CHAPTER THREE

Lipid Rafts, Caveolae, and Their Endocytosis

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Contents

Abstract

Lipid rafts are plasma membrane microdomains enriched in cholesterol and sphingolipids that are involved in the lateral compartmentalization of molecules at the cell surface. Internalization of ligands and receptors by these domains occurs via a process defined as raft-dependent endocytosis. Caveolae are caveolin-1-enriched smooth invaginations of the plasma membrane that form a subdomain of lipid rafts. Endocytosis of rafts, including caveolar but also noncaveolar dynamin-dependent and dynamin-independent pathways, is characterized by its cholesterol sensitivity and clathrin-independence. In this review

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we will characterize lipid rafts and caveolae, their endocytosis and its regulation by the actin cytoskeleton, caveolin-1, dynamin, and cholesterol.

Key Words: Caveolae, Lipid rafts, Endocytosis, Caveolin, Actin, Signaling.

1. Introduction

Caveolae were first identified in the 1950s and defined as smooth invaginations of the plasma membrane devoid of clathrin coat (Palade, 1953). These vesicular carriers represent a subdomain of plasma membrane lipid raft microdomains enriched in cholesterol and sphingolipids. Caveolae are abundant structures associated with expression of the caveolins, proteins that form the caveolar coat, and have been implicated in various biological processes such as endocytosis, transcytosis, and signal transduction. However, raft-dependent endocytosis is not limited to caveolae. Indeed, over the years, many other pathways have been characterized as caveolae-independent/raft-dependent endocytic pathways (Lajoie and Nabi, 2007). Defining caveolae and raft domains and the regulation of their formation and endocytosis by caveolin-1 (Cav1) and other factors is the subject of this review.

2. The Lipid Raft

2.1. Origin of the concept

The observation that glycosphingolipids cluster in the Golgi apparatus before being sorted to the apical surface of polarized cells triggered the emergence of the lipid raft concept (Simons and van Meer, 1988). Further investigation later found that glycosphingolipid clusters are mostly insoluble in detergent at 4 °C, therefore creating detergent-resistant membranes (DRMs). DRMs possess specific properties such as a light buoyant density on sucrose gradients and enrichment in both cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) (Brown, 1994). Indeed, until recently, most of the evidence for rafts as functional domains within the cell came from studies of the DRM association of proteins and the effect of cholesterol-modulating agents on these interactions. However, the use of detergents to identify raft-associated proteins is controversial (Munro, 2003). It has been shown that different detergents are unlikely to reflect the same aspects of membrane organization such that the effect of a given detergent may vary according to cell type (Schuck et al., 2003). Also this approach cannot provide information regarding the preexisting

organization in the multicomponent cell surface, nor a quantitation of its physical characteristics (Rao and Mayor, 2005). While DRMs are still thought by some to be able to give valuable information about raft heterogeneity (Pike, 2004), others do not share that opinion (Lichtenberg et al., 2005). Efforts have been made to develop new technologies to characterize raft behavior without having to use detergent extraction; however, these approaches are also rife with potential problems (Lommerse et al., 2004; Shaw, 2006).

Glycosphingolipid- and cholesterol-rich lipid domain formation may be driven solely by lipid–lipid interactions. In giant unilamellar vesicles (GUVs) containing unsaturated phospholipid, sphingomyelin, and cholesterol, components spontaneously form two distinct phases (Silvius and Nabi, 2006). The liquid disordered state (L_d) is characterized by both highly flexible acyl chains and highly mobile lipid molecules. The liquid ordered state (L_o) contains tightly packed sphingomyelin and cholesterol molecules and presents more restricted motion (Brown and London, 1998). Other evidence for microdomain formation came from studies of GUVs where it was shown that membrane microdomains can form in lipid mixtures resembling those of the plasma membrane (Dietrich et al., 2001; Silvius, 2003).

While segregation of raft domains in model membranes supports the raft hypothesis, difficulties linked to the characterization of membrane microdomains in biological membranes have led to various suggested models. It was proposed that proteins targeted to lipid rafts have a light buoyant density because they are surrounded by cholesterol and sphingolipids (referred to as a lipid shell). Lipid shells are thermodynamically stable structures that have an affinity for preexisting rafts. Hence, they target the protein they encase specifically to these membrane domains (Anderson and Jacobson, 2002). Although the shell is proposed to be stably associated with proteins, its lipid components may interact and even interchange with nonshell lipids (Jacobson et al., 2007). While the existence of a lipid shell can explain the low buoyancy of DRMs, the extent to which stable protein–lipid interactions in biological membranes exist and determine raft association remains to be determined.

2.2. Defining raft domains

Estimates of the size of raft domains are highly variable and have included: $\sim$15 protein molecules by protein cross-linking (Friedrichson and Kurzchalia, 1998); 22 $\pm$ 4 nm diameter by immunoelectron microscopy of Ras-anchored GFP in isolated plasma membrane sheets (Prior et al., 2003); 26 $\pm$ 13 nm diameter by laser trap measurements of local diffusion (Pralle et al., 2000); $<$70 nM diameter and containing fewer than 50 molecules by FRET (Varma and Mayor, 1998); and larger transient confinement domains of hundreds of microns by single molecule tracking

approaches (Dietrich et al., 2002; Schutz et al., 2000). FRET has been observed between lipid-modified raft-associated monomeric GFPs (Zacharias et al., 2002). However, FRET studies of the well-characterized raft component, GPI-APs, generated conflicting data as to whether they cluster within raft domains (Glebov and Nichols, 2004; Kenworthy and Edidin, 1998; Kenworthy et al., 2000; Varma and Mayor, 1998). The conflicting data could be reconciled if a small proportion of the GPI-APs were localized to small clusters (Glebov and Nichols, 2004; Kenworthy et al., 2000). Indeed, fluorescence anisotropy measurements show that the majority of cell surface GPI-APs are present as monomers and that 20–40% are present in small (< 5 nm) clusters (Sharma et al., 2004b). Similarly, single particle tracking analysis of GPI-linked CD59 described rafts as small, unstable structures of only a few molecules with a halftime of less than 1 ms (Subczynski and Kusumi, 2003). Also, further evaluation of the spatial distribution of Ras-anchored GFP by electron microscopy supported the notion of a minimal raft cluster of three to seven protein molecules (Parton and Hancock, 2004). Recent studies using single particle tracking and stimulated emission depletion (STED) have described the cholesterol-sensitive transient anchoring of raft-associated sphingolipids and GPI-APs in domains (Chen et al., 2006; Eggeling et al., 2009). The STED studies showed that transient anchoring occurred for 10–20 ms in domains smaller than 20 nm (Eggeling et al., 2009). Lipid rafts are now universally defined as small (10–200 nm) heterogeneous membrane domains enriched in sphingolipid and cholesterol that are involved in various cellular processes (Pike, 2006).

Using high spatial and temporal FRET microscopy, it was shown that GPI-AP clusters are organized on at least two length scales at steady state: the nanoscale (< 10 nm) and the optically resolvable scale (> 450 nm) (Goswami et al., 2008). The larger complex could correspond to the larger raft domains proposed earlier (Simons and van Meer, 1988). The formation of these cholesterol-sensitive clusters is regulated by cortical actin indicating an active role for the actin cytoskeleton in the dynamics of plasma membrane domain formation (Goswami et al., 2008). Recently, fluorescence photoactivation localization microscopy (FPALM) has localized raft-associated hemagglutinin (HA) to irregular clusters ranging from 40 nm to many μms (Hess et al., 2007). Laminin treatment has been shown to induce the dystroglycan-dependent clustering of the raft-associated GM1 ganglioside into large raft macrodomains. Restricted GM1 diffusion in these domains requiring the actin-associated dystroglycan complex supports a role for organization of raft domains by the extracellular matrix and the underlying actin cytoskeleton (Noel et al., 2009). T and B cell receptor signaling results in the formation of large signaling complexes whose interaction is cholesterol-sensitive and that are associated with DRMs (Cheng et al., 2001; Holowka et al., 2005; Montixi et al., 1998). Raft components such as GM1 are observed by

fluorescence microscopy to cluster together upon receptor ligation in T cells (Gaus et al., 2005; Janes et al., 1999; Viola and Lanzavecchia, 1999) and mast cells (Thomas et al., 1994), a process that involves the actin cytoskeleton (Chen et al., 2007). Extracellular matrix, receptor–ligand interactions and intracellular actin-dependent remodeling can therefore result in the reorganization of raft domains into large optically resolvable structures.

The presence of lipid rafts is not limited to the plasma membrane of the cell. Endoplasmic reticulum (ER) proteins erlin1 and the sigma-1 receptor are found in raft fractions localizing lipid raft microdomains to the ER (Browman et al., 2006; Hayashi and Su, 2003). Due to the increase in sphingolipids and cholesterol along the secretory pathway, lipid rafts are believed to become more abundant in the Golgi complex (Eberle et al., 2002). Cholesterol-sensitive lipid raft microdomains have also been identified on the membrane of endosomes and phagosomes (Dermine et al., 2001; Gagescu et al., 2000; Sobo et al., 2007). Cav1 is also targeted to lipid droplets where it regulates cellular cholesterol homeostasis (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001). Understanding raft domain heterogeneity and organization in the plasma membrane, not to mention their relationship to intracellular raft domains therefore clearly remains a complex and evolving field of study. We can expect the definition of lipid rafts to undergo continuous change as the technology available for their characterization evolves.

3. Caveolae

3.1. Caveolae and caveolin proteins

Caveolae, plasma membrane invaginations of 60–80 nm in diameter, were first identified in the 1950s by electron microscopy (Palade, 1953). Caveolae are expressed in various tissues and cell types such as smooth muscle, fibroblasts, endothelial cells, and adipocytes. The functions of caveolae are diverse and include endocytosis, transcytosis, potocytosis, calcium signaling, and regulation of various signaling events (Parton and Simons, 2007).

The major constituent of caveolae is the protein Cav1 (Rothberg et al., 1992). Cav1 expression in cells devoid of caveolae, such as lymphocytes and CaCo-2 cells, is sufficient to induce caveolae (Fra et al., 1995; Lipardi et al., 1998; Vogel et al., 1998). Mammalian cells express two other isoforms of caveolin (Cav2 and Cav3) with Cav3 being muscle specific. Cav3 is more closely related to Cav1, ~65% identical and ~85% similar, while Cav2 is ~38% identical and ~58% similar to Cav1 (Scherer et al., 1996; Tang et al., 1996). While Cav1 and Cav3 are predominantly expressed on the plasma membrane, Cav2 is localized in the Golgi apparatus and is targeted to the

cell surface and caveolae only upon formation of heterooligomers with Cav1 (Breuza et al., 2002; Li et al., 1998; Mora et al., 1999; Parolini et al., 1999).

All three caveolin proteins share a common topology with N and C termini in the cytoplasm and a long hairpin transmembrane domain (Fig. 3.1). The structure of the three caveolin proteins is almost the same: the amino terminal domain comprises the first 101 amino acid residues in Cav1α and 70–86 residues in Cav1β, Cav2, and Cav3. The transmembrane domain comprises 33 amino acids and the C-terminal part of the protein contains 43–44 amino acids (Fig. 3.2) (Scherer et al., 1995; Williams and Lisanti, 2004). These structural differences impact on protein behavior. Indeed, only Cav1α contain the tyrosine 14 (Y14) residue required for its Src-dependent phosphorylation (Glenney, 1989; Li et al., 1996). Cav1 can also be phosphorylated on serine residue 80 (Ser80) that allows binding of the protein to the ER membrane thereby regulating its entry into the secretory pathway (Schlegel et al., 2001). Cav1 palmitoylation on cysteine residues 133, 143, and 156 allows the protein to oligomerize (Dietzen et al., 1995; Monier et al., 1996). Cav2 is phosphorylated on tyrosine 19 and 27 (Y19 and Y27) (Wang et al., 2004). Cav2 phosphorylation on Y19 upon treatment with insulin increased interaction between pY19Cav2 and pERK while phosphorylation of Cav2 on Y27 prolonged activation of the insulin receptor and promoted nuclear translocation of STAT3 (Kwon and Pak, 2009; Kwon et al., 2009a,b). Cav2 is also phosphorylated

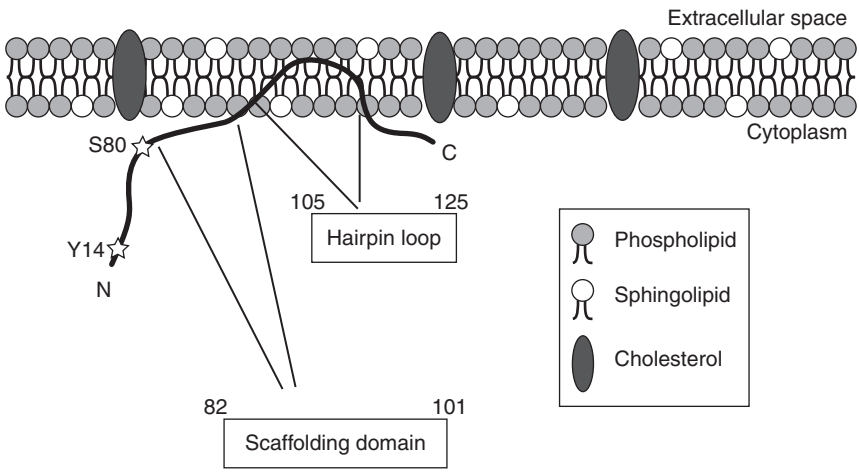

Figure 3.1 Cav1 topology. Cav1 is a 178 amino acid protein inserted into the inner leaflet of the plasma membrane by a hairpin domain resulting in both N- and C-terminal domains facing the cytoplasm. The scaffolding domain (CSD) contains the amino acids 82–101. Cav1 can be phosphorylated on Tyr14 and Ser80.

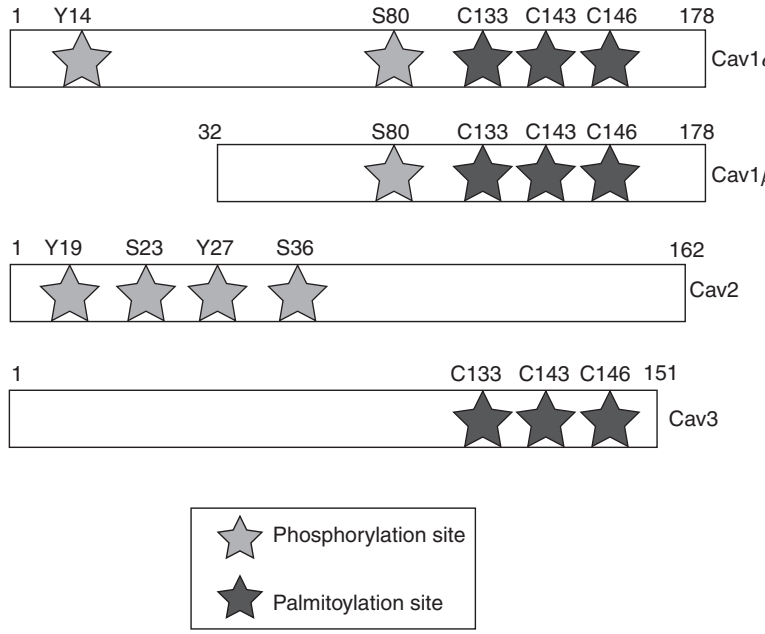

Figure 3.2 Caveolin isoforms. Cav1 is present in two different isoforms: Cav1α and Cav1β. Cav1α contains the full 178 caveolin amino acids while Cav1β is limited to amino acids 32–178. Both isoforms are phosphorylated on S80 and palmitoylated on cysteines C133, C143, and C146. Only Cav1α is phosphorylated on Y14. Cav2 contains amino acids 1–162 (~38% identical) of Cav1 and is phosphorylated on Y19, Y27, S23, and S36. Cav3 contains amino acids 1–151 (65% identical) of Cav1 and is palmitoylated on cysteines C133, C143, and C146.

on Ser23 and Ser36 in a process regulated by protein targeting to lipid raft domains (Sowa et al., 2008).

Caveolin proteins exist as monomers in the Golgi apparatus (Pol et al., 2005) but once in the secretory pathway upon transport to the plasma membrane, they form high-molecular-weight oligomers (Ren et al., 2004). The first step of oligomerization occurs in the ER not long after synthesis of the protein (Monier et al., 1996). Newly synthesized Cav1 is then transported to the Golgi apparatus and at this step, Cav1 association with DRMs has not yet occurred (Pol et al., 2005). Along their subsequent transport through the secretory pathway, Cav1 oligomers form complexes with DRMs, a characteristic of plasma membrane caveolins (Scheiffele et al., 1998). Exit of Cav1 from the Golgi complex is accelerated upon addition of cholesterol (Pol et al., 2005) and inhibited upon glycosphingolipid depletion (Cheng et al., 2006), supporting a role for recruitment to raft domains in Cav1 transport from the Golgi to the cell surface.

Using Cav1–GFP fusion protein, it was shown that Cav1 transits directly from the Golgi complex to the plasma membrane (Tagawa et al., 2005). Based on the size of the structure exiting the Golgi, it was proposed that Cav1 oligomerizes and associates with cholesterol and glycosphingolipids to form exocytic structures similar to mature plasma membrane caveolae. These structures were named ''exocytic caveolar carriers'' (Parton and Simons, 2007). However, the identification of these carriers does not necessarily identify the site of initial formation of caveolae. The slow net transit of caveolin out of the Golgi might be sufficient to allow Cav1 to reach a certain threshold and allow oligomerization and association with lipid rafts (Parton et al., 2006). Certain anti-Cav1 antibodies selectively recognize the Golgi pool of Cav1 but not the protein at the plasma membrane, indicative of a change in Cav1 conformation during its transport to the cell surface. Cholesterol depletion is able to restore this reactivity, suggesting that Cav1 association with rafts may be responsible for differential antibody recognition (Pol et al., 2005).

3.2. Cav1 and the regulation of caveolae formation

The ability of Cav1 to oligomerize is a key determinant of its ability to form caveolae. Cav1 forms higher molecular weight oligomers consisting of 14–16 Cav1 molecules (Monier et al., 1996; Sargiacomo et al., 1995). The 41 residue oligomerization domain preceding the putative transmembrane domain mediates homooligomerization of Cav1 and is required for the formation of large oligomers (Sargiacomo et al., 1995; Schlegel et al., 2000). It is likely that Cav1 oligomerization facilitates the formation of Cav1-enriched microdomains at the cell surface. Tryptophan residues at the membrane interface could further increase cholesterol recruitment and insertion of the scaffolding domain into the membrane, achieving greater expansion of the cytoplasmic leaflet of the caveolar bulb (Parton et al., 2006). Cav2 has also been implicated in caveolae formation, and its serine phosphorylation found to modulate the formation of deep surface attached caveolae versus nonattached caveolar vesicles (Fujimoto et al., 2000; Scheiffele et al., 1998; Sowa et al., 2003). However, while the Cav1 null mouse shows loss of caveolae, caveolae are still present in the Cav2 mouse defining an essential role for Cav1 in caveolae formation in nonmuscle tissues (Drab et al., 2001; Razani et al., 2001, 2002; Zhao et al., 2002). In muscle, Cav3 is essential for caveolae formation and Cav3 mutations and loss of Cav3 and caveolae results in a dystrophic phenotype (Galbiati et al., 2000; McNally et al., 1998; Minetti et al., 1998).

Cav1 expression at the plasma membrane is not sufficient to induce caveolae formation. Cholesterol extraction has been extensively shown to disrupt caveolae at the plasma membrane (Rothberg et al., 1992). Threshold levels of cholesterol are required for caveolae formation and Cav1

incorporation into raft domains at the plasma membrane is a critical determinant of caveolae formation (Breuza et al., 2002; Hailstones et al., 1998). Cav1 binds cholesterol via a putative cholesterol-binding domain and Cav1 has been implicated in cholesterol trafficking and efflux (Fielding and Fielding, 1995; Murata et al., 1995). Interaction of Cav1 with cholesterol may be required for the reorganization of Cav1 oligomers to form invaginated vesicles (Rothberg et al., 1992). Cav1 tyrosine phosphorylation (Y14) has also been recently implicated in caveolae formation in epithelial cells (Orlichenko et al., 2006).

Importantly, another protein, PTRF/cavin-1, is also needed for the formation of caveolae (Fig. 3.3). Cells lacking this protein, but expressing Cav1, present diffuse cell surface Cav1 labeling and fail to express caveolae (Hill et al., 2008). Another member of this protein family, SDPR/cavin-2, is also required for caveolae formation and its overexpression results in the formation of enlarged caveolae and elongated caveolae-derived tubules (Hansen et al., 2009). SRBC/cavin-3 is not required for caveolae formation but does impact on the budding and trafficking of caveolar vesicles (McMahon et al., 2009). A fourth member of the cavin family, MURC/cavin-4, is muscle specific and regulates caveolae formation in muscle (Bastiani et al., 2009). The cavins form a caveolin-associated protein complex (Bastiani et al., 2009; Hansen et al., 2009) and how this protein complex functions to regulate caveolae expression and caveolin function is a field of active investigation that should provide important new insight into caveolae function (Nabi, 2009).

3.3. Cav1 domains outside of caveolae: Cav1 scaffolds

The ability of various modulators and interactors to impact on Cav1 ability to form caveolae indicates that formation of caveolae is a highly complex and regulated cellular process. At the same time, the fact that expression of Cav1 is not sufficient for caveolae formation indicates that Cav1 is present in cells lacking caveolae and should necessarily have functions outside of caveolae. Indeed, there is growing evidence in the literature that Cav1 regulates cellular processes outside of caveolae (Head and Insel, 2007; Lajoie et al., 2009a; Parton and Simons, 2007)

Cav1 contains a scaffolding domain that has the ability to bind various classes of signaling proteins such as G-protein subunits, receptor and non-receptor tyrosine kinases, endothelial nitric oxide synthase (eNOS), and small GTPases (Okamoto et al., 1998; Parton and Simons, 2007). Indeed, interaction of Cav1 with a variety of signaling receptors results in inhibition of their signaling potential (Goetz et al., 2008b). The extent to which this regulatory activity of Cav1 requires expression of caveolae is unclear. Cav1 can regulate epidermal growth factor receptors (EGFR) diffusion and signaling and CT-b diffusion and endocytosis at levels below the threshold

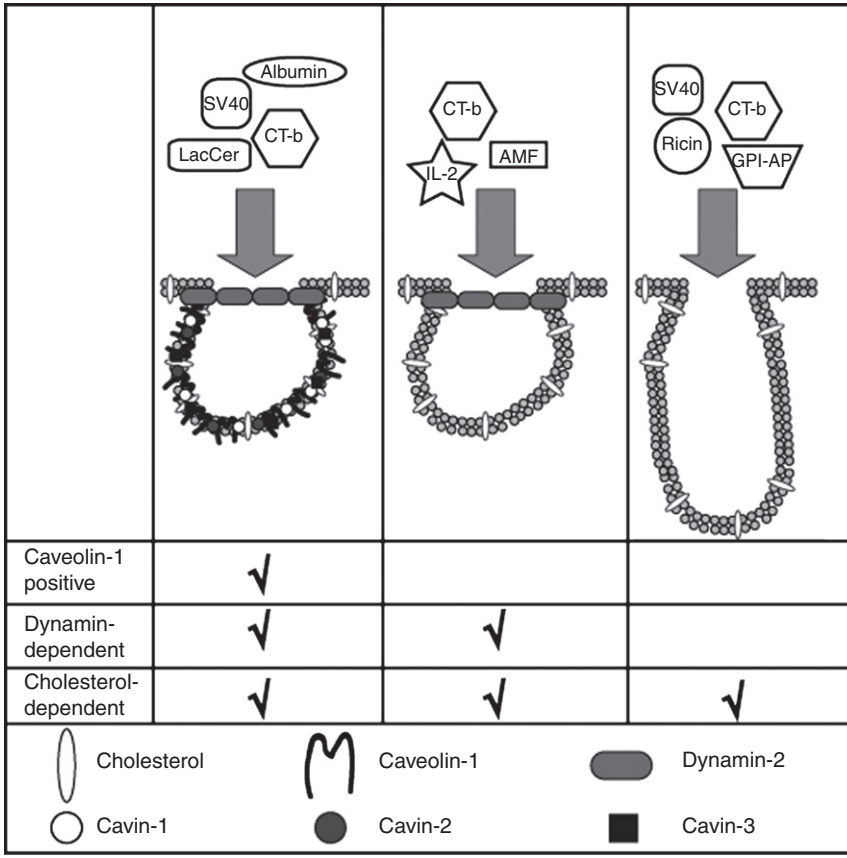

Figure 3.3 Raft-dependent endocytosis encompasses various pathways. Cholesterol-sensitive internalization of lipid rafts can be classified into three major pathways. These include dynamin-dependent endocytosis of caveolae or noncaveolar vesicular carriers as well as dynamin-independent endocytosis via noncaveolar tubular intermediates. Raft-dependent internalization of specific receptors or molecules may occur via multiple pathways, each involving different vesicular intermediates and regulated by specific endocytic machineries.

required for caveolae formation (Lajoie et al., 2007, 2009a). Cav1 can also restrict CT-b diffusion on the endosomal membrane (Pelkmans et al., 2004). Interestingly, endothelial-specific overexpression of Cav1 impaired VEGF signaling without impacting on caveolae number (Bauer et al., 2005). Tyrosine phosphorylated Cav1 has been implicated in focal adhesion organization and dynamics (Gaus et al., 2005; Goetz et al., 2008a; Grande-Garcia et al., 2007; Joshi et al., 2008). These studies describing caveolae-independent functions for Cav1 led to the definition of Cav1 scaffolds, a

term to describe oligomeric Cav1 domains present at the cell surface that do not correspond to invaginated caveolae (Lajoie et al., 2009a). Cav1 and phosphorylated Cav1 may define functionally distinct scaffold domains (Lajoie et al., 2009a). Recently, it was shown that Cav1 and Cav2 form a plasma membrane scaffold containing 15–25 caveolins molecules that can associate with cavins to form caveolae (Hayer et al., 2009). The identification of the cavins may facilitate further study of the selective functions of caveolae and Cav1 scaffolds.

Caveolae therefore represent a subdomain of lipid raft microdomains that are stabilized by caveolin proteins. Lipid rafts are defined as cholesterol- and sphingolipid-enriched membrane domains and numerous studies have attempted the lipid characterization of caveolae and raft domains. Early immunoelectron microscopy studies identified GM1 enrichment in caveolae (Schnitzer et al., 1995). However, GM1 has also been localized to raft domains that associate with nascent clathrin-coated pits (Puri et al., 2005). Analysis of purified caveolae fractions found that the glycosphingolipid GD3 was highly enriched in caveolae, GM3, GM1, and GD1α present inside and outside caveolae membranes and various other glycosphingolipids localized exclusively outside the caveolae fraction (Ortegren et al., 2004). By confocal microscopy, distribution of GM1 and another well-characterized raft marker, GPI-APs, do not overlap with Cav1 (Lajoie et al., 2009b; Noel et al., 2009). Another raft marker, flotillin, defines noncaveolar rafts that can contain GPI-APs and GM1 (Fernow et al., 2007; Glebov et al., 2006; Noel et al., 2009; Stuermer et al., 2001). Raft-associated lipids and proteins therefore exhibit a heterogeneous distribution between caveolar and noncaveolar raft domains.

4. Raft-Dependent Endocytosis

4.1. Multiple rafts for multiple pathways

Internalization of molecules and ligand via clathrin-coated vesicles has been extensively studied and well characterized (Benmerah and Lamaze, 2007; Roth, 2006). However, over the years, many other endocytic routes have been described and do not rely on the formation of clathrin-coated pits. Characterization of these alternate pathways has allowed a better understanding of the complexity of the endocytic process in mammalian cells (Fig. 3.3). Many of these clathrin-independent pathways share an important characteristic: their sensitivity to cholesterol depletion. These pathways rely on internalization of dynamic lipid-rich carriers and are therefore regrouped under the appellation raft-dependent endocytosis.

4.1.1. Caveolae

Although many raft-dependent pathways have been shown to not rely on the presence of caveolin proteins, various molecules or ligand are internalized via caveolae. In endothelial cells, caveolae represent the major route of transcytosis-transporting albumin (Tiruppathi et al., 1997). The albumin docking protein gp60 localizes to caveolae in endothelial cells and thereby activates plasmalemmal vesicle formation and the directed migration of vesicles (Minshall et al., 2000). Modification of plasma albumin on tyrosine residues generates nitrated albumin (NOA) that can be transported across the vascular endothelium via caveolae-dependent transcytosis (Predescu et al., 2002). $G_{\beta\gamma}$ activation of Src kinase signaling induced caveolae-mediated endocytosis and transendothelial albumin transport via transcytosis (Shajahan et al., 2004b) in a process that involves dynamin-2 phosphorylation (Shajahan et al., 2004a). Caveolae-dependent transcytosis of albumin is dependent on the F-actin cross-linking protein filamin A (Sverdlov et al., 2009). Cav1 phosphorylation also regulates H_2O_2-induced pulmonary vascular albumin hyperpermeability via transcytosis (Sun et al., 2009). Increased endothelial caveolae leading to transcytosis of plasma proteins is associated with blood–brain barrier (BBB) breakdown and it is suggested that Cav1 phosphorylation may be one of the key factors involved in this kind of brain injury (Nag et al., 2009). Using intravital microscopy, it was shown that caveolae successfully transport antibody against aminopeptidase P (APP) from the blood across the endothelium into lung tissue (Oh et al., 2007).

Dynamin is a protein involved in the fission of some endocytic vesicles from the plasma membrane (Henley et al., 1999). Caveolae budding from the plasma membrane and subsequent internalization requires dynamin II which is localized at the neck of the vesicle (Oh et al., 1998). Intersectin is another protein that has been shown to be involved in the fission of nascent caveolae from the plasma membrane (Predescu et al., 2007). It was shown that the intersectin is part of a protein complex containing dynamin and SNAP-23 and is localized at the neck of the budding vesicle (Predescu et al., 2003). It was recently shown that intersectin-2L, a guanine nucleotide exchange factor for Cdc42, regulates caveolae endocytosis by regulating actin polymerization and remodeling at the plasma membrane (Klein et al., 2009).

In nonendothelial cells, fluorescent glycosphingolipid analog, BODIPY-lactosylceramide (LacCer) has been shown to be specifically internalized via a caveolae-dependent pathway (Sharma et al., 2003). Caveolae-dependent endocytosis has been shown to be regulated by both gangliosides and β1-integrin expression (Singh et al., 2010). In primary adipocytes, the insulin receptor undergoes rapid internalization via caveolae (Fagerholm et al., 2009). SV40 is endocytosed by caveolae and subsequently targeted to a Cav1 positive endosomal compartment: the caveosome (Pelkmans et al., 2001). However, more recent studies suggest that SV40 is internalized

predominantly via a noncaveolar, dynamin-independent route (Damm et al., 2005; Ewers et al., 2010). Similarly, early studies demonstrated that CT-b may be internalized in a process that involves caveolae (Henley et al., 1998; Oh et al., 1998; Parton et al., 1994). However, more recent studies suggest that the major raft-dependent endocytic route for CT-b is noncaveolar (Kirkham et al., 2005; Lajoie et al., 2009b). The recent demonstration that SV40 binding to GM1 can induce membrane invaginations suggests that interaction of SV40 and potentially cholera toxin with GM1 may induce membrane curvature that promotes endocytic uptake (Ewers et al., 2010). This process does not require Cav1; however, tubule formation by Shiga toxin, whose receptor is the ganglioside GM3, has been shown to derive from caveolae and be modulated by SDPR/cavin-2 defining a potential role for Cav1 and cavins in raft-dependent endocytic processes (Hansen et al., 2009; Romer et al., 2007). Therefore, at least in nonendothelial cells, the role of caveolae as a major endocytic carrier is questionable and other raft-dependent endocytic pathways, independent of caveolae, may play a more predominant role in endocytosis.

Most studies of caveolae endocytosis are based on live cell imaging techniques using Cav1 fluorescent protein chimeras. While useful, fluorescent proteins must be used with care and data analyzed with caution (Snapp, 2009). Cav1–GFP has been shown to induce caveolae and may favor localization of the protein of interest into caveolae. Furthermore, in transiently transfected overexpressing cells, it is unclear how much of the expressed Cav1–GFP protein is actually in caveolae and studies of Cav1 dynamics, and presumably caveolae, may actually be visualizing noncaveolar Cav1–GFP. Photobleaching experiments have revealed that Cav1 is highly stable at the plasma membrane (Thomsen et al., 2002), questioning their role in constitutive endocytosis (Hommelgaard et al., 2005). In contrast, clathrin-coated pits, for instance, are highly mobile and the entire pool of cell surface clathrin-coated vesicles is turned over within 6 min (De Bruyn and Cho, 1987). Indeed, it was proposed that Cav1 may stabilize raft domains at the plasma membrane, thus acting as a negative regulator of raft-dependent endocytosis (Lajoie and Nabi, 2007; Nabi and Le, 2003). Caveolar endocytosis may represent a minor, regulated pathway restricted to specific ligands. Internalization of ligands via caveolae may depend on various factors, such as caveolin, cavins, and ganglioside and cholesterol levels.

4.1.2. Noncaveolar dynamin-dependent endocytosis

Autocrine motility factor (AMF) is another ligand that has been shown to be localized to caveolae and internalized via a dynamin-dependent pathway (Benlimame et al., 1998; Le et al., 2002). Internalization of this protein is enhanced in cells expressing reduced Cav1 levels, indicating that it is internalized via a noncaveolar dynamin-dependent pathway (Kojic et al.,

2007, 2008; Le et al., 2002). Formation of dynamin-dependent smooth plasma membrane vesicles occurs both in the presence or absence of caveolins, indicating that dynamin regulates the formation of raft-derived endocytic carriers independently of Cav1 (Le et al., 2002). Ultrastructural and biochemical experiments showed that clathrin-independent endocytosis of IL2 receptors exists constitutively in lymphocytes and is coupled to their association with DRMs. This raft-dependent pathway requires dynamin and is specifically regulated by Rho family GTPases (Lamaze et al., 2001). Raft-dependent internalization of CT-b from the plasma membrane has also been shown to occur via a noncaveolar dynamin-dependent process (Lajoie et al., 2009b). Flotillin-1 resides in punctate structures within the plasma membrane and in a specific population of endocytic intermediates. These intermediates accumulate both GPI-linked proteins and CT-b. siRNA against flotillin-1 switched the internalization of CT-b from a dynamin-dependent pathway to a dynamin-independent endocytic route (Glebov et al., 2006).

4.1.3. Noncaveolar dynamin-independent endocytosis

Evidence for a clathrin-independent noncaveolar pathway (or clathrin-independent carrier (CLIC)/GPI-AP-enriched early endosomal compartment (GEEC) pathway) came from analysis of cells expressing a temperature-sensitive mutant form of dynamin. Interestingly, blocking clathrin (and presumably caveolae)-mediated endocytosis caused an initial block in fluid phase internalization followed by a gradual compensatory increase. After 30 min fluid phase uptake returned to the same level as before the temperature shift while receptor-mediated endocytosis remained blocked (Damke et al., 1994). Pinocytic fluid phase uptake of multiple GPI-APs was also shown to rely on a noncaveolar raft endocytic pathway independent of dynamin and RhoA (Sabharanjak et al., 2002). This pathway is regulated by ARF1 and CDC42 (Kumari and Mayor, 2008).

More recently, it was shown that the dynamin-independent internalization of CT-b in mouse embryonic fibroblasts occurred predominantly via uncoated tubular or ring-shaped vesicular carriers containing GPI-APs but not Cav1 (Kirkham et al., 2005). Dynamin-independent raft pathways also include a noncaveolar endocytic route for the simian virus SV40 (Damm et al., 2005; Ewers et al., 2010). CT-b endocytosis via a dynamin-independent raft pathway was flotillin-1-dependent, suggesting that flotillin may define raft domains that internalize via this dynamin-independent route (Glebov et al., 2006). Cholesterol is required for the membrane localization of activated Rac1, actin reorganization, membrane ruffling, and macropinocytosis potentially defining another raft-dependent dynamin-independent pathway (Grimmer et al., 2002; Schneider et al., 2007).

Multiple raft-dependent endocytic pathways therefore exist reflecting the heterogeneity of lipid raft microdomains at the cell surface (Hancock,

2006). Many pathways have been characterized and a number of raft endocytic ligands are internalized via different endocytic routes. This varies among cell types and is dependent on receptor expression, ligand concentration, and other factors. For example, transforming growth factor beta (TGFβ) is internalized via both clathrin-coated pits and caveolae (Di Guglielmo et al., 2003). Stimulation with low doses of epidermal growth factor (EGF) leads to receptor internalization via clathrin-mediated endocytosis, while higher dose induces its internalization via a raft-dependent pathway (Sigismund et al., 2005). AMF can be internalized via clathrin-coated pits as well as rafts in NIH-3T3 cells (Benlimame et al., 1998; Le et al., 2000). Internalization via different endocytic pathways is also a characteristic of CT-b. In different cell types, it can be internalized via both clathrin and raft-dependent endocytosis (Pang et al., 2004; Torgersen et al., 2001). Intriguingly, selectivity of GPI-APs for dynamin-independent, noncaveolar raft uptake appears to be related not to the lipid anchor but rather to steric considerations of the anchored protein (Bhagatji et al., 2009). This suggests that multiple elements, including relatively nonspecific ones such as steric bulk, can contribute to selection of the route of endocytosis.

4.2. Regulation of raft-dependent endocytosis

4.2.1. Negative regulation by Cav1

Cav1 is known for its role in vesicle formation and subsequent internalization of cargo via caveolae-mediated endocytosis. However, there is growing evidence that Cav1 can also act as a negative regulator of raft-dependent endocytosis (Lajoie and Nabi, 2007; Nabi and Le, 2003). Indeed, it was shown that Cav1 can reduce or inhibit CT-b internalization via raft pathways (Kirkham et al., 2005; Lajoie et al., 2009b; Le and Nabi, 2003). It was also shown that Cav1 overexpression inhibits AMF uptake (Kojic et al., 2007; Le and Nabi, 2003; Le et al., 2002), dysferlin uptake (Hernandez-Deviez et al., 2008), and raft-dependent integrin endocytosis (Vassilieva et al., 2008). Cav1 overexpression is also associated with inhibition of endocytosis of albumin in HeLa cells (Sharma et al., 2004a).

Interestingly, the ability of Cav1 to inhibit endocytosis is not necessarily caveolae-associated supporting a role for Cav1 scaffolds in this process. In mammary tumor cells, Cav1 levels below the threshold required for caveolae formation is sufficient to reduce CT-b internalization (Lajoie et al., 2009a). Also, overexpression of Cav1 is responsible for inhibition of internalization of dysferlin without direct interaction with the protein (Hernandez-Deviez et al., 2008). These data support the idea of an indirect mechanism for the negative regulatory activity on Cav1. Presence or absence of Cav1 may modulate the protein and lipid composition of cell surface rafts, therefore regulating their endocytic potential. Raft endocytic

ability may be regulated by a dynamic equilibrium between cholesterol, lipids, and protein within these domains.

4.2.2. The role of cholesterol

Cholesterol is a major constituent of lipid rafts and its concentration at the plasma membrane generally regulates raft-dependent phenomena such as signaling and endocytosis. Cholesterol is one of the key factors determining long-range protein mobility at the cell surface (Kenworthy et al., 2004). Cholesterol can regulate caveolae formation and decreased total cellular cholesterol levels were associated with similar decreases in Cav1 protein concentration in cholesterol-depleted cells (Breuza et al., 2002; Hailstones et al., 1998). Cholesterol-modulating agents, including methyl-β-cyclodextrin, nystatin, and filipin, have been variously shown to inhibit both caveolae expression and raft-dependent endocytosis (Damm et al., 2005; Hailstones et al., 1998; Le et al., 2002; Parpal et al., 2001; Schnitzer et al., 1994; Sharma et al., 2004a). Cav1 interacts directly with cholesterol (Fielding et al., 2002; Murata et al., 1995) and cholesterol levels in lipid raft fractions obtained from Cav1 expressing cells were three- to fourfold higher than in matched cells lacking Cav1 (Smart et al., 1996). When cholesterol exit from the ER is inhibited, Cav1 accumulates in the ER identifying a role for cholesterol in Cav1 trafficking to the plasma membrane (Smart et al., 1996). Increased total cholesterol by acute treatment with cyclodextrin/cholesterol or by alteration of growth conditions was shown to significantly stimulate caveolar endocytosis (Sharma et al., 2004a). In heterokaryons containing GFP- and RFP-tagged Cav1, individual caveolar domains undergo little exchange of Cav1. However, when the cells were subjected to transient cholesterol depletion, the caveolar domains were found to exchange Cav1 (Tagawa et al., 2005). Cholesterol therefore represents a major regulator of raft endocytosis and critically impacts on Cav1 expression, dynamics, and ability to form caveolae.

4.2.3. The actin cytoskeleton

Another key regulator of raft-dependent endocytosis is the actin cytoskeleton. In fact, it was shown that complete depolymerization of the actin cytoskeleton with latrunculin A induces rapid and massive movements of caveolin-positive structures toward the centrosomal region of the cell (Mundy et al., 2002). This is in agreement with findings showing that caveolae are stable plasma membrane compartments stabilized by the actin skeleton. Indeed, using fluorescence recovery after photobleaching, it was shown that while intracellular structures labeled with GFP-tagged caveolin were dynamic, GFP-labeled caveolae were highly immobile (Thomsen et al., 2002). Cav1 can bind to actin-associated filamin via the N-terminal region of Cav1 and the C terminus of filamin close to the filamin-dimerization domain

(Stahlhut and van Deurs, 2000). Interestingly, after binding to caveolae, SV40 induced transient breakdown of actin stress fibers. Actin was then recruited to SV40 positive caveolae as actin patches (Pelkmans et al., 2002). More recently, TIRF microscopy was used to show that reversible caveolae budding is limited to the subplasma membrane region by the underlying actin cytoskeleton (Tagawa et al., 2005). Cell detachment from substrate induces clearance of rafts from the cell surface in a Cav1-dependent endocytic process that requires F-actin (Balasubramanian et al., 2007; del Pozo et al., 2005). However, disruption of the actin cytoskeleton by cytochalasin D in A431 cells inhibited alkaline phosphatase uptake via caveolae (Parton et al., 1994). The actin cytoskeleton was also shown to be a critical regulator of the internalization of the antigen–BCR complex (Caballero et al., 2006). The bacteria *Porphyromonas gingivalis* is also thought to be internalized via a pathway requiring both rafts and an intact actin skeleton (Tsuda et al., 2008). Actin depolymerization also induces internalization of tight junction proteins via a caveolae-dependent pathway (Shen and Turner, 2005). Interestingly, when treated with PDGF, 23 proteins show increased raft localization including cytoskeleton proteins such as actin (MacLellan et al., 2005). Using TIRFM, mild cholesterol depletion was found to perturb actin polymerization by preventing CDC42 activation and thereby inhibiting endocytosis of GPI-APs through the GEEC pathway (Chadda et al., 2007). Raft domains therefore function together with the actin cytoskeleton to regulate raft endocytosis.

4.3. Raft endocytosis and modulation of signaling cascades

Many proteins are localized to lipid rafts and these domains have been described as platforms for cellular signaling (Hancock, 2006; Simons and Gruenberg, 2000). Indeed, a quantitative proteomic approach has shown that many signaling molecules are enriched in rafts (Foster et al., 2003). Cav1 possesses a scaffolding domain implicated in the sequestration of receptors and signaling molecules (Liu et al., 2002; Okamoto et al., 1998). In quiescent fibroblasts, EGF and EGFR are initially concentrated in caveolae but rapidly move out of this membrane domain after stimulation with ligand, supporting the idea that caveolae represent a negative regulatory domain (Mineo et al., 1999). EGF signaling was inhibited in senescent cells expressing higher Cav1 levels (Park et al., 2000). In mammary tumor cells, competition exists between various cell surface domains for localization of EGFR and association with Cav1 scaffolds suppresses its signaling potential (Lajoie et al., 2007). A 60-amino acid-long sequence that is continuous with the transmembrane domain is sufficient to target both the transmembrane and cytoplasmic tails of EGFR to caveolae/rafts (Yamabhai and Anderson, 2002). Indeed, Cav1 can induce the

sequestration of the receptor in a cholesterol-sensitive way (Matveev and Smart, 2002). Cav1 also inhibits EGFR-stimulated lamellipod extension and cell migration in metastatic mammary adenocarcinoma cells (MTLn3) (Zhang et al., 2000). However, EGFR was shown to be present in a caveolin-rich fraction from A431 cells, but failed to coimmunoprecipitate with caveolin, indicating that the Cav1 effect on EFGR signaling may be indirect (Waugh et al., 1999). Phosphorylated EGFR also did not coimmunoprecipitate with Cav1, arguing against a caveolar location for EGFR signaling (Waugh et al., 1999). Moreover, it is thought that cholesterol may regulate EGFR signaling independently of Cav1 by modulating the content of lipid rafts (Pike and Casey, 2002; Pike et al., 2005; Westover et al., 2003). Indeed, domain competition for recruitment of EGFR between lipid rafts and Cav1 scaffolds may be regulated by its activation state as well as glycosylation and recruitment to galectin lattice domains (Lajoie et al., 2007; Puri et al., 2005). Similarly, TGFβR internalization via clathrin-dependent endocytosis correlates with subsequent signaling events via Smad2 phosphorylation in EEA1-positive endosomes while its caveolae/raft-dependent internalization is associated with receptor degradation through binding to the smad7–smurf2 complex (Di Guglielmo et al., 2003).

Signaling cascades also impact on raft-dependent endocytosis by regulating the internalization of various endocytic carriers. Inhibition of tyrosine phosphorylation using pharmaceutical agents blocks caveolae endocytosis while treatment of the cells with phosphatase inhibitor okadaic acid triggers endocytosis (Kirkham et al., 2005; Parton et al., 1994; Pelkmans et al., 2002). Treatment of the cells with albumin was shown to induce Cav1 phosphorylation and inhibition of this process was associated with reduced uptake of albumin via transcytosis (Tiruppathi et al., 1997). Inhibition of tyrosine phosphorylation using genistein inhibits uptake of AMF in various tumor cell lines (Kojic et al., 2007, 2008). The same results were described for internalization of lactosylceramide (Sharma et al., 2004a). While the initial recruitment of SV40 to caveolae was not dependent on tyrosine phosphorylation, tyrosine kinase inhibition prevented the dynamin-dependent release of caveolae from the membrane (Pelkmans et al., 2002). Albumin treatment induced Src activation and resulted in Src-dependent tyrosine phosphorylation of dynamin-2. The Y231F/Y597F dynamin-2 mutant expression also resulted in impaired albumin and cholera toxin subunit B uptake and reduced transendothelial albumin transport (Shajahan et al., 2004a). How activation and regulation of endocytic and signaling events within raft domains regulate cellular processes is a subject of intense investigation that may contribute to a better understanding of the functional significance of raft domain localization and endocytosis at the plasma membrane.

5. Conclusion

Lipid rafts are cholesterol- and sphingolipid-rich membrane domains that vary from highly transient nanoscale domains to larger, more stable structures. Caveolin proteins are key regulators of lipid raft domain expression that induce the formation of invaginated caveolae but also noncaveolae scaffold domains that regulate raft function and endocytosis. Raft endocytosis is regulated not only by Cav1 but also by cholesterol and the actin cytoskeleton and may play critical roles in the regulation of cellular signaling.

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