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Review

Intracellular trafficking of raft/caveolae domains: Insights from integrin signaling

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Abstract

Cells have a complex system for delivering and compartmentalizing proteins and lipids in order to achieve spatio-temporal coordination of signaling. Rafts/caveolae are plasma membrane microdomains that regulate signaling pathways and processes such as cell migration, polarization and proliferation. Regulation of raft/caveolae trafficking involves multiple steps regulated by different proteins to ensure coordination of signaling cascades**.** The best studied raft-mediated endocytic route is controlled by caveolins. Recent data suggest integrin-mediated cell adhesion is a key regulator of caveolar endocytosis. In this review we examine the regulation of caveolar trafficking and the interplay between integrins, cell adhesion and caveolae internalization.

Keywords: Integrins; Rafts; Caveolae; Vesicle traffic; Signaling

Contents

Abbreviations: DRM, detergent resistant membranes; ECM, extracellular matrix; Pak, p21 activated kinase; MEF, mouse embryo fibroblast; FRET, fluorescence resonance energy transfer; EM, electron microscopy; GPI-AP, glycosylphosphatidylinositol-achored proteins

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1. Introduction: rafts, caveolae and trafficking

In mammalian cells lipids and proteins are being constantly shipped from one cell compartment to another as a basal mechanism to organize the structure of the cell. In addition, lipid and protein traffic is implicated in the regulation of the intensity and amplitude of many signaling pathways that regulate key processes such as cell migration, cell cycle, and cell polarity. To regulate the spatio-temporal coupling of effectors and activators, the cell ensures compartmentalization and signaling specificity by selectively partitioning proteins and lipids in specific membrane domains. Plasma membrane domains that have attracted attention from many fields are the so-called "lipid rafts". It is difficult to precisely define rafts, although a definition was attempted at a recent Keystone symposium: "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions" [1]. These membrane domains organize proteins and lipids to regulate the intensity of multiple signaling cascades and membrane traffic [2,3]. Besides cholesterol, rafts are also rich in sphingolipids, including ganglioside GM1, and lipid-modified proteins such as caveolins, flotillins, Src-family kinases, and glycosylphosphatidylinositol (GPI)-linked proteins. There is considerable uncertainty about how raft microdomains are formed in cell membranes and how to estimate their exact size, density, composition and stability [4–8]. Indeed, the technical difficulties of visualizing native raft domains in living cells has led to their very existence being questioned [9], whereas the existence of protein-based membrane subdomains is more generally accepted [10,11]. In fact, much of the controversial data could be reconciled by the notion that small and transitory membrane microdomains can be stabilized by proteins or other interactors [12]. Resolution of this complex biological question will undoubtedly require a combination of approaches, including both model membrane systems and powerful imaging techniques applied to membranes in living cells.

One type of raft that is susceptible to fission/fusion with the plasma membrane is exemplified by caveolae [13]. Caveolae are cholesterol and sphingolipid-rich plasma membrane invaginations of a diameter of 60–80 nm, and are distinct from other coated vesicles [13,14]. Biochemically, caveolae partition into raft fractions (the detergent insoluble fractions of cold lysates resolved on sucrose gradients) and contain numerous signaling molecules, including Src family tyrosine kinases, growth factor receptors, GPI-anchored proteins and flotillins [14]. Caveolae are thus enriched in lipids and proteins that partition into raft domains. Caveolin-1 is the main protein component of caveolae, at least functionally speaking, and is required for caveolae biogenesis, since virtually all cell types derived from caveolin-1 knockout mice are devoid of caveolae [15,16]. Caveolin-1, but not caveolin-2, is required for caveolae formation in non-muscle cells, and caveolin-3 is required for caveolae formation in muscle cells. Endocytosis of caveolae is involved in multiple biological processes, including regulation of plasma membrane composition and spatio-temporal regulation of signaling molecules and their effectors [17]. Many signaling molecules are associated with caveolae, suggesting that caveolae compartmentalize signaling molecules and serve as platforms that integrate the regulation of their activity, localization and/or effector coupling [14,18]. In addition, caveolin-1 is postulated to play an important role in anchorage-dependent cell growth. Indeed a putative tumor suppressor action of caveolin-1 is supported by studies in caveolin-1$^{-/-}$ null mice and by the occurrence of caveolin-1 mutations in human breast cancer. However, caveolin-1 may function as a tumor promoter in prostate cancers (reviewed in Ref. [19]). Thus, the effects of caveolin on growth regulatory pathways appear to be multifold and cell-type specific; the solution to this enigma is the subject of very intense research.

Although caveolae are the best characterized vehicle for raft trafficking, other as yet poorly characterized vehicles are beginning to be elucidated. Certain viruses and proteins that were initially believed to enter the cell through caveolae are able of enter cells devoid of caveolins, in a process independent of clathrin and dependent on cholesterol and actin cytoskeleton. These recent findings suggest that there are other entry routes that share many similarities with caveolae-mediated endocytosis [20–23]. We will refer to all these pathways collectively as raft/caveolae-dependent pathways. Many signaling cascades have been shown to be regulated by the localization of key cascade elements in raft/caveolae membrane microenvironments [2,3]. In this way, trafficking of these domains affects the amplitude and the on–off status of many signaling pathways [2,17]. By modulating numerous pathways, these domains contribute to the regulation of cell migration, the cell cycle, cell polarity, apoptosis, and transcription [2,3,24–26].

Understanding raft/caveolae formation and trafficking is crucial to understanding their function in signaling and tumor and cell biology. In this review we will focus on the traffic of rafts and caveolae, the major carrier of lipid domains, its regulation, and the interplay between trafficking of these membrane domains and integrin/cell adhesion.

2. Exocytosis of rafts/caveolae

2.1. Introduction: exocytosis, SNAREs and rafts

Exocytosis consists of the fusion of vesicles with the plasma membrane, allowing the incorporation of proteins and lipids into the plasma membrane and the secretion of the vesicle contents from the cell to the extracellular medium [27]. Exocytosis can occur constitutively or in a tightly regulated way. Constitutive exocytosis operates in all cells and the vesicles derive from the *trans*-Golgi network (TGN). Regulated exocytosis takes place in many cell types upon receipt of a specific stimulus, such as a local and transient increase in calcium levels. "Kiss-and-run" exocytosis occurs in a transient fusion pore, through which only part of the vesicle content is released, after which the pore is closed and the vesicle is released back to the cytoplasm.

Exocytosis requires the participation of a large number of proteins, which are highly conserved in eukaryotic cells [27].

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins mediate membrane fusion and are essential for exocytosis. SNARE proteins present on opposing vesicle and plasma membranes form four helical bundles through their SNARE motifs. This protein association brings the membranes into close apposition and supplies the force required for fusion. The many different SNARE proteins localize to specific membrane compartments, contributing to the specificity of membrane fusion. SNARE proteins are strictly regulated through interactions with regulator proteins in a spatially and temporally controlled manner (for a detailed review see Refs. [28,27]).

2.2. Role of rafts in SNARE-mediated exocytosis

The central questions that remain unanswered are whether there are preferential plasma membrane regions for vesicle fusion and how exocytotic vesicles are targeted to the right place. It has been proposed that proper spatiotemporal regulation of membrane fusion is partially determined by cholesterol and sphingolipid-rich microdomains, that is rafts [29]. The possible role of rafts in regulated exocytosis is suggested by the distribution of SNARE proteins at detergent resistant membranes (DRM-s). The association of SNARE proteins with rafts has been documented in Madin-Darby canine kidney (MDCK) cells [30], in PC12 cells [29], and in 3T3-L1 adipocytes, RBL mast cells, HeLa cells, and brain sinaptosomes [31,32]. These studies showed that SNARE proteins are clustered in raft domains, possibly defining the exocytic plasma membrane sites [27]. In addition, cholesterol depletion blocks the formation of secretory vesicles, both regulated and constitutive, by the TGN of neuroendocrine AtT-20 cells [33], and sphingolipid depletion results in the missorting of prohormone convertase 2, a protease of the regulated secretory pathway involved in the intracellular maturation of prohormones [34]. These studies strongly suggest that the integrity of rafts is important for exocytosis. However, other studies have questioned the presence of SNAREs in rafts, since they cluster in cholesterol-rich microdomains that are soluble in cold Triton-X-100 and do not co-localize with raft markers such as GPI-linked proteins or sphingomyelin [35]. These studies also showed that cholesterol depletion inhibits exocytosis, indicating that the cholesterol-dependent SNARE clusters define the vesicle docking sites and are required for membrane fusion and exocytosis. The requirement for SNARE clusters suggests that a high local concentration of SNARE monomers is necessary to establish the fusion site. Four different possibilities for raft function in regulated exocytosis have been proposed: (i) exocytosis occurs exclusively in raft domains; (ii) exocytosis occurs exclusively in nonraft domains; (iii) raft and nonraft domains support different types of exocytosis (full fusion vs. kiss-and-run exocytosis); (iv) the different domains regulate the fusion of different types of specific vesicle. The molecular mechanism underlying domain-specific fusion might involve the intrinsic lipid composition, the protein accumulation/exclusion and/or SNARE conformation [27]. However, the exact contribution of rafts to exocytosis is unknown and deserves further research.

2.3. Sorting of rafts/caveolae

Rafts are one of the mechanisms employed by cells to distribute membrane proteins. Indeed, the raft hypothesis was originally proposed to explain the segregation of lipids and membrane proteins during their distribution in polarized epithelia. Their role has been documented both in the basolateral distribution from the Golgi complex and in the transport of proteins from the TGN to the apical plasma membrane in polarized MDCK [36]. Experiments showing that cholesterol depletion decreases apical transport capacity without affecting basolateral sorting suggested that rafts were mainly involved in apical transport [37]. However, rafts have also been found in basolateral membranes. Most caveolae localize to the basolateral domain of epithelial cells, giving rise to one of the most notable ultrastructural differences between the apical and basolateral plasma membranes in polarized MDCK cells. The basolateral localization of caveolae has been corroborated in experiments demonstrating that caveolin-2 localizes only to the basolateral surface, whereas caveolin-1 is present both at apical and at basolateral poles. Caveolin-1 and -2 are both sorted to basolateral vesicles, whereas caveolin-1 is also targeted to the apical pole from the TGN, probably traveling in as-yet uncharacterized non-caveolar carriers, as recently proposed by Ref. [13]. Caveolin is cotranslationally translocated into the ER membrane and its ability to form caveolae is acquired through post-translational modification during its intracellular transport. Oligomerization of caveolin, which is required for caveolar biogenesis, begins in the ER. Caveolin complexes subsequently undergo a number of changes during their transport to the cell surface, including increases in caveolin oligomer size, a decrease in solubility owing to the association with cholesterol and sphingolipids, and an increase in the phosphorylation of caveolin-2 [38,39]. This agrees with evidence that neither caveolae nor rafts are present in the ER. In fact, assembled caveolar domains first appear in the Golgi complex [40]. The behaviour of raft proteins on the surface of polarized MDCK cells has also been analyzed by antibody cross-linking [41]. Antibody cross-linking of raft proteins at the apical plasma membrane induced only small clusters, in contrast with the large clusters produced at the basolateral plasma membrane.

2.4. Regulation of caveolar exocytosis

The SNARE protein Syntaxin-6 has been shown to regulate transport from the Golgi complex to the plasma membrane of caveolin-1, GPI-anchored protein and GM1 [42]. In fact, caveolin-1 and Syntaxin 6 co-localize at the Golgi in human skin fibroblasts, while in endothelial cells, caveolin-1 co-clusters with the *t*-SNARE proteins SNAP-23 and Syntaxin-4 at the plasma membrane (Fig. 1). Formation of *t*-SNARE clusters depends on cholesterol and caveolin-1. Co-localization of caveolin-1 with the organized *t*-SNARE clusters defines the sites of fusion of caveolae with the plasma membrane [43].

Involvement of caveolae has also been documented in kiss-and-run exocytosis with the plasma membrane; this occurs in an

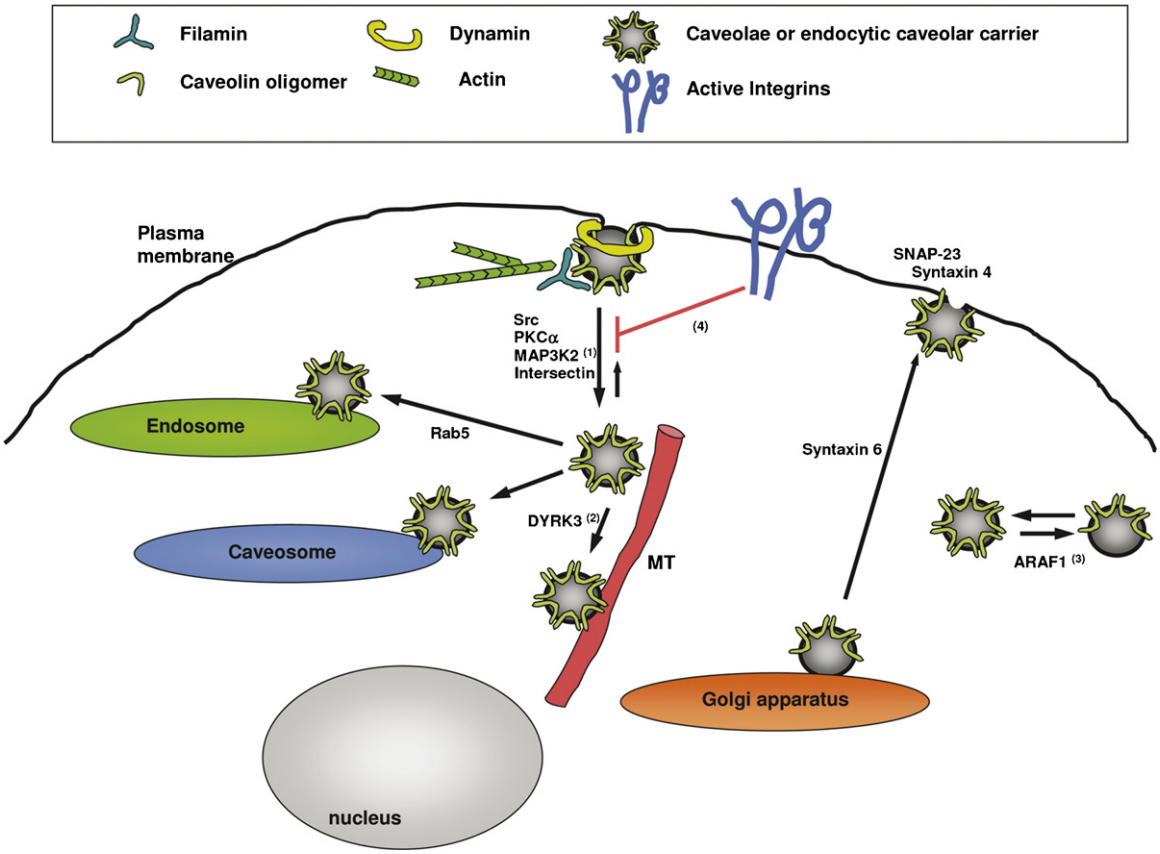

Fig. 1. Regulation of the caveolar cycle. The figure depicts the different stages of caveolae trafficking and the currently known proteins regulating each step. At the plasma membrane, caveolae are linked to the actin cytoskeleton through interaction with filamin. Dynamin plays a role in the initial steps of endocytosis. (1) MAP3K2 has been implicated in the regulation of kiss-and-run cycles. Src, PKCα and intersectin regulate caveolar internalization. Rab5 has been implicated in the regulation of endocytic caveolar carrier traffic towards the endosome. (2) Dyrk3 plays a role in the regulation of long-range traffic mediated by microtubules (MT). Syntaxin 6 regulates the traffic of caveolar components to the cell surface, and Syntaxin-4 and SNAP-23 regulate the fusion of endocytic caveolar carriers with the plasma membrane. (3) The caveolar coat is regulated by ARAF1. Integrins negatively regulate caveolae internalization (4).

N-ethylmaleimide (NEM)-sensitive manner and is mediated by single caveolar vesicles. Caveolae-mediated kiss-and-run exocytosis is regulated by at least two serine/threonine kinases (KIAA0999 and MAP3K2). It appears that caveolae undergo a continuous short-range cycle of docking, fusion and fission. During this cycle caveolae remain near the plasma membrane, although they can move between the plasma membrane and intracellular pools [44]. These findings establish that caveolar endocytosis and exocytosis are tightly coupled. The physiological role of this tight coupling is currently unknown.

2.5. Role of caveolin in transcytosis

Since rafts are involved both in endocytic and in exocytic pathways, it is not surprising that they have been proposed to be involved also in transcytosis. This process, which occurs in polarized cells, is a transcellular transport consisting of endocytosis of cargo by the fission of a vesicle from one side of the plasma membrane, its traffic across the cell in vesicle carriers, and its delivery to the other side of the cell by fusion with the plasma membrane [45,46]. In endothelial cells, caveolae have been suggested to carry out this process [13]; however, there has been no clear demonstration of this as yet [13]. Transcytosis is not only used to transport external substances, it is also involved in targeting newly synthesized proteins from the basolateral membrane to the apical surface [45,46]. Newly synthesized membrane proteins are first segregated into apical or basolateral exocytic pathways. Then, after endocytosis, they are either recycled to the same plasma membrane domain or transported to the opposite cell surface by transcytosis [45]. Contrary to earlier results [47] transcytosis of proteins (gp114) from the apical side to caveolae at the basolateral membrane has been detected [41]. Recently, a role for caveolin-1 has been described in transcellular migration of leukocytes across the endothelium after the induction of transient ICAM-1 clustering [48]. The significance of this large-scale transcytosis mechanism in vivo remains to be explained.

3. Endocytosis of rafts/caveolae

Mammalian cells have evolved complex and diverse strategies to internalize molecules or particles from the plasma membrane and outside the cell. Particles are endocytosed by phagocytosis and solutes and fluids by pinocytosis. Pinocytosis can be divided into at least four types: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis,

and clathrin- and caveolin-independent endocytosis, and the regulation of these pathways involves shared and specific pathways [49]. The best characterized form of raft internalization is mediated by caveolae, but other types of endocytosis depend on raft components, indicating that caveolae are not the only pathways that the cell has evolved to internalize rafts. Many pathogens use these pathways to enter the cell, and the mechanisms that regulate these entry routes have been identified through the study of pathogens such as SV40, polyoma virus, coxsackievirus and *Vibrio cholerae.*

There are few studies implicating clathrin-mediated endocytosis – the best characterized form of endocytosis – in the traffic of raft markers. Anthrax toxin receptor enters the cell through a clathrin and raft-dependent mechanism [50]. In addition, acute cholesterol depletion blocks clathrin-mediated endocytosis [51,52], although this may be through indirect effects on PIP2-mediated actin regulators [53]. Like caveolae-mediated endocytosis, clathrin-mediated endocytosis requires dynamin and the actin cytoskeleton. Although these examples show that there is some cross-talk between clathrin-mediated endocytosis and raft traffic, it appears that most raft trafficking occurs via other routes. The following sections discuss the current understanding of these routes and their regulation.

3.1. Caveolin-independent raft endocytosis

Several studies show that some proteins and viruses are internalized through a pathway that is independent of both clathrin and caveolae [20–23,54]. These entry routes have been classified as raft pathways because they are either cholesterol-depletion sensitive, use GM1 as a receptor, or the proteins that internalize through this pathway reside in DRM domains. The molecules that regulate these pathways are mostly unidentified. However, the regulation of the entry process in these pathways involves raft components, small Rho GTPases and actin, showing that much of the molecular machinery is shared.

3.1.1. Dynamin-independent, Cdc42-mediated raft endocytosis

In caveolin-1 null MEFs, the cholera toxin B subunit enters through a clathrin and dynamin-independent mechanism that is partly sensitive to cholesterol depletion [21]. This entry route is mediated by uncoated tubular or ring-shaped structures [55]. These structures are very similar to Cdc42-dependent endocytic structures used by GPI-anchored proteins, which enter the cell through a clathrin- and dynamin-independent and Cdc42-dependent mechanism [55]. A well known user of the caveolae entry route, the SV40 virus, can also use this entry route in vitro. Cells lacking expression of caveolin-1 are able to engulf SV40 in a cholesterol and tyrosine kinase-dependent manner, but independently of dynamin and Arf6 [20]. Whether SV40 can enter through this pathway in vivo is unknown. The facts that these pathways are used by cholera toxin B subunit and GM1 and are sensitive to cholesterol depletion suggest that they involve the internalization of non-caveolae raft domains.

Little is known about the proteins involved in this entry route. A recent study showed that Flotillin-1 is a putative key player in a clathrin- and caveolin-independent entry route that it is used by GPI-AP [56]. It will be interesting to determine whether flotillins are required for SV40 infection and cholera toxin B subunit uptake in caveolin-1 knockout MEFs. Future studies are needed to determine whether these phenomena are indeed related to the same entry route or whether significant differences exist in terms of the lipid and protein composition and regulation of these enigmatic endosomes.

3.1.2. Dynamin-dependent and RhoA-mediated endocytosis

Two studies have shown that some receptors, including the interleukin 2 receptor (IL-2R) and the γc cytokine receptor, use a similar entry route that is independent of caveolin and clathrin, but is dependent on dynamin [22,23]. The requirement for dynamin suggests that at least part of the machinery of caveolae- and clathrin-mediated endocytosis is involved in the regulation of this entry route. As predicted, this entry route is sensitive to cholesterol depletion.

Endocytosis of the γc cytokine receptor also appears to be highly dependent on the actin cytoskeleton. Souvonnet et al. showed that siRNA-mediated knock-down of cortactin, an actin cytoskeleton regulator implicated in clathrin-mediated endocytosis [22,57], severely reduced γc cytokine receptor uptake. These results show that several features of clathrin-mediated endocytosis, including dependence on dynamin and actin reorganization, are shared by various entry routes.

3.1.3. Macropinocytosis

Macropinocytosis produces large endocytic vesicles that form when membrane protrusions fuse with another region of the plasma membrane to engulf large amounts of fluid into macropinosomes. Macropinosome formation is preceded by membrane ruffling, and signals that regulate ruffling are important for this type of endocytosis [49]. While the role of Rac GTPase in macropinocytosis is clearly established, there is some evidence for a role for rafts in this process. Macropinocytosis is blocked by cholesterol depletion [58], and membrane ruffles are enriched in certain raft markers [59]. In addition, some proteins enter the cell via raft macropinocytosis [60,61]. However, although macropinocytosis may be dependent on raft components, it is unlikely that the resultant large vesicles would specifically trigger the internalization of raft components.

3.2. Caveolin-dependent endocytosis

3.2.1. Induction of caveolae internalization

The internalization of caveolae has been a focus of interest for many years. It is widely accepted that caveolae can be endocytosed; however, the evidence for its extent is conflicting. Some studies report limited movement of plasma membrane caveolae; for example, FRAP studies indicate that plasma membrane caveolae are highly immobile vesicles [62–64]. In contrast, other studies show that caveolae can be highly mobile and rapidly internalized and cleared from the plasma membrane [18,65], and a recent study has shown that caveolae are highly mobile in the lung endothelium in vivo [66]. These discrepancies are most likely due to the different cell types or conditions used for anal-

ysis. Whatever the explanation, caveolar endocytosis appears to be a highly regulated process (Fig. 1).

A great deal of information about caveolae internalization has been gathered from experiments with viruses such as SV40, polyoma virus, echovirus 1 and respiratory syncytial virus [67]. Binding of SV40 to the cell surface induces a number of changes that trigger caveolae internalization. SV40 internalization requires clustering of the ganglioside GM1 (the SV40 receptor), tyrosine phosphorylation and the actin cytoskeleton [68], which suggested that these process might be important for caveolar endocytosis. Changes in lipid composition have strong effects on caveolar dynamics. Caveolar endocytosis is increased by exogenous glycosphingolipids (GSLs) and by elevated GM1 or cholesterol, without significantly affecting the internalization rate of other endocytic routes [69]. Furthermore, Caveolin-1-GFP motility is also significantly enhanced by exogenous GSL. In contrast, overexpression of caveolin-1 reduces internalization rates [70,71], a phenotype reversible by addition of GSLs [69]. In some systems, however, overexpression of caveolin-1 has been shown to increase internalization [72]. These results suggest that there is a tight balance between lipid components of caveolae and caveolin, and the maintenance of this balance is an important regulator of caveolae internalization. Pharmacological inhibition of phosphatases also induces caveolae internalization, and similar effects are achieved by insults such as oxidative stress, heat and hyperosmotic shock [73,74]. The physiological role of internalization in response to stress is unclear. An important regulator of caveolae internalization is integrin signaling; sudden loss of cell adhesion induces striking effects in caveolae internalization [18]. This pathway will be discussed in Section 3.2.2.3.

3.2.2. Cellular pathways regulating caveolae internalization

The regulation of caveolae internalization is likely to be very complex and highly regulated. The importance of understanding the mechanisms that govern trafficking of these domains and of non-caveolar caveolin is highlighted by the disruption of normal caveolin trafficking in limb girdle muscular dystrophy and breast cancer associated with mutations in caveolin-3 and caveolin-1, respectively [75–78].

Numerous studies have established that dynamin regulates an early step in caveolar endocytosis [79,80]. Interference with dynamin function by RNAi or transfection with dominant negative forms inhibits caveolae internalization and SV40 infection [68] and integrin-mediated caveolin-1 internalization [17]. Minshall and co-workers studied caveolae-mediated albumin and cholera toxin B subunit transport in microvascular endothelial cells, and concluded that Src-mediated dynamin phosphorylation was required for proper internalization of caveolae [81]. Src family kinases appear to be key players in this endocytic route, since Src regulates both albumin uptake by endothelial cells and GSL-induced caveolae internalization [69,71,81]. Similarly, PKCα activity is required for regulated internalization of caveolae [69,82]. Another protein implicated in caveolae internalization in endothelial cells is intersectin, a scaffolding protein involved in clathrin-mediated endocytosis. Intersectin localizes to the neck of caveolae and interacts with dynamin, suggesting that is involved in the fission process [83].

A recent study examined the effect on SV40 infection of suppressing the expression of 590 individual kinases; 19% of the kinases affected infection, and of these, 34 specifically regulate the caveolae trafficking [84]. A number of steps in the cycling of plasma membrane associated caveolae have been described; caveolae appear to undergo cycles of fission and fusion with the plasma membrane, and a switch activates trafficking further into the cytoplasm [44]. Several kinases that regulate different aspects of caveolar biology have been described, including Src tyrosine kinase, MAP3K2, Dyrk3, and ARAF1 [44] (Fig. 1).

3.2.2.1. Role of cytoskeleton in caveolar endocytosis. The actin cytoskeleton and microtubules are intimately involved in caveolae biology. Invaginated caveolae are often detected close to actin bundles by electron microscopy [85], and caveolin-1-GFP partially co-localizes with stress fibers in CHO cells [63]. A yeast two-hybrid strategy showed that caveolin-1 interacts with filamin, which may act as a physical linker between actin and caveolae [86]. These studies also demonstrated the role of RhoA and stress fibers in organizing caveolae. In differentiated adipocytes caveolin is tightly associated with the actin cytoskeleton and both molecules form the rossete-like cav-actin structure [87]; however, the functional significance of this tight association remains undefined.

The exact role of the actin cytoskeleton in caveolae internalization and/or formation is a matter of debate. It has been proposed that cortical actin constrains the movement of caveolae. This would explain the increased fluorescence recovery time of photobleached caveolin-1-GFP and its cytosolic accumulation in cells treated with the inhibitor of actin polymerization latrunculin A [40,63]. Similarly, cytochalasin D treatment increases the lateral motility and clustering of GFP-caveolin-1 [62], and under basal conditions cytochalasin D appears to induce caveolae internalization [73]. However, earlier studies reported that internalization requires an intact actin cytoskeleton [74]. Knock-down of the human kinome showed that SV40 infection is modulated by actin cytoskeleton regulators, with actin polymerization and depolymerization correlating with decreased and increased SV40 infection, respectively [84]. A more detailed analysis of the role of the actin cytoskeleton in SV40 infection revealed that a cycle of actin depolymerization followed by polymerization is required for proper infection, involving the disruption of stress fibers first, and then the formation of actin tails [68]. It is clear that the actin cytoskeleton plays an important role in caveolae dynamics and internalization, but more studies are required to determine its exact role in the complete cycle of caveolae endocytosis.

There is greater consensus on the role of microtubules in caveolae traffic. Initial studies showed that accumulation of internalized caveolin near the microtubule organizing center (MTOC) or the Golgi apparatus was dependent on intact microtubules [74,88]. Further studies have confirmed the requirement for microtubules in long-range caveolae movement [40,63]. Interestingly, disruption of microtubules increases the proportion of invaginated caveolae at the plasma membrane,

presumably as a result of impaired long-range movement. If this is the case, it follows that pinching-off of caveolae requires intact microtubules. The proteins that link caveolae and microtubules are currently unknown.

3.2.2.2. Role of caveolin-1 tyrosine phosphorylation in caveolar endocytosis. Caveolin-1 tyrosine 14 plays an important role in caveolae dynamics. Several tyrosine kinases phosphorylate this residue, including c-Abl, Src, and Fyn [89–92]. In addition, phosphorylation of this residue is induced by a range of stimuli, including EGF, insulin, Angiotensin II, hydrogen peroxide, adrenocorticotropin and integrin activation [17,90,93–97]. Dephosphorylation of tyrosine 14 is regulated by PTP1b phosphatase [98].

Initial observations showed that tyrosine phosphorylation of caveolin-1 correlated with increased caveolae internalization, as shown by decreased numbers of invaginated caveolae and increased caveolin-1 staining in the cytoplasm [99]. Similar findings have been reported for caveolin-1 phosphorylation induced by EGF. Caveolin-1 is enriched at cell–cell borders in various cell types [100]. Stimulation with EGF induces tyrosine phosphorylation of caveolin-1, a loosening of cell–cell junctions and movement of caveolin-1-GFP from the cell–cell borders to the cell interior [100]. In addition, integrin-mediated caveolae internalization is rescued by re-expression of caveolin-1 in caveolin-1 null MEFs, but not by a non-tyrosine phosphorylatable caveolin-1 mutant [17] (see below).

Viruses that enter through caveolae also require tyrosine phosphorylation for efficient infection. For example, coxsackievirus requires phosphorylation of caveolin-1 at tyrosine 14 by Fyn tyrosine kinase for entry into epithelial cells [101] and SV40 requires Src tyrosine kinase activity [68].

The exact role of tyrosine 14 phosphorylation in caveolar dynamics is still unknown. C-terminal Src kinase (Csk), tumor necrosis factor-α-receptor associated factor 2 (TRAF2) and grb7 bind specifically to tyrosine 14 phosphorylated caveolin-1 [93,102]. However, the role of these proteins in caveolar traffic remains unexplored.

3.2.2.3. Integrin-regulated caveolae internalization. Integrins are the main receptors of the extracellular matrix (ECM). They are heterodimers formed by an α and a β subunit. Integrins are important for an enormous range of biological processes, including cell adhesion to the ECM, cell–cell interactions, migration, proliferation and survival [103]. Activation and/or ligand binding induces integrin clustering, which leads to the recruitment of signaling molecules and actin filaments to the generally short cytoplasmic tail [103]. Numerous proteins interact with integrin cytoplasmic tails, including talin, filamin and FAK [104,105]. The recruitment of signaling molecules and cytoskeletal elements initiates multiple signaling cascades that can result in changes in cell polarity, cell migration, cell cycle progression, gene expression and survival [106,107]. Integrin activation regulates many signaling intermediates, including Erk, PI 3-kinase, FAK, Src family tyrosine kinase and small Rho GTPases [106,107]. In addition, integrin signals are required for coupling of growth factor receptors to downstream effectors [107]. In the case of the small Rho GTPase Rac, integrins regulate not only its activity, but also, and in contrast to growth factor receptors, Rac targeting to the plasma membrane, where it can bind effectors and trigger downstream signaling [108]. The specific plasma membrane sites to which integrins target Rac are rafts [109]. Other Rho family GTPases such as Rho and Cdc42 also bind to rafts in an integrin-dependent manner [110–112]. Further studies have shown that integrins control membrane binding by GTPases by regulating the internalization of their membrane binding sites [109].

Several raft markers, including GM1, GPI-anchored proteins, caveolin-1 and cholesterol itself are rapidly internalized in a specific and reversible manner upon loss of integrin-mediated adhesion. Similarly, the amount of cholesterol-enriched plasma membrane domains decreases after detachment of senescent cells, and accompanying this change, caveolin, Fyn and GM1 move away from raft fractions [113]. Therefore, raft internalization occurs upon cell detachment, providing an explanation for the disruption of Rac targeting and signaling in detached cells [25]. This notion is confirmed by experiments in which GM1 domains are artificially held in the membranes of suspended cells. Holding GM1 domains in the surface of detached cells induces retention of plasma membrane Rac, which in turn is able to activate PAK even in non-adherent cells. These studies suggest a model in which integrin-mediated cell adhesion permits targeting of Rac to the plasma membrane and its coupling to PAK by preventing internalization of Rac binding sites contained in rafts. A more recent study by Gaus et al., using the reporter molecule Laurdan and two-photon microscopy, showed that integrins and phosphocaveolin both contribute to increased order at the plasma membrane, since liquid-ordered domains are internalized and membrane organization is drastically perturbed upon cell detachment. Maximum order was detected in this study at focal adhesions, where phosphocaveolin is localized and recruits membrane components that induce order, such as cholesterol [114]. Moreover, membrane order in cells lacking caveolin-1 was restored by re-expression of caveolin, but not a non-phosphorylatable caveolin mutant. This study thus underscores the role of integrins in regulating plasma membrane domains.

Raft clearance from the plasma membrane upon cell detachment appears to be mediated by the caveolar pathway [17]. Detachment triggers a rapid clearance of caveolin-1 and GM1 from the plasma membrane and increases caveolin-1 colocalization with GM1 in an internal compartment. The principal evidence supporting the involvement of caveolin-1 in membrane domain clearance after cell detachment comes from studies in cells lacking caveolin-1 expression [115]. Fibroblasts derived from caveolin-1 knockout mice are not able to internalize GM1 in suspended cells or to shut down the Rac signaling pathway. The wild type phenotype is rescued by expression of caveolin-1. Similarly, melanoma-derived M21L cells that lack expression of caveolin-1 are unable to internalize GM1 after detachment. Therefore, uncoupling of integrins from intracellular signaling triggers the clearance of some enriched cholesterol and GM1 membrane domains from the plasma membrane by a caveolae-dependent mechanism. Other studies have underscored the

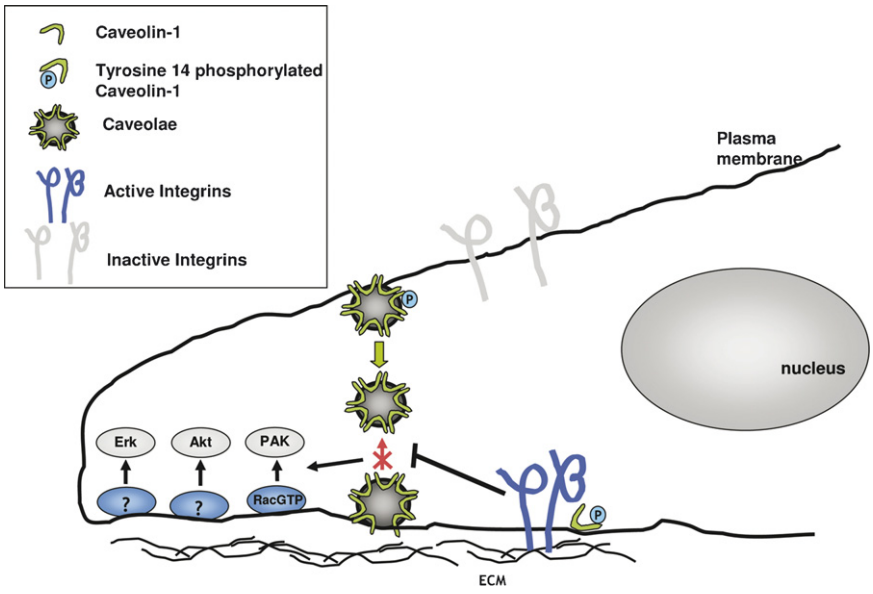

Fig. 2. Proposed model for integrin-mediated caveolae traffic. The proposed model for the integrin-mediated regulation of caveolae traffic is depicted. In adherent cells, active integrin signaling prevents caveolae internalization. This is achieved, at least in part, by the localization of phospho-caveolin-1 to focal adhesions. This allows the coupling of plasma membrane localized-RacGTP and other unknown proteins to their downstream effectors, including PAK1, Akt and Erk. In the absence of active integrin signaling, i.e., in non-adherent cells, phosphorylated caveolin-1 is relocated to caveolae and this induces their internalization, which uncouples Rac and other proteins from their effectors and thus accelerates the shutting down of multiple signaling pathways.

importance of integrin signaling in caveolin-mediated endocytosis [84,116,117]. For example, addition of GSL-s induced an increased internalization of caveolar markers, which coincided with partial cell detachment [117]. Little is known about the mechanism of caveolae internalization. However, studies indicate that tyrosine phosphorylation of caveolin-1 after caveolae formation is required for this process [17,117]. This is striking, since phosphorylated caveolin-1 represents less than 1% of the total caveolin-1 pool [17]. This suggests that, rather than total levels of phospho-caveolin-1, a specific pool of caveolin-1, in a specific cell compartment, is important for regulation. In adherent cells, pY14caveolin-1 is located at focal adhesions but rapidly moves to caveolae when cells are suspended, inducing internalization of caveolae. These data are also supported by sucrose gradient fractionation studies, which detect phospho-caveolin in heavy fractions in adherent cells but in light fractions in suspended cells [17]. These results suggest that pY14caveolin-1 is required for integrin-regulated caveolae internalization.

All these observations have led to the elaboration of a model for integrin-mediated caveolin internalization (Fig. 2) [115]. In adherent cells, integrins sequester pY14caveolin-1 at focal adhesions, which leaves Rac and other signaling proteins free to bind to their binding sites at the plasma membrane, thus permitting their signal transduction activity. When cells are detached, integrins are inhibited, and pY14caveolin-1 moves to caveolae, inducing their internalization so the binding sites disappear; Rac and other signaling proteins are thus uncoupled from their effectors, blocking signal transduction. This model predicts that signaling will be increased by the inhibition of caveolae internalization, and this could contribute to cell transformation. Some of the signaling pathways regulated by integrin-mediated adhesion, including Ras-Erk and PI3K-Akt, are also regulated by caveolin, and their activity is indeed retained in suspended caveolin-1$^{-/-}$ cells [17]. Moreover, alterations to these pathways are frequent in cancer cells. Normal cells require integrin-ECM mediated signals to properly proliferate: their growth is anchorage-dependent. In contrast, cancer cells bypass this requirement and grow in an anchorage-independent manner. The recent studies reviewed here reveal a fascinating connection between caveolar endocytosis and integrin-ECM mediated cell adhesion. Defining the pathways that regulate integrin-dependent caveolae internalization will undoubtedly reveal novel molecular mechanisms implicated in cell proliferation and cell migration, and could identify potential targets for therapeutic intervention.

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