

## Regulation of raft-dependent endocytosis

P. Lajoie, I. R. Nabi \*

Department of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia, Vancouver, Canada

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### Abstract

Raft-dependent endocytosis is in large part defined as the cholesterol-sensitive, clathrin-independent internalization of ligands and receptors from the plasma membrane. It encompasses the endocytosis of caveolae, smooth plasmalemmal vesicles that form a subdomain of cholesterol and sphingolipid-rich lipid rafts and that are enriched for caveolin-1. While sharing common mechanisms, like cholesterol sensitivity, raft endocytic routes show differential regulation by various cellular components including caveolin-1, dynamin-2 and regulators of the actin cytoskeleton. Dynamin-dependent raft pathways, mediated by caveolae and morphologically equivalent non-caveolin vesicular intermediates, are referred to as caveolae/raft-dependent endocytosis. In contrast, dynamin-independent raft pathways are mediated by non-caveolar intermediates. Raft-dependent endocytosis is regulated by tyrosine kinase inhibitors and, through the regulation of the internalization of various ligands, receptors and effectors, is also a determinant of cellular signaling. In this review, we characterize and discuss the regulation of raft-dependent endocytic pathways and the role of key regulators such as caveolin-1.

**Keywords:** raft-dependent endocytosis • caveolin-1 • caveolae • cholesterol • cellular signaling

### Introduction

Identified in the 1950s, caveolae are 50–80 nm diameter plasma membrane invaginations that are morphologically distinct from clathrin-coated pits [1]. Caveolae are cholesterol- and sphingolipid-rich and considered a subdomain of plasma membrane microdomains or lipid rafts. Lipid rafts have been defined as ‘small (10–200 nm) heterogeneous membrane domains enriched in sterol and sphingolipids that are involved in the compartmentalization of various cellular processes’ [2].

Multiple studies have described the role of caveolae and rafts in the endocytosis of various ligands (for reviews see refs. [3–5]). Several raft-dependent pathways have been described and raft ligands are quite liberal in their selectivity for a particular route of entry into the cell. The extent and nature of raft-dependent endocytosis is regulated by various cellular components that include caveolin-1 (Cav1), cholesterol and dynamin as well as regulators of the actin cytoskeleton.

\*Correspondence to: Dr Ivan R. NABI  
Department of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia, 2350

Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3.  
Tel.: (604) 82 2-70 00; Fax: (604) 82 2-23 16  
E-mail: ivan.robert.nabi@ubc.ca

Cav1 is the major component of caveolae and its expression is essential for the formation of caveolar vesicles. An absence of caveolae is noted in cells that do not express Cav1 and its reintroduction into these cells induces caveolae formation at the plasma membrane [6]. Two other proteins of the same family also exist. Caveolin-2 (Cav2) facilitates but is not essential for caveolae formation [7–9]. Caveolin-3 (Cav3) is specifically expressed in muscle [10]. Cav1 is a scaffolding protein that oligomerizes at the plasma membrane [11]. Caveolae are highly immobile at the plasma membrane [12] and Cav1 has been proposed to be a negative regulator of raft-dependent endocytosis [13, 14]. However, upon activation by SV40, Cav1 mobility at the cell surface is greatly increased [15]. There is also evidence for raft-dependent endocytic pathways independent of Cav1 that are mediated by distinct carrier vesicles [16–19]. In this review, we will characterize the various raft-dependent endocytic pathways and discuss their regulation.

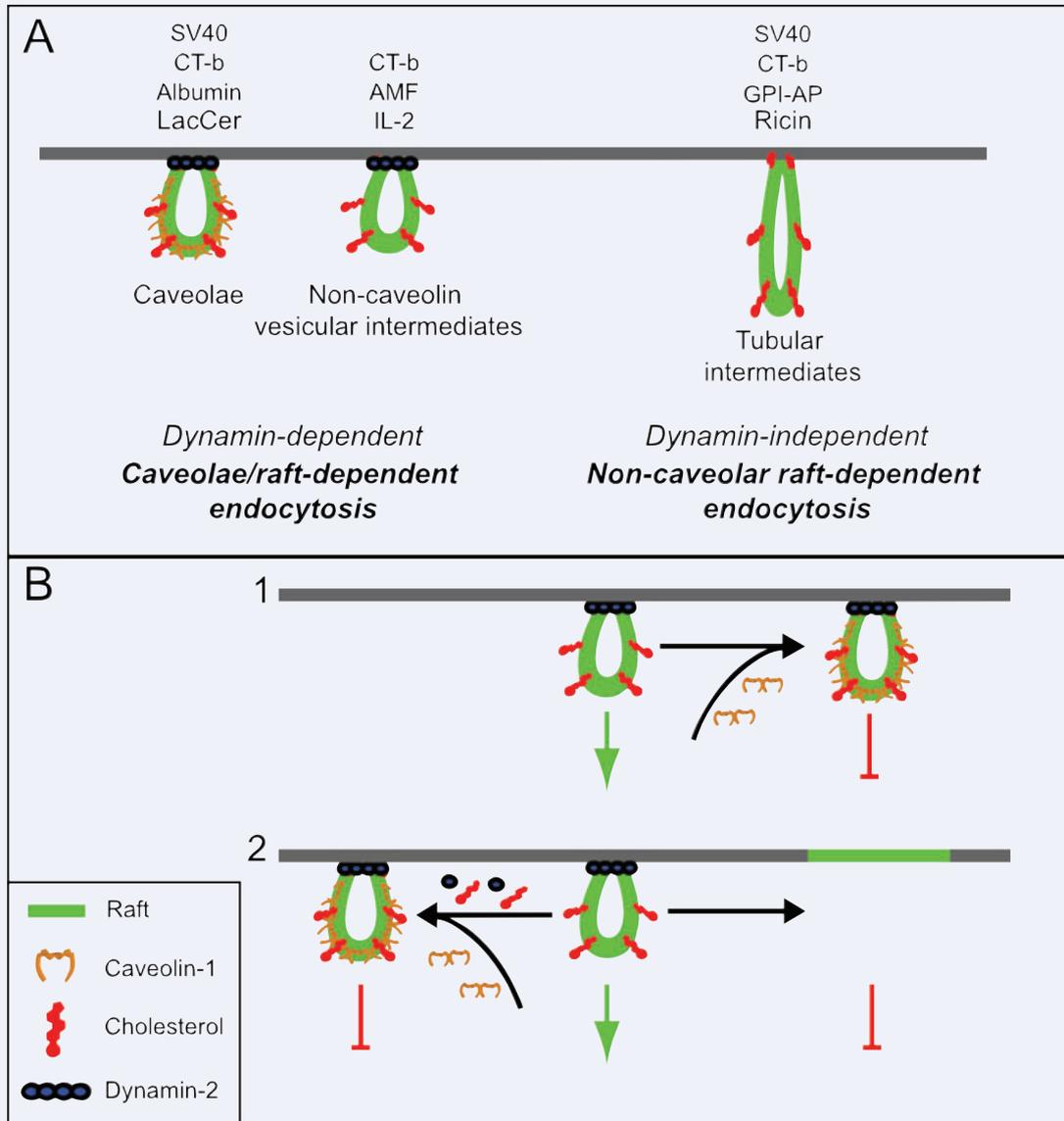
## Raft-dependent endocytosis encompasses various pathways

Internalization of molecules *via* clathrin-coated pits is the best studied endocytic pathway [20]. Various other pathways, commonly referred to as clathrin-independent, have been identified and are under intense investigation. Some of these pathways are cholesterol-sensitive and therefore considered to be raft-mediated. It is important to recognize that clathrin-mediated endocytosis is also sensitive to acute depletion of cholesterol [21, 22] and that raft recruitment has been shown to precede clathrin-dependent endocytosis for EGFR, BCR and anthrax toxin [23–25]. In addition, macropinocytosis, involving Rac1-dependent membrane ruffling at the plasma membrane, can be cholesterol-sensitive, potentially defining another dynamin-independent raft pathway [26, 27]. However, macropinocytosis has been shown to be dynamin-dependent in NIH-3T3 and HUVEC cells [28, 29]. The dynamin- and raft-dependence of macropinocytotic pathways may be cell type and cargo-specific. For the purposes of this review, raft-dependent endocytic pathways will be defined by their clathrin-independence and cholesterol-sensitivity and will not include macropinocytosis.

A characteristic of some of these raft-dependent pathways is their dependence on dynamin, a molecule involved in vesicular fission from the plasma membrane [30, 31]. The formation of dynamin-dependent smooth plasma membrane vesicles, or caveolar invaginations can occur both in the presence or absence of caveolins [13]. Similarities between the caveolae and non-caveolin dynamin-dependent raft endocytic pathways led us to refer to them inclusively as caveolae/raft-dependent endocytosis [14]. Dynamin-independent raft pathways have been described that are caveolin-independent and invoke tubular intermediates [16, 17]. While the heterogeneity of raft domains [32] is certainly indicative of higher orders of complexity and regulation of their endocytosis, to a large extent, and at least for now, raft-dependent endocytic pathways can be classified based on their caveolin- and dynamin-dependence (Fig. 1A). This classification is based on mechanistic similarities of the raft-dependent internalization of select ligands at the plasma membrane. Indeed, different cargoes that use similar raft endocytic mechanisms may be internalized *via* distinct raft domains and targeted to different intracellular sites [14, 33].

Some ligands enter the cell *via* a caveolae-dependent pathway. The simian virus SV40 follows a dynamin-dependent, caveolae-mediated pathway that targets a caveolin-positive endosome, the caveosome, before being delivered to the smooth endoplasmic reticulum [34]. When stimulated by SV40, caveolin, dynamin and actin are recruited sequentially to the caveolae [34]. The raft-dependent endocytic pathway of cholera toxin b-subunit (CT-b) has also been characterized as a dynamin-dependent, caveolar pathway [19, 35–37]. Albumin is internalized *via* a dynamin-dependent pathway that requires caveolin [38]. In lymphocytes lacking Cav1, endocytosis of the interleukin-2 receptor occurs *via* a clathrin-independent, cholesterol-sensitive pathway that requires dynamin and is regulated by the RhoA GTPase [39]. In NIH-3T3 cells, the autocrine motility factor receptor is localized to caveolae and internalization of its ligand, AMF, is cholesterol and dynamin-dependent and negatively regulated by Cav1 expression [13, 40].

A raft-dependent, dynamin-independent pathway has also been described for CT-b and SV40 [16, 17] that exhibits similarity to a Cdc42-dependent pathway followed by GPI-anchored proteins (GPI-AP) and fluid phase markers [41]. In fibroblasts from Cav1 knockout mice, SV40 exploits an alternate,



**Fig. 1** Raft-dependent endocytosis and its regulation by Cav1. **(A)** Several endocytic pathways are characterized as raft-dependent and mediate the uptake of various ligands, including but not limited to those indicated. These include dynamamin-dependent pathways that invoke caveolae or non-caveolin vesicular intermediates and that can be referred to as caveolae/raft-dependent endocytosis [14]. Dynamamin-independent pathways invoke non-caveolar tubular intermediates. While similar mechanisms control the uptake of the indicated raft-dependent ligands, they are not necessarily internalized by the same raft domains or follow similar intracellular targeting routes. **(B)** Cav1 may negatively regulate uptake via the dynamamin-dependent, non-caveolin pathway by either stabilizing raft invaginations at the cell surface (1) or by sequestering key components, including cholesterol, dynamamin and others, required for raft-dependent uptake (2). Cholesterol is not shown in the flat portion of the membrane to simplify the diagram. LacCer: lactosylceramide; CT-b: cholera toxin b subunit; GPI-AP: glycosylphosphatidylinositol-anchored proteins; AMF: autocrine motility factor; IL-2: interleukin-2; SV40: simian virus 40.

Cav1-independent pathway that is cholesterol and tyrosine kinase dependent but independent of clathrin, dynamin-2 and ARF6 [16]. A similar pathway has also been described for CT-b in Cav1<sup>-/-</sup> fibroblasts where it is ARF6-dependent [17]. This pathway invokes not caveolar invaginations but the formation of uncoated tubular endocytic structures and an intracellular dynamin-dependent step for delivery to endosomes and the Golgi apparatus [17]. Internalization of CT-b has also been shown to occur *via* a dynamin-independent pathway defined not by caveolin but by flotillin, another raft component [42].

CT-b therefore provides an example of an endocytic ligand internalized by several pathways including clathrin-coated pits and both dynamin-dependent and independent raft pathways [43]. A recent study showed that 50% of CT-b enters the cell *via* clathrin-coated pits with the remainder internalized *via* dynamin-independent, caveolin-independent uncoated tubules. In the same study, the authors showed that about only 2% of the total pool of Cav1 positive caveolae contributes to the internalization of CT-b, suggesting that internalization of CT-b *via* caveolae represents only a minor contribution [17]. However, CT-b internalization was found to be deficient in immortalized Cav1<sup>-/-</sup> MEF-derived cell lines [44] contrasting with the demonstration that primary Cav1<sup>-/-</sup> MEFs show no difference in CT-B uptake compared to wild-type MEFs [17]. In HeLa cells, depletion of flotillin by siRNA prevents its uptake *via* a dynamin-independent route and switches it to a dynamin-dependent route [42]. Variable cell surface expression of the CT-b receptor, GM1 ganglioside, impacts on the extent of its raft-dependent endocytosis [45]. In addition, CT-b concentrations used vary significantly (from 0.05 to 10 µg/ml) between studies from different laboratories [17, 42, 45, 46]. Interestingly, in studies defining the dynamin-independent raft pathway, both CT-b and dextran concentrations were relatively low [17, 18]. Variable factors, ranging from expression of ligand receptors to raft components, may impact not only on the extent of CT-b uptake but also on its route of entry into different cells or clonal populations of the same cell type.

Raft-dependent endocytosis is therefore a highly complex process in which the same cargo can follow various entry routes and in which different cargo can use similar entry routes with different molecular regulation. This complexity should not preclude efforts to classify these pathways based on common denomi-

nators, as proposed in Figure 1A. Further characterization of the cargo-specificity and molecular regulation of raft-dependent pathways will lead to a better understanding of what are clearly intricate mechanisms regulated by multiple factors.

## Cav1 and the regulation of raft-dependent endocytosis

Fluorescence recovery after photobleaching (FRAP) experiments have shown that movement of Cav1 at the cell surface is restricted by cortical actin as well as through interaction with the actin-binding protein filamin [12, 47]. Caveolar stability at the plasma membrane suggests that rapid, constitutive internalization and turnover of caveolae is unlikely to occur. Rapid, reversible budding of caveolae, or potocytosis, was originally suggested to regulate folate internalization [48]. More recently, TIRF microscopy was used to show that reversible caveolae budding is limited to the subplasma membrane region by the underlying actin cytoskeleton [15]. Disruption of the actin cytoskeleton induces rapid internalization of caveolar vesicles [49, 50]. Recruitment of SV40 to caveolae induces the transient, localized breakdown of the actin cytoskeleton [51]. Actin depolymerization also induces internalization of tight junction proteins *via* a caveolae-dependent pathway [52]. However, earlier work showed that disruption of the actin cytoskeleton by cytochalasin D in A431 cells inhibited alkaline phosphatase uptake *via* caveolae [37]. The submembrane actin cytoskeleton would therefore appear to be a critical regulator of the endocytic potential of caveolae.

Several raft-dependent endocytic pathways are regulated *via* Rho family GTPases. GPI-anchored proteins have been shown to be internalized *via* a Cdc42-regulated pathway that is independent of Rho and Rac [41]. RhoA regulates IL-2 receptor internalization [39] and CT-b endocytosis to the Golgi apparatus in Cos-1 cells is dependent on RhoG [53]. The Menkes disease ATPase (ATP7A) uptake can be inhibited by a Rac-1 dominant negative mutant [54]. Constitutively active Rac and RhoA also downregulate clathrin-dependent endocytosis [55]. Differential expression and local activation of Rho family GTPases may be a key determinant of the cell type specificity of raft-dependent endocytosis.

Threshold levels of Cav1 and cholesterol regulate caveolae formation [56, 57]. Various cholesterol modulating agents, including methyl- $\beta$ -cyclodextrin, nystatin and filipin, have been shown to inhibit both caveolae expression and raft-dependent endocytosis [13, 16, 57–60]. Caveolar endocytosis of various ligands can be significantly increased by addition of cholesterol or glycosphingolipid to human fibroblasts [60]. Using heterokaryons expressing both Cav1-GFP and Cav1-RFP, it has been shown that cholesterol depletion increased exchange between otherwise stable Cav1 positive structures [15]. Cav1 interacts directly with cholesterol [61, 62] and cholesterol levels in lipid raft fractions obtained from Cav1 expressing cells were 3–4-fold higher than in matched cells lacking Cav1 [63]. It is possible that Cav1 regulation of raft endocytosis is linked to its ability to sequester cholesterol in raft domains (Fig. 1B). In biological membranes, the ratio between cholesterol and phospholipids is maintained slightly below 1:1 [64]. When in excess and therefore free from interaction with phospholipids, cholesterol shows a higher chemical activity [65]. This pool has been described as active cholesterol [65–68]. Cav1 may therefore regulate cholesterol-dependent processes, such as raft endocytosis, through sequestration of active cholesterol.

Caveolae budding from the plasma membrane and subsequent internalization requires dynamin II [36]. Expression of the K44A dynamin mutant increases the number of caveolae in caveolin-expressing NIH-3T3 cells as well as the formation of morphologically similar invaginations in Ras and Abl-transformed NIH-3T3 cells expressing little caveolin [13]. Indeed, caveolae-like structures in cells devoid of caveolin have been reported [69]. Several studies have shown that overexpression of Cav1 is associated with reduction, even inhibition of raft-dependent endocytosis [13, 17, 38, 46]. Cav1 overexpression was also found to inhibit the non-caveolar, dynamin-independent endocytosis of CT-b [17]. Reduction of Cav1 levels in mammary tumor-derived cell lines is associated with both increased plasma membrane mobility and raft-dependent uptake of CT-B to the Golgi apparatus. Interestingly, regulation of CT-b mobility and endocytosis in these cells occurred at Cav1 levels below the threshold for caveolae formation (Lajoie, Nim and Nabi, unpublished). This suggests that Cav1 may act indirectly to regulate raft-dependent endocytosis by impacting on the composition and endocytic potential of non-caveolar raft domains

(Fig. 1B). Indeed, the idea of dynamic exchange between raft domains is consistent with the ability of raft components, such as Cav1 or flotillin, to impact on the raft-dependent endocytosis of select ligands by modulating the endocytic potential of distinct raft domains.

## Signaling and raft-dependent endocytosis

Treatment of cells with tyrosine kinase inhibitors blocks caveolae endocytosis while addition of the phosphatase inhibitor okadaic acid triggers endocytosis [17, 37, 51, 70]. Indeed, the use of the non-specific tyrosine kinase inhibitor genistein is generally recognized as a selective inhibitor of raft-dependent endocytic pathways. Cav1 is phosphorylated by Src kinase at tyrosine 14 [71]; however, the role of Cav1 phosphorylation in raft endocytosis is still unclear. The predominant cellular location of tyrosine phosphorylated Cav1 is in focal adhesions. Redistribution of tyrosine phosphorylated Cav1 from focal adhesions to caveolae upon cell detachment from the extracellular matrix triggers raft-dependent endocytosis and plasma membrane depletion of Rac [72]. Activation of v-Src in Rat-1 cells is responsible for Cav1 phosphorylation and is associated with loss of plasma membrane caveolae [73]. In addition, Cav1 phosphorylation on tyrosine 14 is associated with flattening, aggregation and fusion of caveolae vesicles [74]. However, in pancreatic cancer cells, EGF stimulation of Src-mediated Cav1 phosphorylation leads to a marked increase in the number of assembled caveolae at the cell surface [75]. Src kinase regulation of transcytosis of albumin across the endothelial cell monolayer is associated with Cav1 phosphorylation [70]. Src kinase activity is also required for stimulation of caveolae internalization by glycosphingolipids and cholesterol [60]. However, whether Cav1 tyrosine phosphorylation is a critical regulator of caveolae internalization remains to be determined.

SV40 recruitment to caveolae stimulates local tyrosine phosphorylation. Tyrosine phosphorylation inhibitors do not prevent SV40 recruitment to caveolae but do prevent recruitment of dynamin to caveolae suggesting that tyrosine phosphorylation is crucial for

dynamamin-dependent caveolae budding [51]. Similarly, the Src-dependent internalization of albumin *via* a Gi-coupled pathway requires interaction of its receptor, gp60, with Cav1 [38, 76]. Dominant negative Src reduces phosphorylation of dynamamin-2 and dynamamin-2 association with Cav1 resulting in reduced albumin uptake [77]. This suggests that tyrosine phosphorylation regulates caveolar budding by controlling dynamamin recruitment to caveolae. However, the requirement for tyrosine kinases in the raft-dependent uptake of AMF in cancer cells expressing low levels of Cav1 [78] and in the dynamamin-independent raft uptake of SV40 in Cav1<sup>-/-</sup> cells [16] is indicative of further complexity for the role of tyrosine phosphorylation in raft-dependent endocytosis. A siRNA screening approach of kinase inhibitors identified a large group of 208 human kinases as regulators of SV40 entry and 39 of them were involved in caveolae/raft trafficking [79]. Application of a similar approach to other raft ligands may identify common and, potentially, distinct kinases that control raft-dependent endocytosis of various raft ligands.

Cav1 has a well-established scaffolding function implicated in the sequestration of cytokine receptors and lipid-anchored signaling intermediates as well as cholesterol [80, 81]. Sequestration of EGFR and TGFβR to caveolae and interaction with Cav1 is associated with inhibition of signaling capacity [76, 82–84]. These studies were later confirmed when it was shown that Cav1 was able to induce sequestration of the receptor [85] and to directly bind EGFR [86, 87]. Moreover, the second cysteine region of EGFR contains sequences that target the receptor to caveolae/raft domains [88]. Upon stimulation with EGF, EGFR is no longer localized in low density raft fractions, consistent with its migration from caveolae to clathrin coat pits upon stimulation [83]. Alternatively, Cav1 may indirectly regulate EGFR signaling through regulation of the cholesterol content of lipid rafts [89]. When stimulated with a high EGF dose, EGFR is internalized *via* a caveolae/raft-dependent pathway associated with ubiquitination of the receptor [90]. Similarly, clathrin-dependent uptake of TGFβR is associated with subsequent signaling events *via* Smad2 phosphorylation in EEA1-positive endosomes while its caveolae/raft-dependent is associated with receptor degradation through binding to the smad7-smurf2 complex [82]. Raft-dependent endocytosis is therefore both regulated by and impacts on cell signaling.

## Conclusion

Raft-dependent endocytosis includes various cholesterol-sensitive endocytic routes, distinct from clathrin-mediated endocytosis, which can be classified based on their dependence on Cav1 and dynamamin. These pathways share sensitivity to cholesterol depletion as well as to other more selective regulators whose cell-specific expression may impact on the endocytic pathway followed by multiple raft-dependent ligands. By impacting indirectly on raft domain organization, various raft components, including cholesterol, Cav1 and flotillin, regulate raft-dependent endocytosis. Cav1 acts as a determinant of raft-dependent endocytosis by stabilizing rafts at the cell surface, *via* receptor recruitment or through sequestration of cholesterol and other critical determinants of raft-dependent endocytosis. Further study of raft-dependent endocytosis should lead to the further classification and identification of specific regulators of the endocytic potential of these varied pathways. Open questions that remain relate to the molecular regulation of raft-dependent endocytosis and how the heterogeneous composition of raft domains, including but not limited to cargo, impacts and determines their endocytic potential, mechanism of internalization and intracellular targeting.

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