrane proteins into vesicle buds that can pinch off from early endosomes. Membrane is retrieved by clathrin-mediated endocytosis to an EE (Fig. 4), and this endosome is capable of sorting synaptic membrane proteins into different SVs that repopulate the SV pool (Morgan et al., 2013). Caveolin-1 (Cav1)-dependent lipid rafts can be incorporated into SVs during SV endocytosis, and palmitoylated Cav1 is able to interact with endocytic components, which modulates SV endocytosis (Koh et al., 2021). The size of SVs (35–55 nm) is comparable to the size of small exosomes. Exosomes can be released from the presynaptic terminal through the fusion of MVBs with the presynaptic membrane (Korkut et al., 2009; Koles et al., 2012; Janas et al., 2016); at the neuromuscular junction, exosomes are released from the presynaptic terminal at periactive zone regions, where these vesicles gain access to the subsynaptic reticulum.

Rafts and EVs

EVs can be classified into four types (Jeppesen et al., 2019) – exosomes (nanovesicles of endosomal origin), microvesicles formed by plasma membrane budding-out, apoptotic bodies secreted by the cell during apoptosis, and autophagic EVs (nanovesicles of autophagosome origin). Intraluminal vesicles (ILVs) are formed by budding into the MVB membrane. Following the fusion of MVBs with the cell membrane, ILVs are released as exosomes (40–150 nm) into the intercellular environment. In contrast, microvesicles (100–1000 nm) arise from the outward budding and shedding from the plasma membrane. Autophagosome fusion with MVB generates an amphisome, whose fusion with the plasma membrane secretes autophagic EVs (40–1000 nm). Apoptotic bodies are the largest type of EVs (1–5 µm) and are shed from cells during apoptosis.

Lipid rafts have been found to be involved in the generation of microvesicles from various cell types, for example, erythrocytes (Salzer et al., 2002), monocytes (Del Conde et al., 2005) and endothelial cells (Burger et al., 2011). The role of rafts in the secretion of vesicles with exposed PS from platelets has been investigated, and it was found that disturbing the raft phase by cholesterol depletion inhibits their secretion (Wei et al., 2018). These authors also noticed the binding of the lipid raft marker Cholera enterotoxin subunit B (CTxB), which specifically interacts with raft ganglioside GM1 (Sapoń et al., 2020a), to platelet-derived vesicles. This confirms the hypothesis that microvesicles are released from raft areas (Wei et al., 2018). These reports suggest that microvesicle budding from plasma membrane can be raft dependent. However, there is a lipidomics report showing that adipocyte-derived large EVs that are enriched in microvesicles contain a much lower percentage of cholesterol in comparison with

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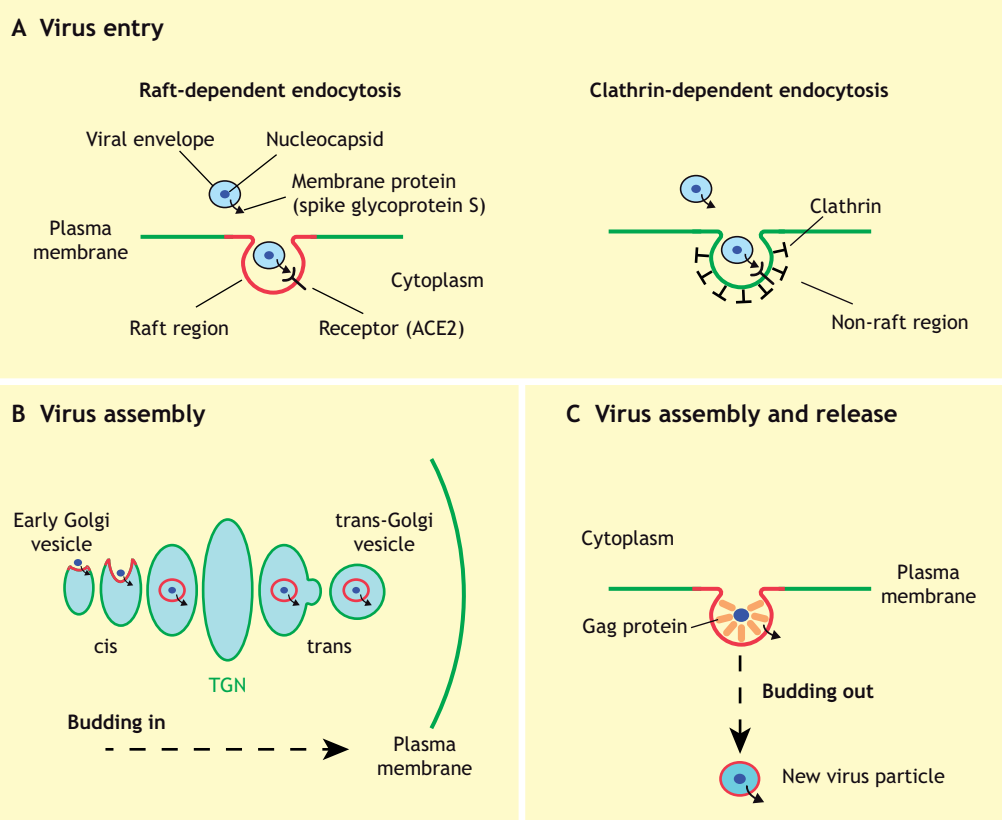

Fig. 6. Role of rafts in enveloped virus infection. Rafts are involved in virus entry into the host cell and its assembly inside the host cell, as well as release form the plasma membrane. (A) SARS-Cov-2 enters human cells by binding of virus spike glycoprotein (S) to its receptor, angiotensin-converting enzyme 2 (ACE2) in the plasma membrane of the host cell; this entry occurs either at rafts in a caveolin- and clathrin-independent manner, or via clathrin-dependent, raft-independent endocytosis. (B) SARS-Cov-2 assembly is thought to involve raft-dependent budding-in of early Golgi membrane (or the membrane of intermediate compartment between the ER and the Golgi), thus enclosing the nucleocapsid. After maturation within the trans-Golgi network (TGN), the enveloped nucleocapsid enters the cytoplasm within a trans-Golgi vesicle through budding-out. (C) In the case of HIV, virus assembly occurs at the plasma membrane and is mediated by the interaction of the viral structural protein Gag with lipid rafts at the cytoplasmic site, before the newly formed enveloped virus is released through budding out from the plasma membrane.

adipocyte-derived small EVs, enriched in exosomes, suggesting that regions outside of rafts can be also involved in microvesicle budding (Durcin et al., 2017). Thus, budding of microvesicles from the plasma membrane can be raft dependent or independent (Fig. 3B–D).

Membrane rafts found in the membrane of MVBs are important for the formation of ILVs, which eventually become exosomes, and loading of their cargo. Inside an MVB, ILVs can be formed essentially in two ways, either requiring endosomal sorting complex required for transport (ESCRT) or independent of it (Rajagopal and Harikumar, 2018). For ESCRT-independent ILV formation, the importance of both lipid raft domains and the shape of lipid molecules have been highlighted (Fig. 5). Here, the presence of ceramide molecules in the outer leaflet of the MVB membrane, as well as that of lysophospholipids and glycosphingolipids in the intracellular leaflet, cause the spontaneous inward budding of the MVB raft region, resulting in ILV formation (Janas et al., 2015).

Rafts within the MVB membrane are also likely to be involved in the sorting of cargo into the forming ILVs, although the exact mechanism is not yet completely understood. In the case of loading RNA, RNA is transported from the cytoplasm by RNA-binding proteins (RBPs) to the outer surface of the MVB membrane, and there is recent evidence for a role of RBPs in RNA sorting and loading into EVs (Corrado et al., 2021). RNA loading might involve interaction of the inward-budding raft domain with the RNA–RBP complex through either direct RNA interaction with the raft region or/and indirect interaction via RBPs (Fig. 5). The first mechanism is supported by studies on the binding of RNA aptamers to lipid-raft-containing and exosomal liposomes (Janas et al., 2016; 2021; Mańka et al., 2021), which showed lipid-raft-specific and RNA-sequence-specific RNA–liposome complex formation. Furthermore, bioinformatic analysis had identified raft RNA motifs (RAFT motifs) frequently in the exosomal pro-tumoral miRNAs transferred from cancer cells to immune cells (Janas et al., 2020). Finally, miRNAs also possess sorting sequences that determine their sorting into exosomes or small EVs (EXO motifs) (Villarroya-Beltri et al., 2013; Garcia-Martin et al., 2022).

Rafts and enveloped viruses

Lipid rafts have also been reported to act as platforms used by viruses for their assembly and budding from host cells, for instance for the human immunodeficiency virus type 1, HIV-1 (Fig. 6C) (Ono and Freed, 2001; Thornhill et al., 2020), the Ebola virus, the Marburg virus (Bavari et al., 2002) and the measles virus (Manié et al., 2000). The envelope of HIV is enriched with sphingolipids and cholesterol compared with the plasma membrane, and binding of *in vitro*-translated HIV Gag protein to liposomal membranes occurs preferentially in cholesterol-rich membrane domains (Dick et al., 2012), indicating that rafts are the regions where viruses bud out from the host cell. Furthermore, multimerization of HIV Gag at the assembly site occurs in a liquid-ordered lipid phase that is enriched in cholesterol and sphingolipids (Sengupta and Lippincott-Schwartz, 2020). Proteins with affinity for this lipid environment partition into it, resulting in selective incorporation of proteins into the nascent viral membrane (Sengupta and Lippincott-Schwartz, 2020).

Recently, considerable attention has focused on the role of lipid rafts in the lifecycle of coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes COVID-19, and lipid rafts have been suggested as significant factors in the infection of host cells. Specifically, cholesterol- and sphingolipid-rich lipid raft microdomains in the plasma membrane have been

shown to be involved in SARS-CoV entry through an endocytic pathway that is independent of clathrin and caveolae, but is dependent on rafts and involves viral attachment to angiotensin-converting enzyme 2 (ACE2) in the host cell plasma membrane (Wang et al., 2008; Palacios-Rapalo et al., 2021) (Fig. 6A). SARS-CoV-2 requires protease activity for entry into the cell and binds to the ACE2 receptor; sialic acid in ganglioside-enriched membrane domains might facilitate contact between the virus and its receptor (Avota et al., 2021). If the target cell expresses insufficient transmembrane protease, serine 2 (which cleaves the viral spike, S, protein at the multibasic site, thus initiating the membrane fusion process) or if a virus–ACE2 complex does not encounter this protease, the virus is internalized not through fusion, but via CME (Inoue et al., 2007; Bayati et al., 2021; Jackson et al., 2022). A study using SARS-CoV-2 pseudoviruses, which are deprived of critical virulence genes, indicates that virus infection is highly dependent on rafts, as cholesterol depletion of cell membranes using methyl-β-cyclodextrin resulted in a reduction of pseudovirus infection, which was re-instated upon cholesterol supplementation (Li et al., 2021). In addition, it has been suggested that lipid rafts concentrate the ACE2 receptor on host cell membranes, thereby facilitating its interaction with the viral envelope spike protein and so promoting infection (Sorice et al., 2021).

The effect of S-acylation on membrane lipid organization of SARS-CoV-2 and its infectivity (Mesquita et al., 2021) suggests that lipid rafts are also involved in virus assembly (Fig. 6B). S-palmitoylation of the spike protein not only results in the formation of viruses with enhanced fusion capacity, but also facilitates the formation of localized, ordered cholesterol and sphingolipid-rich lipid nanodomains in the early Golgi (or the intermediate compartment between the ER and the Golgi), where viral budding occurs, as well as generating cholesterol-rich lipid domains within viral envelopes (Mesquita et al., 2021). It has been suggested that acyl moieties recruit cholesterol into close proximity to the spike protein, thus triggering the formation of ordered nanodomains (Mesquita et al., 2021).

Exosomes, SVs and enveloped HIV virus particles are enriched (relative to the parent cell total membrane lipids and parent cell plasma membrane lipids) in the typical raft lipids, namely, cholesterol and sphingomyelin (Puchkov and Haucke, 2013; Lorizate et al., 2013; Janas et al., 2016; Skotland et al., 2020), thus supporting the notion that membrane raft microdomains are involved in the budding process.

Conclusions and future perspectives

Since their discovery, there has been a continuing interest in lipid rafts, cholesterol-enriched membrane domains, and numerous studies have suggested that they have a role in cell signaling, pathogen invasion, development of certain diseases and membrane transport. One of the aspects of the latter, the role of rafts in vesicle formation, has been the focus of this Review.

As discussed here, rafts are involved in the formation of a whole range of cellular vesicles, include vesicles that transport cargo between organelles, endocytic vesicles and secretory vesicles, such as SVs and EVs. Raft-mediated vesicle formation also includes interactions with cargo of sorting vesicles and membrane receptors, as well as other regulatory proteins. Rafts also mediate vesicle transport through facilitating the generation of actin comets, as well as promoting vesicle anchoring and fusion with their target membrane. Several mechanisms through which rafts act have been proposed, including formation of platforms for the selective localization of proteins for their association with coat proteins and subsequent formation of a budding vesicle.

The initial view of rafts as distinct and stable regions has evolved into that of a more flexible, transient, nano-sized domain, and our understanding of these cholesterol-rich domains in both physiological and pathological conditions is increasing. Nonetheless there is still a need to further study the functional complexity of rafts and their exact involvement in cellular vesicle formation. Although the molecular mechanisms of enveloped virus entry into host cells are mostly known, how viruses assemble remains elusive. There is another puzzling process, ILV assembly during inward budding of the MVB membrane; in both cases lipid rafts appear to be involved. One of the intriguing questions is whether virus RNA can directly interact with membrane lipid rafts in the host cytoplasm, thus facilitating virus assembly, or whether virus RNA internalization during enveloped virus assembly involves an indirect interaction involving an RBP. A corresponding question for ILV assembly is whether cytoplasmic RNAs can directly interact with membrane lipid rafts within the MVB membrane, thus implicating lipid shape within the raft region in RNA binding, and thus in vesicle formation.

Competing interests

The authors declare no competing or financial interests.

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