Caveolin-1 regulation of dynamin-dependent, raft-mediated endocytosis of cholera toxin–B sub-unit occurs independently of caveolae

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Received: September 8, 2008; Accepted: January 3, 2009

Abstract

Ganglioside GM1-bound cholera toxin–B sub-unit (CT-b) enters the cell via clathrin-coated pits and dynamin-independent non-caveolar raft-dependent endocytosis. Caveolin-1 (Cav1), associated with caveole formation, is a negative regulator of non-caveolar raft-dependent endocytosis. In mammary epithelial tumour cells deficient for Mgat5, Cav1 is stably expressed at levels below the threshold for caveole formation, forming stable oligomerized Cav1 microdomains or scaffolds that were shown to suppress EGFR signalling and reduce the plasma membrane diffusion rate of both EGFR and CT-b. Below threshold levels of Cav1 also inhibit the dynamin-dependent raft-mediated endocytosis of CT-b to the Golgi indicating that Cav1-negative regulation of raft-dependent endocytosis is caveole independent. Inhibition of CT-b internalization does not require Cav1 phosphorylation but does require an intact Cav1 scaffolding domain. By flow cytometry, both over-expression of Cav1 and the dynamin K44A mutant block CT-b internalization from the plasma membrane defining a dynamin-dependent raft pathway for CT-b endocytosis in these cells. However, only minimal co-localization between CT-b and Cav1 is observed. These results suggest that Cav1 regulates raft-dependent internalization of CT-b indirectly via a mechanism that requires the Cav1 scaffolding domain and the formation of oligomerized Cav1 microdomains but not caveolae.

Keywords: caveolin-1 • raft endocytosis • cholera toxin b subunit • dynamin • caveole

Introduction

Lipid rafts are small heterogeneous cholesterol and sphingolipid-enriched membrane domains involved in various biological processes [1]. Partition of molecules in plasma membrane lipid rafts favours their internalization via a process called raft-dependent endocytosis, generally characterized as clathrin-independent and sensitive to cholesterol depletion [2–4]. However, substantial heterogeneity in the regulation of raft-dependent endocytic pathways exists, in particular with respect to their dynamin dependence and the role of caveolae and the caveolar coat protein, caveolin-1 (Cav1) [4].

Dynamin-dependent caveolea-mediated endocytosis has been implicated in the internalization of simian virus 40 (SV40), lactosylceramide, albumin and GM1-ganglioside binding cholera toxin–B sub-unit (CT-b) [5–13]. However, more recent studies have proposed that CT-b is internalized from the cell surface predominantly via clathrin-dependent endocytosis and dynamin-independent raft endocytosis [14–16]. In Cav1−/− mouse embryo fibroblasts (MEFs), CT-b and SV40 are internalized via a dynamin independent, non-caveolar raft pathway that invokes tubular carriers [15, 17]. In wild-type MEFs, 50% of CT-b was internalized via clathrin-dependent endocytosis and essentially the rest by a dynamin-independent, non-caveolar pathway [15] that may involve flotillin-defined raft domains [16]. Interestingly,
dynamin-regulated delivery of CT-b from the tubular carriers to the Golgi and not internalization at the cell surface questioning the extent to which CT-b is internalized via caveolar and dynamin-dependent raft endocytic pathways [15].

Cav1 over-expression prevents internalization of raft-dependent ligands such as CT-b, albumin, dysferlin and autocrine motility factor [15, 18–22]. Cav1 expression may negatively regulate raft-dependent endocytosis by sequestering ligands and their receptors in stable cell surface caveolae [3, 15, 20]. However, we recently demonstrated that Cav1 expression below the threshold required for caveolea formation can suppress EGFR signalling and cell surface diffusion of both EGFR and CT-b describing required for caveolae formation can suppress EGFR signalling [23].

Mammary epithelial tumour cells derived from wild-type PyMT transgenic mice express high levels of Cav1 and caveolae, whereas tumour cells from mice deficient for Golgi N-acetylglucosaminyltransferase V (Mgat5–/−) express Cav1 at levels below the threshold for caveolae formation. PyMT Mgat5–/− tumours generally grow more slowly, but a minority ‘escape’ the Mgat5 null phenotype (Mgat5–/−ESC) and show rapid growth and loss of Cav1 expression [23, 24]. In this cell model, oligomerized Cav1 microdomains, independently of caveolae formation, were found to be sufficient to impose growth restrictions on cells deficient for Mgat5 [23]. Here, using these cell lines, we show that Cav1 regulates the dynamin-dependent, raft-mediated internalization of CT-b under conditions where it is not associated with caveolae formation.

Materials and methods

Cell culture

Mgat5+/+, Mgat5−/− and Mgat5−/−ESC cells were derived from wild-type PyMT transgenic mice expressing high levels of Cav1 and caveolae, whereas tumour cells from mice deficient for Golgi N-acetylglucosaminyltransferase V (Mgat5–/−) express Cav1 at levels below the threshold for caveolae formation. PyMT Mgat5–/− tumours generally grow more slowly, but a minority ‘escape’ the Mgat5 null phenotype (Mgat5–/−ESC) and show rapid growth and loss of Cav1 expression [23, 24]. In this cell model, oligomerized Cav1 microdomains, independently of caveolae formation, were found to be sufficient to impose growth restrictions on cells deficient for Mgat5 [23]. Here, using these cell lines, we show that Cav1 regulates the dynamin-dependent, raft-mediated internalization of CT-b under conditions where it is not associated with caveolae formation.

Flow cytometry

Mgat5−/−ESC cells were treated with mβCD or infected with adenoviruses encoding for clathrin-hub, dynamin-wt, dynamin K44A and Cav1 [20]. After 48 hrs, cells were incubated for 30 min. at 37°C with either 1 μg/ml CT-b-FITC or 15 μg/ml Tf-FITC. To remove surface-bound fluorescent ligand, cells were rapidly chilled on ice and washed with ice-cold acidic buffer (0.5 mM NaCl, 0.2 mM acetic acid, pH 2) [26]. For flow cytometry, at least 50,000 cells were acquired and analyzed using FACSCalibur and Cellquest software (BD Biosciences, San Jose, CA, USA). Data are the means ± S.E. of triplicates and are representative of three separate experiments.

Results

Reduced Cav1 increases dynamin-dependent, raft-mediated endocytosis of CT-b to the Golgi

As previously reported [23], quantitative immunofluorescence shows that Mgat5−/− cells show approximately 50% reduction in Cav1 levels, below the threshold for caveolae formation, compared with Mgat5+/+ cells, whereas Mgat5−/−ESC cells display significantly reduced Cav1 expression compared with both cell lines (Fig. 1A). Rescue of Mgat5−/− cells (Rescue) with an Mgat5 expressing retrovirus restored Cav1 expression to levels observed in Mgat5+/+ cells. However, upon rescue, Mgat5−/−ESC cells (ESC-Rescue) still showed reduced Cav1 levels (Fig. 1A). GM1 expression impacts on CT-b uptake [27] and we verified that all cell lines displayed similar surface GM1 levels by labelling with CT-b-FITC at 4°C (Fig. 1A). However, following addition of CT-b at 37°C, only Mgat5−/−ESC and ESC-Rescue cells, which express reduced Cav1 levels, showed increased CT-b endocytosis to the Golgi (Fig. 1B). These results are consistent with our previous demonstration that loss of Cav1 in
Mgat5−/−ESC cells was associated with increased cell surface diffusion of CT-b [23]. The fact that ESC-Rescue cells displayed the same elevated CT-b uptake as Mgat5−/−ESC cells indicates that Mgat5 expression does not impact on CT-b internalization (Fig. 1B).

Internalization of CT-b to the Golgi in Mgat5−/−ESC cells was sensitive to cholesterol depletion with methyl-β-cyclodextrin (mβCD), inhibited by adenoviral expression of the dynamin K44A mutant but not wild-type dynamin and not affected by...
over-expression of the clathrin-hub (Fig. 2A). CT-b is therefore delivered to the Golgi apparatus of Mga5^{−/−}-ESC cells via a clathrin-independent, dynamin-dependent raft pathway. Over-expression of Cav1 has previously been shown to inhibit CT-b internalization [15, 19]. Consistent with these results, CT-b internalization to the Golgi was greatly reduced in Mga5^{−/−}-ESC cells infected with Cav1-expressing adenovirus (Fig. 2A).

Cav1-negative regulation of raft-dependent endocytosis is caveolea-independent and scaffolding domain-dependent

Cav1 contains a scaffolding domain implicated in Cav1 oligomerization and receptor sequestration as well as a Src-kinase tyrosine

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**Fig. 2** Cav1 regulation of CT-b endocytosis requires an intact scaffolding domain but not phosphorylation on tyrosine 14. (A) Mga5^{−/−}-ESC cells were treated with mβCD for 30 min. or infected with adenoviruses encoding for clathrin-hub, dynamin-wt, dynamin K44A or Cav1 and then incubated with CT-b-FITC (green) for 30 min. at 37°C. Cells were then fixed and labelled with GM130 (red). Co-localization of CT-b and Golgi was quantified for the indicated experimental conditions. (B) Mga5^{−/−}-ESC cells were transfected with Cav1-wt or Cav1 Y14F and F92A/V94A mutants and processed for endocytosis. Cells were then fixed and labelled with anti-GM130 and anti-myc antibodies to identify transfected cells and CT-b-FITC/Golgi co-localization was quantified. *P < 0.01 relative to control cells.
phosphorylation site (Y14) that is associated with EGF-induced caveole formation and focal adhesion turnover [28–31]. The ability of Cav1 to interact with EGFR and restrict the EGF signalling response was found to be dependent on the scaffolding domain and not Cav1 tyrosine phosphorylation [23]. Mga5<sup>−/−</sup> ESC cells transfected with Cav1 mutant in the Y14 tyrosine phosphorylation site (Cav1Y14F) mutant displayed a similar reduction in CT-b internalization to the Golgi as cells expressing wild-type Cav1 (Fig. 2B). However, the Cav1F92A/V94A scaffolding domain mutant was unable to significantly reduce CT-b internalization in Mga5<sup>−/−</sup> ESC cells (Fig. 2B). These data indicate that the Cav1 scaffolding domain, but not its tyrosine phosphorylation, is critical for Cav1-negative regulation of CT-b endocytosis.

Conversely, siRNA-mediated knock-down of Cav1 in Mga5<sup>+/+</sup> and Mga5<sup>−/−</sup> cells, resulting in decreased Cav1 expression, significantly increased uptake of CT-b to the Golgi (Fig. 3A and B). Uptake of CT-b in Mga5<sup>−/−</sup> cells following Cav1 siRNA treatment was dynamin- and cholesterol-dependent and clathrin-independent (Fig. 3C) showing that Cav1 is negatively regulating the raft-dependent endocytosis of CT-b. The fact that reduction of Cav1 levels in Mga5<sup>−/−</sup> cells, lacking caveolae [23], increases CT-b uptake demonstrates that Cav1 regulates raft-dependent endocytosis independently of caveolae.

**Cav1 regulation of CT-b endocytosis occurs indirectly at the cell surface**

CT-b has been shown to follow a two-step path to the Golgi apparatus via caveosomes with dynamin blocking CT-b delivery to the
Golgi at an intracellular site [11, 15]. We therefore established whether inhibition of CT-b endocytosis by dynamin and Cav1 occurs at the cell surface or intracellularly. After incubation with CT-b-FITC at 37°C, cells were acid washed to eliminate any cell surface label and intracellular CT-b-FITC measured by flow cytometry. CT-b-FITC labelling at 4°C provided a control for efficacy of cell surface removal of CT-b-FITC by acid washing and use of the same conditions on clathrin-dependent transferrin (Tf) uptake showed specificity for raft-dependent endocytosis (Fig. 4). mβCD treatment and over-expression of Cav1 and dynamin K44A completely inhibited cellular uptake of CT-b to levels observed at 4°C. Conversely, infection with the clathrin hub adenovirus did not impact on intracellular accumulation of CT-b-FITC but did block Tf-FITC uptake (Fig. 4B).

No significant colocalization was observed between Cav1 and CT-b either in caveolae-expressing Mgat5+/+ cells or in Mgat5−/− cells expressing oligomerized Cav1 microdomains but not caveolae (Fig. 5). Although some co-localization between CT-b and Cav1 could be observed in Mgat5−/−ESC cell transfected with Cav1-mRFP, the fact that the inhibition of CT-b endocytosis by Cav1 over-expression was not associated with recruitment of CT-b to cell surface Cav1 domains is indicative of an indirect effect of Cav1 on CT-b endocytosis (Fig. 5).

Discussion

In the mammary epithelial tumour cells used here, CT-b is shown to be internalized predominantly via a cholesterol- and caveolin-1-sensitive, dynamin-dependent raft pathway. Inhibition of CT-b endocytosis by mβCD, the dynamin K44A mutant and Cav1 was shown to occur at the plasma membrane. This suggests that CT-b internalization in these cells follows an endocytic route different from the dynamin-independent pathway involving tubular intermediates reported in MEFs [15]. This study therefore further highlights the varied routes of endocytosis followed by CT-b in different cell types (reviewed in [4]). The basis for the choice of major pathways remains to be determined.

Fig. 4 Cav1 and dynamin regulate raft-dependent CT-b endocytosis at the cell surface. (A) Mgat5−/−ESC cells were not treated (Control), treated with mβCD or infected with adenoviruses encoding for clathrin-hub, dynamin K44A and Cav1 and incubated with CT-b-FITC at 37°C for 30 min. and cell surface CT-b removed by acid washing. Alternatively, cells were not incubated with CT-b (Untreated), not acid-washed (wo A.W.) or incubated with CT-b-FITC at 4°C. Prior to flow cytometry, cells were incubated with propidium iodide (PI) to identify dead cells. Representative flow cytometry profiles are shown for PI and CT-b-FITC intensities under the various conditions, as indicated. (B) Percent positive cells was determined following incubation with CT-b-FITC (left) or Tf-FITC (right) for the conditions indicated earlier. Cells were acid washed in order to remove surface-bound CT-b-FITC or Tf-FITC (black bars) except where indicated (wo A.W., white bars).
endocytic route followed by CT-b in different cells remains uncertain. The internalization of CT-b via caveolae [10, 12–14, 32], the fact that Cav1−/− MEFs show a switch in CT-b uptake from the clathrin pathway to a dynamin-independent raft pathway [15] and the ability of Cav1 expression to regulate the dynamin-dependent raft uptake of CT-b shown in this study argues that expression of Cav1 is one, but certainly not the only, determinant of the endocytic route followed by CT-b.

The ability of siRNA-mediated reduction of Cav1 levels in Mgat5−/− cells, that express few or no caveolae [4], to increase CT-b delivery to the Golgi indicates that Cav1 regulation of endocytosis is independent of caveolae formation. Although Mgat5−/− cells express Cav1 at levels below the threshold for caveolae formation, they still express oligomeric Cav1 that recruits EGFR and functionally regulates cell surface CT-b and EGFR diffusion [23]. Indeed, the scaffolding domain of Cav1 was crucial for Cav1-negative regulation of CT-b endocytosis as a scaffolding domain mutant, but not mutation of the Y14 tyrosine phosphorylation site, inhibited CT-b uptake to the Golgi apparatus. Similarly, mutation of the Cav1 scaffolding domain, but not the Y14 tyrosine phosphorylation site, inhibited EGFR signalling [4]. However, minimal CT-b co-localization with Cav1 contrasts the recruitment of EGFR to Cav1 microdomains previously observed in the Mgat5−/− cells [23]. Similarly, negative regulation by caveolin of dysferlin endocytosis requires the scaffolding domain but not dysferlin association with caveolins or caveolae [22]. The Cav1 scaffolding domain mediates interaction with various receptors, the formation of Cav1 oligomers and Cav1 interaction with cholesterol [29]. The ability of reduced levels of Cav1 to regulate raft-dependent CT-b internalization together with its minimal co-localization with uninternalized cell surface CT-b leads us to believe that expression of Cav1 and of oligomerized Cav1 microdomains impact at distance on raft functionality.

Cav1 interacts with dynamin-2 [33] and Cav1 could potentially regulate raft endocytosis by sequestering dynamin-2 away from rafts. Although such sequestration of dynamin-2 should also affect clathrin-dependent uptake, Cav1 does not affect Tf uptake (Fig. 4). Alternatively, Cav1 may function as a store of lipid raft components such as cholesterol or sphingolipids that may be released under specific conditions [34]. Indeed, cholesterol and glycosphingolipids can override Cav1-dependent negative regulation of the caveolar uptake of lactosyl ceramide [21] and plasma membrane sphingomyelin is required for CT entry into cells [35]. Cav1 is a cholesterol-binding protein [36] and its sequestration of cholesterol may impact on the functionality, including the endocytic potential, of non-caveolar raft domains. Indeed, whether raft domains are small, transient and dynamic [37, 38], stable Cav1 expressing microdomains with the capacity to interact with and sequester raft components will necessarily impact on their composition, functionality and endocytic potential. Indirect regulation of raft-dependent endocytosis by oligomerized Cav1 microdomains, or scaffolds, therefore represents a regulatory mechanism distinct from sequestration of endocytic ligand by invaginated caveolae. This provides a novel functional role for oligomerized Cav1 microdomains outside caveolae.
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