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Cholesterol regulation of rab-mediated sphingolipid endocytosis

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Abstract Despite a tight regulation of its intracellular content, cholesterol is found accumulated in pathological conditions such as sphingolipidosis as well as after cell treatment with drugs like hydrophobic amines. Furthermore, cellular cholesterol increases when cultured cells approach confluence. Under these conditions, the endocytic pathways of plasma membrane sphingolipids are differently affected. In this short review, we will summarize recent results from our laboratory as well as those of other groups, indicating that the intracellular accumulation of cholesterol inhibits the dissociation of rab GTPases from the target membranes, causing the alteration of rab-mediated membrane traffic.

Keywords Glycosphingolipid · Sphingomyelin · Fluorescent lipid analog · Endosome · Sphingolipidosis · Cell confluency · Guanine nucleotide dissociation inhibitor

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Introduction

Sphingolipids are membrane lipid constituents that are accumulated in the plasma membrane and the endocytic compartments of eukaryotic cells [1, 2]. In the plasma membrane, sphingolipids form specific lipid domains [3, 4] that are involved in signal transduction and membrane trafficking. Sphingolipids are also the reservoir of lipid second messengers such as ceramide and sphingosine-1-phosphate [5]. The endocytosis of sphingolipids regulates the amount of lipids on the cell surface as well as the formation of lipid second messengers via lipid degradation in the endosomes/lysosomes. The endocytic pathways of cell surface sphingolipids are not fully characterized.

Sphingolipids are composed of glycosphingolipids and sphingomyelin. Glycosphingolipids bind a variety of sugars to the ceramide backbone, whereas sphingomyelin is a conjugate of ceramide and phosphocholine. Studies using fluorescent sphingolipid analogs revealed different endocytic pathways of glycosphingolipids and sphingomyelin [6] that are regulated by different rab small GTPases.

In a similar way to sphingolipids, cholesterol is enriched in the plasma membrane and the endocytic compartment [7]. It has been reported that sphingolipids preferentially form a complex with cholesterol [8, 9]. Cellular content of cholesterol is stringently regulated [10]. However, cells accumulate cholesterol in genetic diseases such as sphingolipid storage diseases [11–13]. In cultured cells, cellular cholesterol is increased when cells approach confluence [14–16]. The endocytic pathways of fluorescent sphingolipids are differently affected under these conditions. Recent results from our laboratory as well as those of other groups suggest that cholesterol regulates the intracellular distribution of rab proteins and thus affects membrane traffic.

Endocytic pathways of cell surface sphingolipids

Little is known about the endocytic pathways of endogenous sphingolipids, because of the technical difficulties. Instead, the endocytic pathways of cell surface sphingolipids have been studied using either fluorescent sphingolipid analogs [17, 18] or toxins that bind specific sphingolipids [19, 20]. However, the molecular size of fluorophores that bind to fluorescent lipids is often as big as the lipid itself. In addition, the fluorescent lipids have to be more hydrophilic than their natural counterpart to favor efficient insertion into the plasma membrane. It has been reported that the fate of endocytosed fluorescent sphingomyelin is dependent on its acyl chain length [21–23]. Recently, Kuerschner *et al*. have developed novel polyene–lipid analogs [24]. These lipids exhibit a structural similarity to natural lipids and thus are expected to behave like natural lipids.

Since proteins are much bigger than lipids, the binding of lipid-specific protein toxins may alter the endocytic pathway of endogenous lipids. Thus the endocytosis of fluorescent lipid analogs and lipid-binding proteins has to be carefully evaluated. Nevertheless, these data provided fundamental information of lipid endocytosis, such as the rate of bulk plasma membrane recycling [25] as well as polarized transport routes of epithelial cells [26].

In fully polarized human hepatoma HepG2 cells, 6-[*N*-(7-nitrobenz-2-oxa-1,3 diazole-4-yl)amino]hexanoyl-glucosylceramide (C6-NBD–GlcCer) is recycling between the subapical compartment (SAC) and the apical bile canalicular membrane. By contrast, C6-NBD–sphingomyelin initially accumulates in the SAC, but is ultimately transported to the basolateral membrane [27, 28]. In normal human skin fibroblasts, boron dipyrromethenedifluoride–lactosylceramide (BODIPY–LacCer) and –globoside (BODIPY–Globoside) are internalized by a clathrin-dependent mechanism and targeted to the Golgi apparatus [6]. The endocytosis of fluorescent sphingolipids are regulated by several rab proteins. Golgi targeting is inhibited in cells expressing dominant-negative rab7 and rab9, but not rab11 constructs [29]. Rab7 mediates early to late endosomes, and late endosomes to lysosome transport, whereas rab9 is involved in late endosomes to Golgi transport [30]. On the other hand, rab11 regulates transport from recycling endosomes to the plasma membrane. These results suggest that BODIPY–glycolipids are targeted to the Golgi apparatus through late endosomes. In addition to Golgi targeting, endocytosed BODIPY–LacCer is recycled back to the plasma membrane. This recycling is inhibited by dominant-negative rab4, but not by rab11 [31]. Rab4 is suggested to play a role in fast recycling from early endosomes [32, 33].

In contrast to BODIPY–glycolipids, BODIPY–sphingomyelin utilizes both clathrin-dependent and -independent endocytic pathway in normal human skin fibroblasts [6]. In subconfluent Chinese hamster ovary (CHO) cells, C6-NBD– and BODIPY–sphingomyelin are endocytosed to recycling endosomes identified by co-labeling with endocytosed transferrin [16, 25]. In contrast, BODIPY–LacCer is accumulated into perinuclear Golgi region [16]. A detailed kinetic study indicates that 42–62% of the internalized C6-NBD–sphingomyelin returns to the plasma membrane with a half-time of 1–2 min [34]. Similar results are obtained using Hep2 and nonpolarized Madin–Darby canine kidney cells.

Cholesterol accumulation alters the endocytic pathway of fluorescent sphingolipids

Cells acquire cholesterol as cholesteryl ester in the form of low density lipoprotein (LDL). LDL is endocytosed to late endosomes/lysosomes, where cholesteryl ester is hydrolyzed to free cholesterol. Free cholesterol is then released to plasma membrane and the endoplasmic reticulum [35]. The exit of cholesterol from late endosomes is blocked in cells from patients of genetic diseases such as sphingolipid storage diseases (SLSD) [36, 37]. Those cells accumulate free cholesterol in the late endosomes/lysosomes. Several hydrophobic amines are reported to mimic SLSD [38–40]. It is suggested that the specific endosomal lipid domains containing the unique phospholipid, bis(monoacylglycero) phosphate (BMP, also called as lysobisphosphatidic acid, LBPA) is involved in the cholesterol exit from late endosomes [37, 41–44].

SLSDs generally result from a defective lysosomal hydrolase or activator-protein cofactors, which lead to accumulation of LDL-derived lipids in late endosomes/lysosomes [45, 46]. In addition, lipid accumulation in Niemann–Pick type C (NPC) and mucolipidosis type IV diseases arises from defects in transport of cholesterol to or from late endosomes/lysosomes [47–49]. In fibroblasts from SLSD patients, BODIPY–LacCer is targeted predominantly to late endosomes/lysosomes instead of going to the Golgi apparatus [36]. Depletion of cholesterol from SLSD cells restores normal BODIPY–LacCer targeting to the Golgi, whereas overloading normal cells with cholesterol redirects the lipid to the late endosomes/lysosomes. In contrast, overloading with cholesterol did not eliminate Golgi targeting of BODIPY–sphingomyelin [6]. Similar to BODIPY–LacCer, cholera toxin B-subunit (CtxB), which specifically binds ganglioside GM1, is targeted to the Golgi apparatus of normal cells, but punctate endosomal structures in SLSD cells. It is also reported that dialkylindocarbocyanine lipid–mimetic analogs that recycle efficiently from early endosomes in wild-type cells are targeted to late endosomes in NPC cells [50].

In cultured endothelial cells and CHO cells, cellular cholesterol is reported to increase when cells become confluent

[14–16]. Fluorescence labeling of CHO cells by cholesterol marker, filipin, indicates cholesterol accumulation in recycling endosomes as well as in other cellular compartments [16] (Fig. 1a). Under these conditions, the endocytic pathway of BODIPY–sphingomyelin, but not BODIPY–LacCer, is altered. Whereas BODIPY–sphingomyelin is accumulated in recycling endosomes in subconfluent cells, this lipid analog is distributed into early endosomes in confluent cells (Fig. 1b). Similar to BODIPY–sphingomyelin, fluorescent poly(ethylene glycol)-derivatized cholesterol (PEG-chol) is accumulated in recycling endosomes in subconfluent cells and is concentrated in early endosomes in confluent cells. PEG-Chol preferentially partitions to cholesterol-rich membrane domains [51]. Since the bulk PEG moiety of PEG-Chol prevents transbilayer movement of the molecule, PEG-Chol could selectively monitor the endocytic pathway of cholesterol-rich domain of plasma membrane. These results suggest that cellular cholesterol content controls the endocytic pathway of plasma membrane cholesterol as well as sphingolipids.

Cholesterol alters the membrane–cytosol cycle of rab proteins

Rab proteins and their effectors coordinate consecutive stages of transport, such as vesicle formation, vesicle and organelle motility, and tethering of vesicles to their target compartments [30]. Efficient membrane transport relies on the cycling of active GTP form and inactive GDP form of rab proteins. In addition, GDP form of the protein has to be released from the target membrane by GDP dissociation inhibitor (GDI) for reutilization [52, 53]. Thus, both GTP-GDP cycle and membrane–cytosol cycle of the proteins are required for proper rab-mediated transport.

Accumulating evidence suggests that cholesterol alters the endocytic pathway of sphingolipids through modulating the activity of rab proteins. Overexpression of wild-type rab7 and rab9 in NPC cells restores Golgi targeting of BODIPY–LacCer and CtxB [29]. Niemann–Pick type A (NPA) is a deficiency in acid sphingomyelinase, resulting in

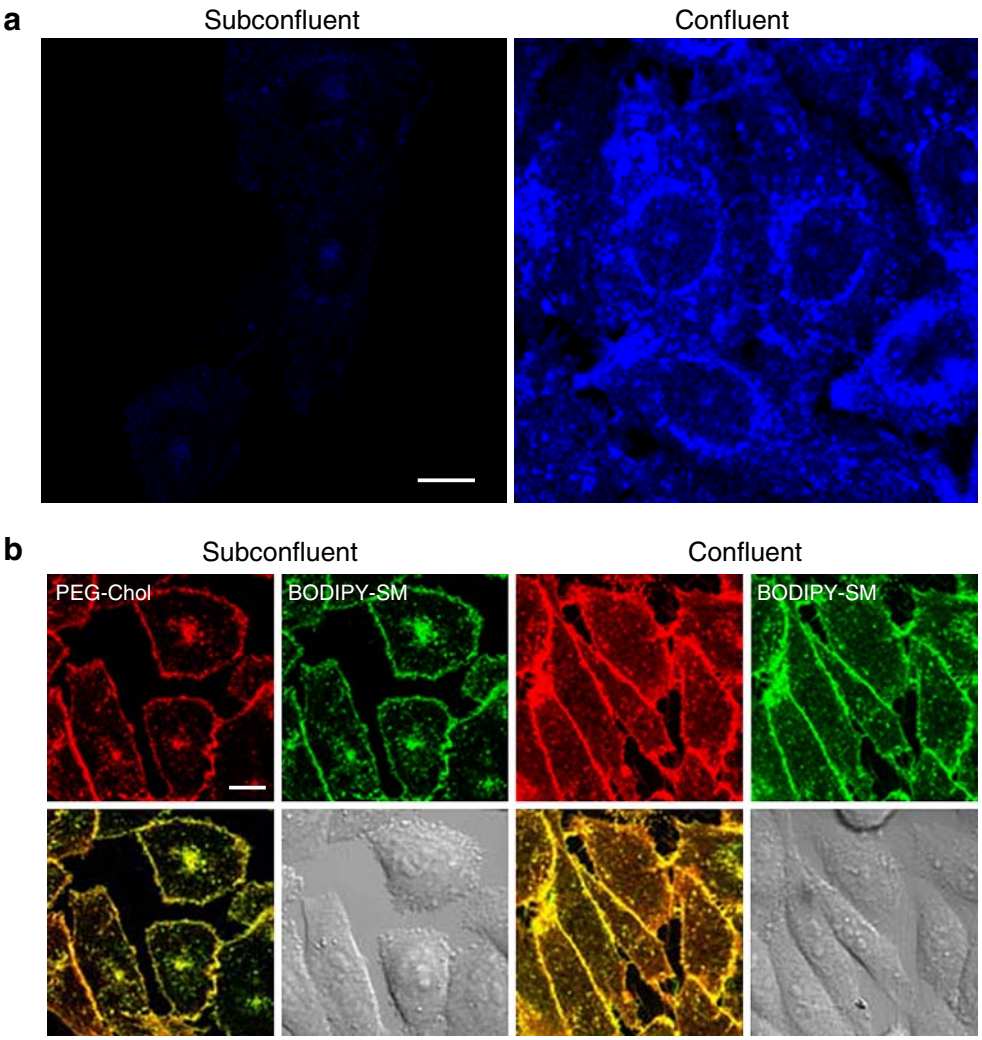

Fig. 1 Intracellular cholesterol distribution and endocytosis of fluorescent PEG-Chol and BODIPY–sphingomyelin in subconfluent and confluent CHO cells. **a** Intracellular distribution of free cholesterol was monitored by cholesterol-specific antibiotic, filipin, in subconfluent and confluent cells. Bar=10 μm. **b** Subconfluent and confluent cells were incubated with TRITC-PEG-Chol and BODIPY–sphingomyelin for 15 min at 37°C. Bar=10 μm

late endosomal and lysosomal accumulation of sphingomyelin and cholesterol [54]. NPA and NPC cells accumulate cholesterol not only in BMP/LBPA- and rab7-positive late endosomes, but also in rab4-positive early endosomes. In addition to endosome targeting, recycling of BODIPY–LacCer, which is mediated by rab4, is delayed in NPA and NPC cells [31]. In normal human skin fibroblasts, endogenous rab4 is often found in tubular extensions projecting from early endosome antigen (EEA1)-positive globular structures. In contrast, rab4 and EEA1 extensively colocalized and are found mainly in globular structures in NPA and NPC cells. Cholesterol depletion in these cells restores lipid recycling and rab4 distribution.

Using C6-NBD–sphingomyelin, it is suggested that the rab11-dependent and recycling endosome-mediated lipid recycling is delayed in confluent cells. In contrast, the rab4-dependent and early endosome-mediated recycling is not affected [16]. In subconfluent cells, endogenous rab11 is distributed in the perinuclear Golgi region and in the cytoplasm, whereas in confluent cells, rab11 is accumulated in recycling endosomes [16] (Fig. 2). In contrast, cell confluency did not alter GTP form/GDP form ratio of rab 11. When the constitutive, active GTP form of rab11 is overexpressed in confluent cells, the endocytosed fluorescent PEG-Chol is accumulated in the recycling endosomes. Constitutive, active rab11 was localized to recycling endosomes as observed in the case of endogenous rab11 in confluent cells. Dominant negative rab11, localized in cytoplasm, did not alter the internalization of lipid probes. These results suggest that in confluent cells rab11 is localized in the recycling endosomes as inactive form. Thus, the release of inactive rab11 from the recycling endosomes is delayed in confluent, cholesterol-rich cells. This idea is supported by the observation that the depletion of cholesterol in confluent cells partially re-distributes rab11 to the cytoplasm and redirects BODIPY–sphingomyelin to recycling endosomes.

When cholesterol is accumulated in late endosomes/lysosomes, membrane associated rab7, but not rab5 is increased [55]. Rab7 is poorly extracted by GDI from membrane fractions prepared from cholesterol-accumulated cells. In addition, increase of cholesterol content in membrane fractions prepared from control cells also inhibits rab7 extraction by GDI. It is also reported that the extraction of rab9 by GDI is reduced in NPC membranes [56]. Similarly, in vitro extraction of rab4, but not rab11, with GDI is severely attenuated in endosomal fractions from NPA and NPC cells [31]. The membrane bound form of rab11 was decreased by cholesterol depletion and the overexpression of GDI in confluent CHO cells [16]. The effect of cholesterol on GDI-mediated rab protein extraction is reconstituted in liposomes loaded with prenylated rab9 [56]. These results suggest that cholesterol directly affects the membrane–cytosol cycle of rab proteins.

Conclusion and perspectives

The above results indicate that the accumulation of cholesterol alters the endocytic pathways of fluorescent sphingolipid analogs by inhibiting the extraction of rab proteins from endosome membranes. Recently, it is shown that the intracellular distribution of cholesterol is also under the control of rab proteins [29, 57–59]. These results suggest that rab proteins are potential targets for treatment of SLSDs. Furthermore, the difference of cell density is observed in other patho-physiological conditions *e.g.* during wound healing. It is speculated that alteration of cholesterol metabolism and sphingolipid endocytosis could occur during wound healing in connection to the cholesterol

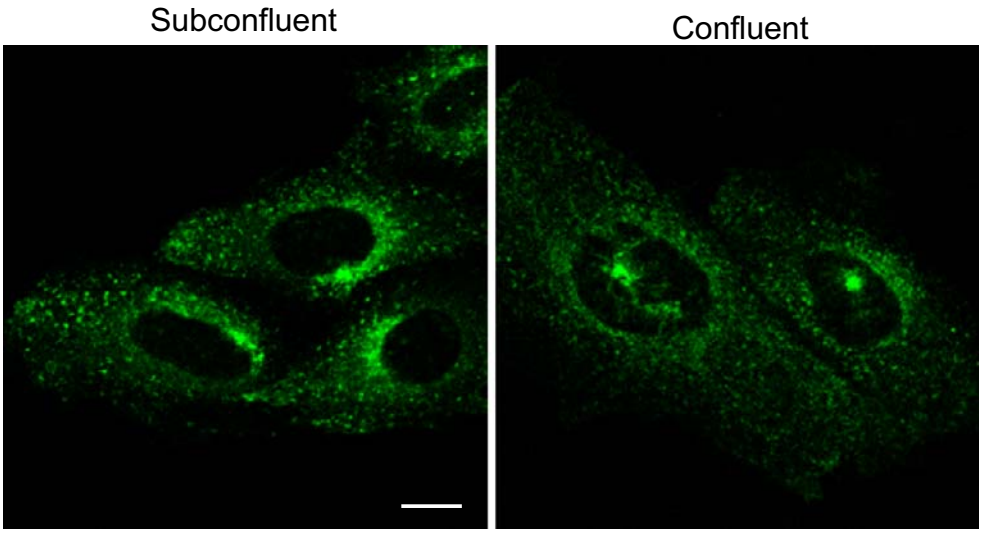

Fig. 2 Intracellular distribution of rab11 in subconfluent and confluent cells. Bar=10 μm

regulation of membrane polarization during cell movement [60, 61].

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