

Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways

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Sphingolipids (SLs) are plasma membrane constituents in eukaryotic cells which play important roles in a wide variety of cellular functions. However, little is known about the mechanisms of their internalization from the plasma membrane or subsequent intracellular targeting. We have begun to study these issues in human skin fibroblasts using fluorescent SL analogues. Using selective endocytic inhibitors and dominant negative constructs of dynamin and epidermal growth factor receptor pathway substrate clone 15, we found that analogues of lactosylceramide and globoside were internalized almost exclusively by a clathrin-independent (“caveolar-like”) mechanism, whereas an analogue of sphingomyelin was taken up approximately equally by clathrin-dependent and -independent pathways.

We also showed that the Golgi targeting of SL analogues internalized via the caveolar-like pathway was selectively perturbed by elevated intracellular cholesterol, demonstrating the existence of two discrete Golgi targeting pathways. Studies using SL-binding toxins internalized via clathrin-dependent or -independent mechanisms confirmed that endogenous SLs follow the same two pathways. These findings (a) provide a direct demonstration of differential SLs sorting into early endosomes in living cells, (b) provide a “vital marker” for endosomes derived from caveolar-like endocytosis, and (c) identify two independent pathways for lipid transport from the plasma membrane to the Golgi apparatus in human skin fibroblasts.

Introduction

Sphingolipids (SLs)* are synthesized in the endoplasmic reticulum and Golgi apparatus of eukaryotic cells and are subsequently transported to the plasma membrane (PM), where they are highly enriched (Schwarzmann and Sand-

hoff, 1990; van Meer and Holthuis, 2000). Some PM SLs may not be homogeneously distributed in the plane of the membrane bilayer, but are thought to be concentrated, along with cholesterol, in membrane microdomains (Edidin, 1997; Rietveld and Simons, 1998; Brown and London, 2000). In principle, SLs at the PM may be internalized by one or more endocytic mechanisms, either as part of membrane recycling or remodeling, or as a consequence of specific endocytic events induced by binding of ligands to PM receptors. Once internalized from the PM, lipids can be transported to other intracellular destinations (e.g., lysosomes or the Golgi complex); however, the specific pathways for internalization and intracellular targeting of PM SLs are not well characterized, primarily because of methodological limitations in studying the transport of endogenous lipids (see Pagano, 1990). Consequently, the movement of PM lipids along the endocytic pathway has been examined by using (a) labeled (fluorescent, biotinylated, spin labeled, or radiolabeled “short chain”) lipid analogues (Pagano and Sleight, 1985; Schwarzmann and Sandhoff, 1990; Hoekstra

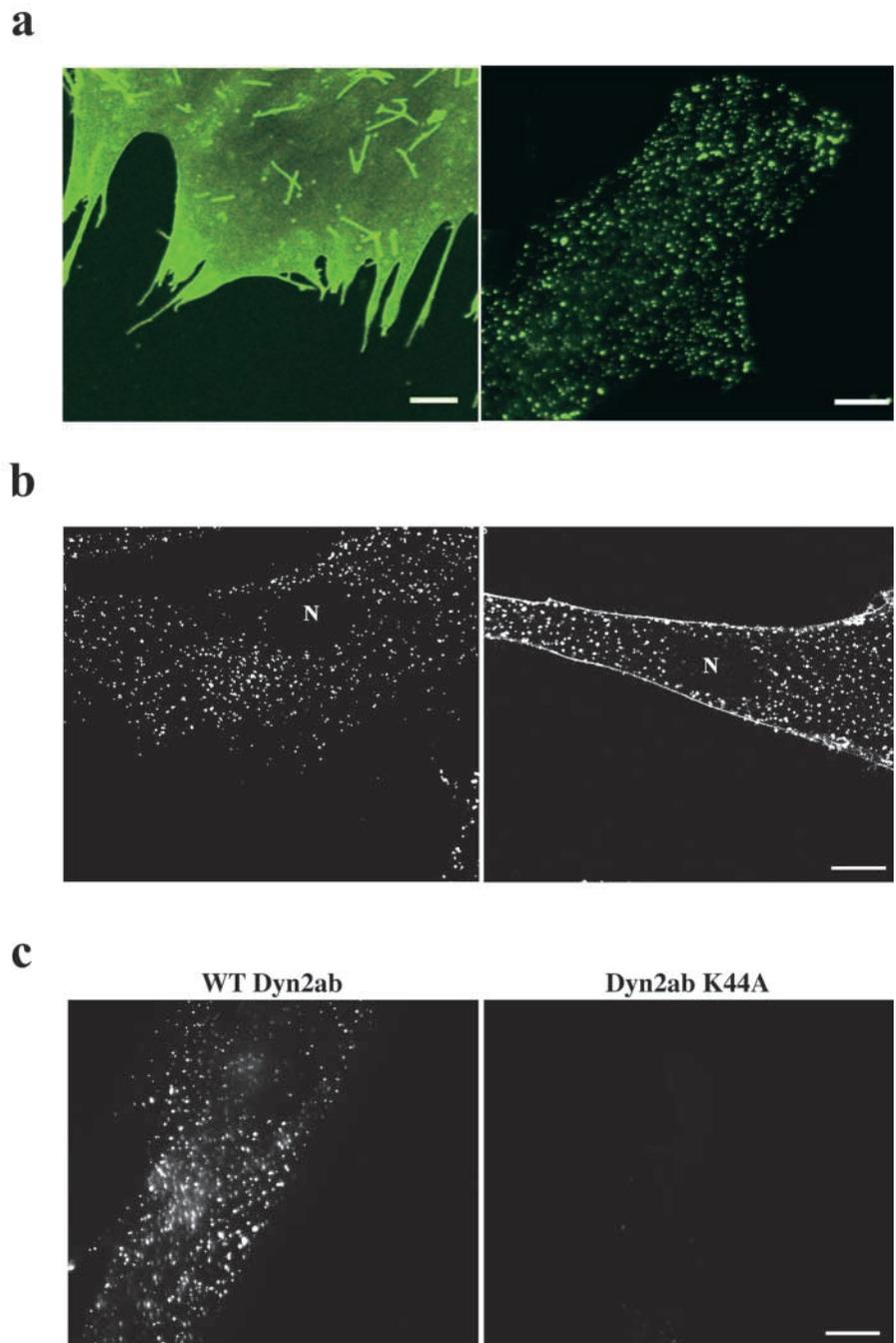
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*Abbreviations used in this paper: BODIPY, boron dipyrromethenedifluoride; CtxB, cholera toxin, B-subunit; DF-BSA, defatted bovine serum albumin; Dyn2, dynamin 2; Eps15, EGFR pathway substrate clone 15; GSL, glycosphingolipid; LacCer, lactosylceramide; m β -CD, methyl- β -cyclodextrin; PM, plasma membrane; SL, sphingolipid; SLSL, sphingolipid storage disease; SM, sphingomyelin; StxB, shiga toxin, B-subunit. Key words: endocytosis; caveolae; cholesterol; Eps15; lipid storage diseases

Figure 1. Internalization of fluorescent LacCer from the plasma membrane of human skin fibroblasts into very early endosomes. (a) Cells were incubated for 30 min at 10°C with 2 μ M BODIPY-LacCer, washed, and immediately observed (left) or warmed for 30 s at 37°C and back exchanged (right) before observation under the fluorescence microscope at green wavelengths. (b) Cells were incubated for 30 min at 10°C with 2 μ M BODIPY-LacCer, washed, warmed for 30 s at 37°C, back exchanged, and observed by confocal microscopy. Note the discrete punctate fluorescent structures which outlined the region of the nucleus (N). The right panel shows a specimen which was warmed for 30 s, but back exchanged for a shorter period of time than the left, so that the outline of the cell could also be seen. (c) Cells were transfected with GFP-tagged wild-type (WT Dyn2ab) or mutant (Dyn2ab K44A) dynamin 2 constructs and subsequently pulse labeled with BODIPY-LacCer as in panel a. Transfected cells were identified by GFP-fluorescence; BODIPY-lipids were observed at red wavelengths. No red fluorescence could be detected in transfected cells which were not treated with the fluorescent SLs. Virtually identical results to panels a–c were also obtained using BODIPY-SM (data not shown). Bars, 10 μ m.



and Kok, 1992), or (b) labeled toxins which bind to certain endogenous SLs and can be used to trace lipid distribution and transport in cells (Sandvig and van Deurs, 1996; Johannes and Goud, 1998).

Using these methods, several fluorescent SL analogues and SL-binding toxins have been shown to be endocytosed by temperature- and energy-dependent processes (Koval and Pagano, 1989, 1990; Schwarzmann and Sandhoff, 1990; Hoekstra and Kok, 1992; Martin and Pagano, 1994). Recycling of fluorescent sphingomyelin (SM) (Koval and Pagano, 1989, 1990; Mayor et al., 1993) and glucosylceramide (GlcCer) (Kok et al., 1991, 1992) between intracellular membranes and the PM has been studied extensively in a number of cell types, including human skin fibroblasts,

CHO cells, and polarized cells. In addition to recycling, internalized lipids may be specifically targeted to other intracellular compartments, such as late endosomes/lysosomes and the Golgi apparatus, and evidence for endocytic sorting of lipids between these compartments has been provided by several groups (Kok et al., 1991; Mukherjee et al., 1999; Puri et al., 1999). Evidence that some SLs are targeted to the Golgi apparatus after endocytosis comes from the use of biotinylated, fluorescent, or nondegradable SL analogues, or the labeled B-subunits of cholera toxin (CtxB) or shiga toxin (StxB) which bind to GM₁ ganglioside and globoside, respectively (Schwarzmann and Sandhoff, 1990; Schwarzmann et al., 1995; Chen et al., 1998; Puri et al., 1999; Grimmer et al., 2000; Sandvig and van Deurs, 2000).

Table I. Inhibition of sphingolipid endocytosis by biochemical treatments and dominant negative constructs

	Clathrin-independent internalization			Clathrin-dependent internalization			Clathrin-dependent and -independent
	Nystatin ¹	Genistein ¹	m β -CD ¹	Chlorpromazine ¹	K ⁺ depletion ¹	Eps15 mutant ²	Dynamin mutant ²
	%	%	%	%	%	%	%
Rh-CTXB	74.9 \pm 7.53 ³	80.8 \pm 2.8	81.4 \pm 16.9	0.8 \pm 0.1	2.6 \pm 1.6	–	–
Dil-LDL	2.6 \pm 1.4	3.3 \pm 2.3	1.8 \pm 0.4	78.3 \pm 18.2	76.0 \pm 2.3	–	–
BODIPY-LacCer	73.9 \pm 5.0	82.0 \pm 4.5	81.0 \pm 14.9	0.7 \pm 0.1	7.0 \pm 0.0	2.6 \pm 0.2	94.3 \pm 4.0
BODIPY-Globoside	–	81.6 \pm 5.5	83.8 \pm 14.9	0.9 \pm 0.2	0.0 \pm 0.0	–	–
BODIPY-SM	48.8 \pm 3.1	48.8 \pm 4.3	44.9 \pm 10.7	50.1 \pm 13.8	51.2 \pm 3.6	49.0 \pm 13.2	95.2 \pm 2.4

¹Cells were pretreated with or without the indicated inhibitor (see Materials and methods) and subsequently incubated at 10°C with Dil-LDL, Rh-CtxB, or BODIPY-SL. The samples were then warmed for 5 min at 37°C to allow endocytosis to occur and then back exchanged to remove fluorescent lipid remaining at the PM. Samples were then observed under the fluorescence microscope and the percentage of internalization of the marker or lipid analog was determined by image analysis of the fluorescently labeled cells. Image analysis results were validated for BODIPY-LacCer and -SM by lipid extraction and analysis (data not shown).

²Cells were transfected with plasmids expressing GFP-tagged mutant or wild-type constructs of Dyn2ab or Eps15 (See Materials and methods), incubated with BODIPY-LacCer or -SM as in footnote 1, and observed under the fluorescence microscope. Transfected cells were identified by GFP fluorescence. BODIPY-SLs were observed at red wavelengths. No red fluorescence could be detected in transfected cells which were not treated with fluorescent SLs.

³Percentage inhibition is relative to internalization in untreated control cells for drug treatments, or relative to internalization using wild-type constructs for Eps15 or dynamin. Values are mean \pm SD of at least 10 cells in each of three independent experiments.

The relative importance of specific mechanisms for the endocytosis and intracellular targeting of PM SLs are not known. Studies using StxB bound to the cell surface show that this lipid–toxin complex is internalized via clathrin-dependent endocytosis (Johannes and Goud, 1998). In addition, a fluorescent analogue of SM partially colocalizes with endocytosed transferrin receptor within seconds of internalization from the PM, suggesting that at least a portion of the SM analogue is endocytosed via the clathrin pathway (Chen et al., 1997). A second potential mechanism for endocytosis of SLs is internalization via caveolae. Endocytosis through caveolae has been best characterized as a mechanism for the entry of small molecules such as folic acid; however, CtxB bound to GM₁ ganglioside, endothelin and growth hormone receptors, antibody–cross-linked alkaline phosphatase, SV-40 virus, and bacteria have all been reported to be internalized via caveolae or “caveolar-like” processes (Parton, 1994; Orlandi and Fishman, 1998; Chen and Norkin, 1999; Lobie et al., 1999; Okamoto et al., 2000; Shin et al., 2000; Pelkmans et al., 2001). Finally, in addition to the clathrin and caveolar endocytic mechanisms, caveolar-like endocytosis is exhibited by certain cell types that lack caveolin-1, and some reports suggest the existence of additional nonclathrin, noncaveolar endocytic processes in some cells (Orlandi and Fishman, 1998; Sandvig and van Deurs, 2000).

In the current study, we examine the mechanism of endocytosis of several different SL analogues from the PM of human skin fibroblasts and provide evidence that two glycosphingolipid (GSL) analogues were selectively internalized via a clathrin-independent pathway, whereas another SL, SM, was internalized by both clathrin-dependent and -independent mechanisms. Furthermore, we show that although both internalization pathways led to Golgi targeting of SLs in normal cells, the initial mode of internalization was a major factor in determining the subsequent targeting of the SLs to late endosomes/lysosomes versus the Golgi apparatus in SL storage disease (SLSD) fibroblasts. These findings were confirmed using SL binding toxins which monitor the traf-

ficking of endogenous SLs at the PM, demonstrating that our results were not limited to fluorescent SL analogues.

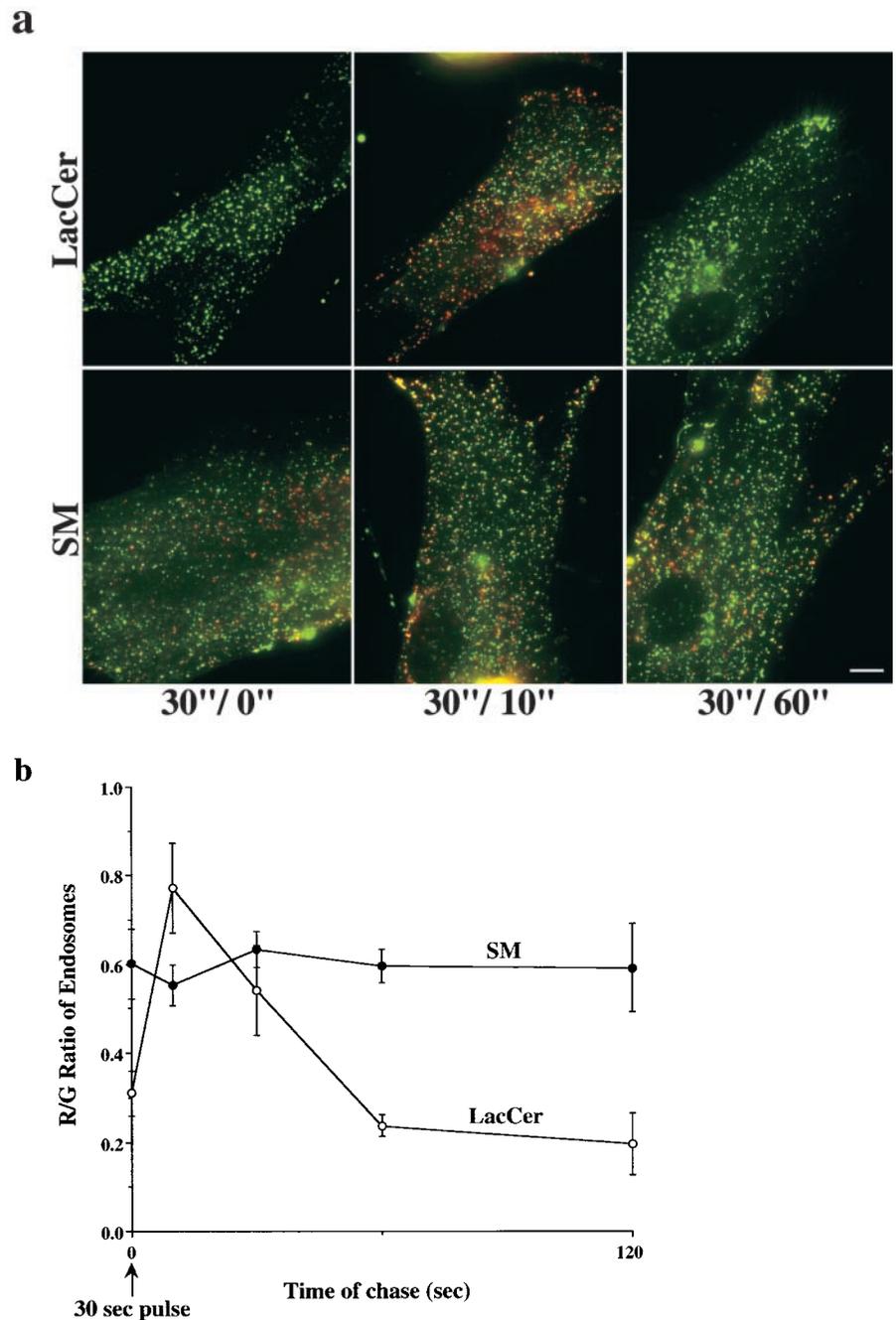
Results

Initial pathway of SL internalization in normal human skin fibroblasts

Our studies used fluorescent analogues of SM, lactosylceramide (LacCer), and globoside containing a boron dipyrromethenedifluoride (BODIPY)-labeled fatty acid (Kang and Haugland, 1989; Pagano et al., 1991). The fluorescence emission of these analogues changes from green (G) to red (R) wavelengths with increasing concentration in membranes due to excimer formation, and measurements of the “R/G ratio” by quantitative fluorescence microscopy can give information about the concentration of an analogue within an intracellular organelle in living cells (Pagano et al., 1991; Chen et al., 1997). When human skin fibroblasts were incubated with BODIPY-LacCer or -SM for 30 min at 10°C, washed, and observed by conventional or confocal fluorescence microscopy, only PM labeling was seen and the fluorescence appeared to be uniformly distributed (LacCer, Fig. 1 a, left; SM, data not shown). Using R/G ratios of fluorescence images, as well as lipid extraction and analysis, we found that a 30 min pulse at 10°C with 2 μ M BODIPY-LacCer or 1 μ M BODIPY-SM resulted in “equal loading” of the SL analogues in the PM (2.92 \pm 0.11 vs. 2.99 \pm 0.29 nmol/mg protein, respectively) and an R/G ratio of 1.0.

When the labeled cells were then incubated for 30 s at 37°C to allow internalization of the fluorescent analogues to occur and then back exchanged at 10°C in the presence of metabolic inhibitors to block further endocytosis and to remove any fluorescent SL remaining at the PM (Chen et al., 1997), numerous fluorescent punctate structures were observed which were widely scattered throughout the cytoplasm, as shown in Fig. 1 a (right) for the LacCer analogue. Confocal microscopy demonstrated that most of the fluorescent lipid which was resistant to back exchange was intracel-

Figure 2. Internalization of fluorescent SL analogues from the plasma membrane of human skin fibroblasts into very early endosomes. (a) Cells were incubated with BODIPY-LacCer or -SM at 10°C using SL concentrations that resulted in incorporation of equal amounts of the analogues in the PM. The cells were then washed, warmed for 30 s at 37°C, back exchanged, chased for 0, 10, or 60 s at 37°C in the absence of inhibitors, and observed under the microscope at green + red wavelengths. (b) Cells were pulse-labeled as in panel a and the R/G ratio (a measure of the molar density of the SL analogue) of ≥ 500 endosomes in 3–5 cells was quantified for each data point. Values are mean \pm SD. Bar, 10 μ m.



ular and not continuous with the cell surface. This was clearly shown by the presence of discrete punctate fluorescent structures outlining the region of the nucleus (N) in optical sections through the center of the cell after 30 s of internalization (Fig. 1 b, left). An image is also presented of a cell after incomplete back exchange so that the PM can be readily discerned in relation to endocytic vesicles (Fig. 1 b, right). When identical experiments were carried out using human skin fibroblasts expressing a dominant negative mutant of dynamin (van der Blik et al., 1993), no internalization of BODIPY-LacCer into punctate structures could be detected (Fig. 1 c). In contrast, the punctate structures visualized after 30 s at 37°C appeared identical to those shown in Fig. 1 a when cells were transfected with a wild-type dynamin (Fig. 1 c). Expression of the dominant negative dy-

namin construct also completely blocked the internalization of BODIPY-SM (data not shown). For convenience, we hereafter refer to the punctate structures derived from fluorescent SL analogue internalization as “endocytic vesicles” or “endosomes,” but do not mean to infer that these vesicles represent a discrete endosomal compartment.

We next studied the fate of BODIPY-LacCer and -SM present in “30 s endosomes” after various periods of further chase at 37°C (Fig. 2). There were no obvious differences in the number or distribution of endosomes formed from LacCer versus SM after the initial 30 s of internalization. However, at this time point virtually all of the endosomes appeared green in color when LacCer was used, whereas in the case of SM, numerous red/orange endosomes (indicative of high BODIPY-SM concentrations) as well as green endo-

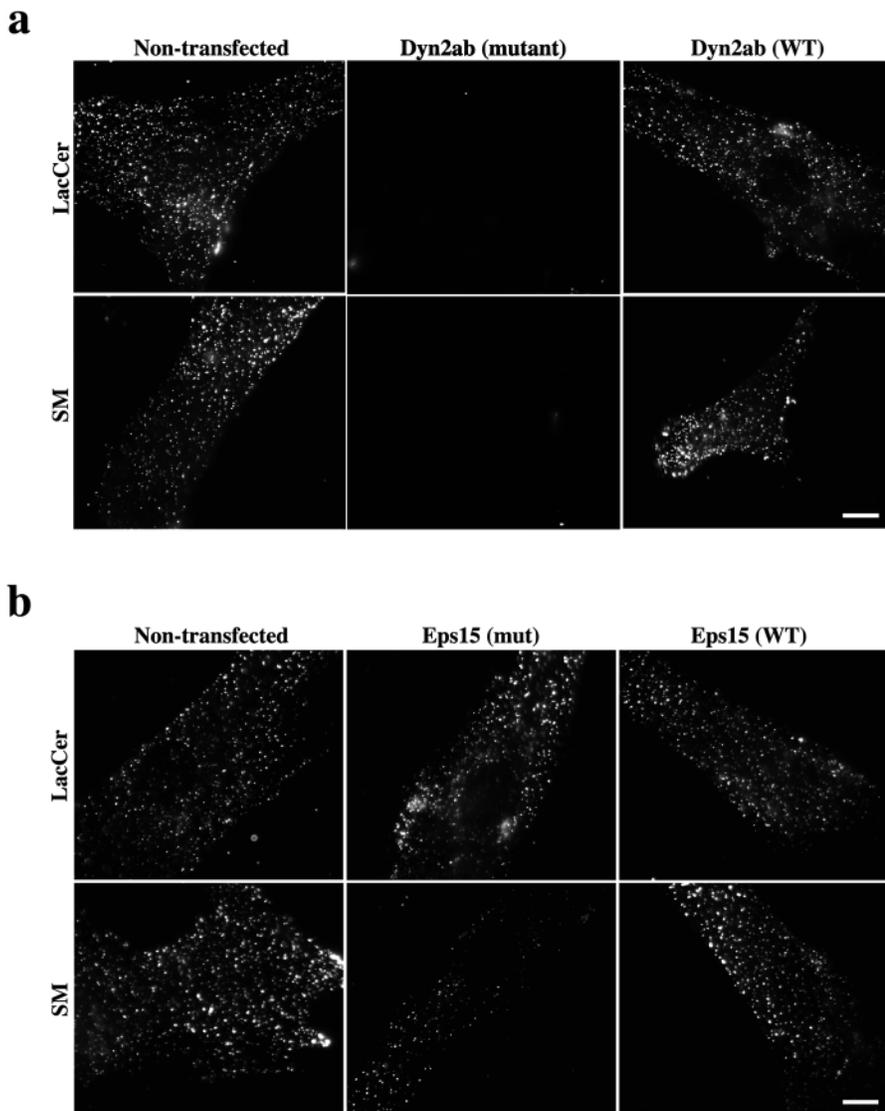


Figure 3. Role of dynamin and Eps15 in the internalization of BODIPY-LacCer and -SM from the PM. Human skin fibroblasts were transfected with plasmids expressing GFP-tagged mutant or wild-type (WT) constructs of (a) Dyn2ab or (b) Eps15. Cells were subsequently incubated for 30 min at 10°C with 5 μ M BODIPY-LacCer or -SM, washed, warmed for 5 min at 37°C, back exchanged, and observed under the fluorescence microscope. GFP and BODIPY-SLs were visualized as in Fig. 1. Bars, 10 μ m.

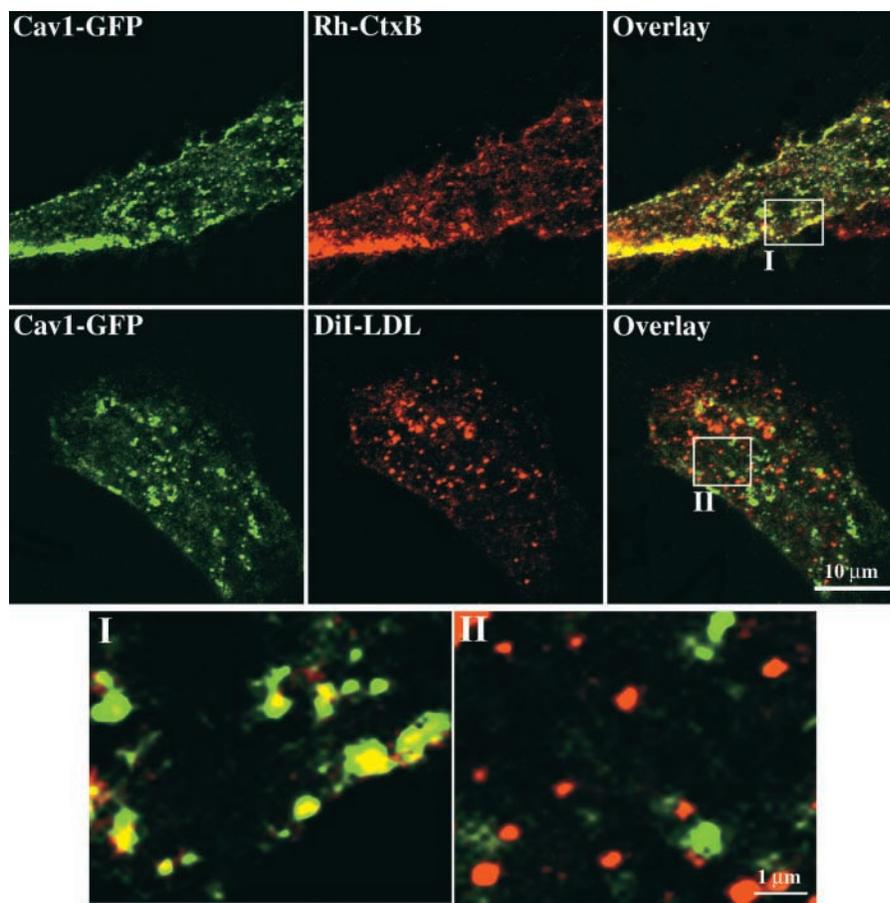
somes were seen (Fig. 2 a, 30''/0''). After further incubation at 37°C in the absence of metabolic inhibitors, there was no obvious qualitative change in the appearance of the SM-containing endosomes during 10–60 s of chase (Fig. 2 a). However, for LacCer, many red/orange endosomes were observed at 10 s, but at later times of chase all endosomes became green in color (Fig. 2 a). Analogous experiments were carried out using BODIPY-globoside, which behaved similarly to the LacCer analogue (data not shown). The changes in R/G ratios of SM- and LacCer-containing endosomes over time are also shown quantitatively (Fig. 2 b). These results show that LacCer, but not SM, was transiently concentrated in early endosomes and suggested that the BODIPY-LacCer and -SM analogues might be internalized by different mechanisms and/or sorted differently after internalization.

To further test this hypothesis, we used a series of pathway-specific inhibitors of endocytosis to learn whether there was a differential effect on the internalization of the LacCer and SM analogues from the PM (Table I). We first evaluated various concentrations of these inhibitors to maximize their specificity and eliminate toxic side effects. Under optimized conditions, chlorpromazine and K⁺ depletion inhib-

ited the internalization of fluorescent LDL (DiI-LDL), a marker for the clathrin pathway, by >75%, but had no effect on the uptake of rhodamine-labeled CtxB (Rh-CtxB), a marker for caveolar and caveolar-like internalization (Parton, 1994; Orlandi and Fishman, 1998). Conversely, treatment with methyl- β -cyclodextrin (m β -CD), nystatin, or genistein significantly inhibited (~75–81%) Rh-CtxB internalization, but had no effect on DiI-LDL uptake. Internalization of BODIPY-LacCer (at 5 min) was almost completely inhibited by pretreatment of cells with m β -CD, nystatin, or genistein, but was unaffected by chlorpromazine or K⁺ depletion (Table I). BODIPY-globoside internalization was similarly inhibited by drugs which affect the caveolar-like pathway. In contrast, internalization of BODIPY-SM was inhibited 45–50% using each inhibitor, suggesting that BODIPY-SM is internalized by both clathrin-dependent and clathrin-independent mechanisms.

To complement the experiments using inhibitors, we next expressed recombinant proteins reported to inhibit specific endocytic mechanisms and determined their effects on the internalization of BODIPY-LacCer and -SM. For these studies, green fluorescent protein (GFP)-tagged constructs

Figure 4. Colocalization of Rh-CtxB and DiI-LDL with caveolin-1-GFP in human skin fibroblasts. Cells were transfected with a plasmid expressing caveolin-1-GFP (COOH-terminal; see Materials and methods) and subsequently incubated with 0.2 μ M Rh-CtxB or 0.5 μ g/ml DiI-LDL for 30 min at 10°C, washed, and warmed for 5 min at 37°C. The samples were then observed by confocal microscopy. Note the extensive colocalization of Rh-CtxB (but not DiI-LDL) with caveolin-1-GFP. No crossover between red and green fluorescence channels could be detected in control cells labeled with marker alone (i.e., Rh-CtxB or DiI-LDL) or in caveolin-1-GFP-labeled cells containing no marker.



were used so that transfected fibroblasts could be readily identified by green fluorescence, whereas internalization of BODIPY-lipids was visualized in the red region of the spectrum. First, human skin fibroblasts were transfected with constructs of dynamin, a protein previously shown to be involved in endocytosis from both clathrin-coated pits and caveolae (Herskovits et al., 1993; van der Blik et al., 1993; Henley et al., 1998; Oh et al., 1998). No internalization of BODIPY-LacCer or -SM was seen after 5 min at 37°C in cells expressing a dynamin K44A mutant, whereas SL internalization in cells transfected with the wild-type dynamin construct was indistinguishable from that in nontransfected cells (Fig. 3 a; Table I). When cells were transfected with a dominant negative EGFR pathway substrate clone 15 (Eps15) mutant which inhibits clathrin-mediated endocytosis (Benmerah et al., 1999), LacCer internalization was unaffected, whereas SM internalization was significantly inhibited (Fig. 3 b; Table I), consistent with our inhibitor results showing partial inhibition of SM internalization by chlorpromazine and K^+ depletion. Lipid internalization in cells transfected with wild-type Eps15 was indistinguishable from nontransfected cells (Fig. 3 b).

We next carried out colocalization studies using the fluorescent SL analogues and DiI-LDL or Rh-CtxB as endocytic markers. Preliminary studies in which cells were doubly labeled with fluorescent LDL and CtxB at 10°C and subsequently incubated for 5 min at 37°C showed almost no colocalization of these markers (data not shown), consistent with their initial internalization by different pathways

(clathrin-dependent versus clathrin-independent). Next, we examined the overlap of Rh-CtxB and DiI-LDL with caveolin-1-GFP, shown previously to behave the same as endogenous caveolin-1 (Pelkmans et al., 2001). We found that Rh-CtxB (but not DiI-LDL) extensively colocalized with caveolin-1-GFP after 5 min at 37°C (Fig. 4). This result suggests that in human skin fibroblasts, CtxB is a valid marker for caveolae as defined by the presence of caveolin-1-GFP. Finally, there was extensive colocalization (>80%) of BODIPY-LacCer with Rh-CtxB but not with DiI-LDL, whereas BODIPY-SM showed partial overlap with both markers (Fig. 5).

Together, the results in Figs. 1–5 demonstrate that (a) all the fluorescent SL analogues were internalized from the PM by a vesicle-mediated process, (b) the LacCer and globoside analogues were endocytosed almost exclusively by a clathrin-independent (caveolar-like) mechanism, and (c) the SM analogue was internalized approximately equally by both clathrin-dependent and -independent mechanisms.

Internalization and subsequent targeting of SL analogues in storage disease fibroblasts and in normal cells overloaded with cholesterol

BODIPY-LacCer is internalized from the PM and transported predominantly to the Golgi complex of normal cells, whereas in numerous SLS cell types, the LacCer analogue accumulates in punctate cytoplasmic vesicles which partially colocalize with fluorescent dextran and lysotracker dyes as a

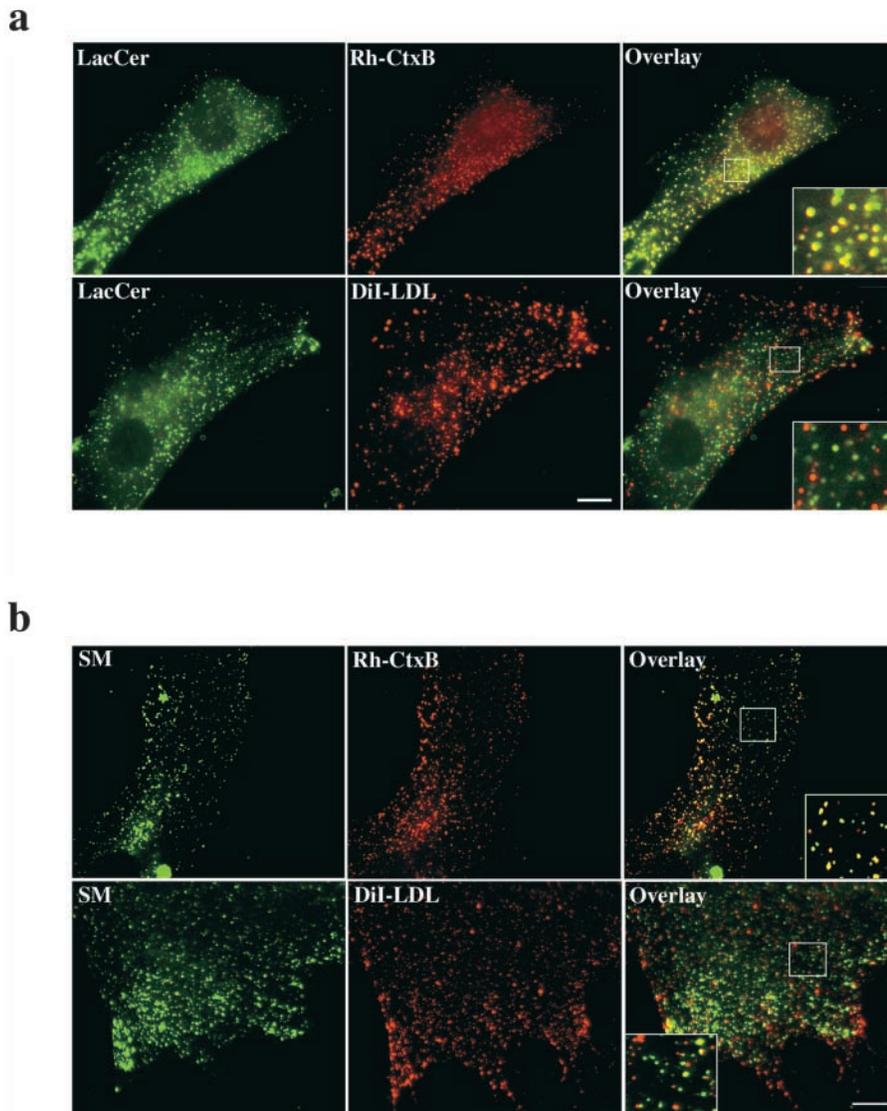


Figure 5. Colocalization of endosomes labeled by fluorescent LacCer or SM with endocytic markers. Cells were incubated with 0.5 μ M (a) BODIPY-LacCer or (b) BODIPY-SM, and 0.2 μ M Rh-CtxB or 0.5 μ g/ml DiI-LDL for 30 min at 10°C, washed, warmed for 30 s at 37°C, back exchanged, and chased for 30 s at 37°C in the absence of inhibitors before fluorescence microscopy. (a) Note the extensive colocalization of BODIPY-LacCer and Rh-CtxB, visible as yellow orange fluorescence, whereas little colocalization of BODIPY-LacCer was seen with DiI-LDL. (b) Note the partial overlap of BODIPY-SM with both Rh-CtxB and DiI-LDL. Quantitative measurements of the overlap of BODIPY-LacCer or BODIPY-SM with Rh-CtxB and DiI-LDL, determined by image processing, were: LacCer/Rh-CtxB, $83.1 \pm 2.3\%$; LacCer/DiI-LDL, $5.7 \pm 1.1\%$; SM/Rh-CtxB, $40.6 \pm 3.2\%$; SM/DiI-LDL, $54.1 \pm 2.5\%$. Values are the mean \pm SD for ≥ 200 endosomes in 3–5 cells. Bars, 10 μ m.

result of elevated intracellular cholesterol (Chen et al., 1998, 1999; Puri et al., 1999; unpublished data). Therefore, it was of interest to learn whether the initial internalization mechanism for LacCer was altered in SLSD cells. We found that pretreatment of SLSD cells with m β -CD followed by pulse-labeling with the LacCer analogue eliminated nearly all intracellular labeling, whereas chlorpromazine had no effect using Niemann Pick Type C (NP-C) (Fig. 6 a), Niemann Pick Type A, GM₁- or GM₂-gangliosidosis cells (data not shown). Transfection with dominant negative Eps15 also had no effect on LacCer internalization or targeting in these cell types (data not shown). Thus, BODIPY-LacCer was internalized via a chlorpromazine- and Eps15-insensitive mechanism in SLSD cells as well as in normal cells, even though Golgi targeting is disrupted in these cells. Furthermore, BODIPY-globoside was also targeted to endosomes/lysosomes in SLSD cells and this internalization was similarly m β -CD-sensitive and chlorpromazine-insensitive (data not shown).

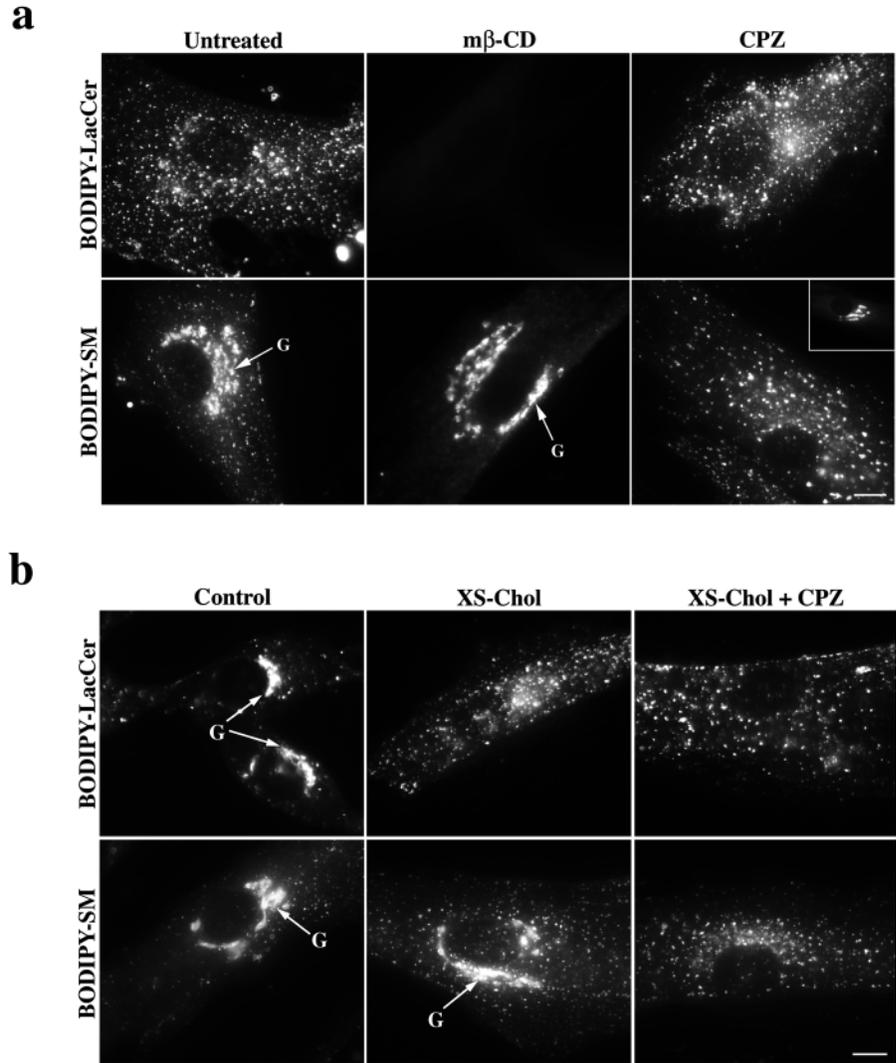
In contrast, the SM analogue was targeted to both the Golgi complex and to punctate structures in the cytoplasm of SLSD cells (e.g., NP-C) (Fig. 6 a, BODIPY-SM and Untreated), whereas in normal cells it was transported primarily

to the Golgi apparatus (Fig. 6 b, Control). Importantly, m β -CD treatment selectively blocked targeting of BODIPY-SM to the endosomes/lysosomes, whereas chlorpromazine treatment (Fig. 6 a) and mutant Eps15 (data not shown) blocked transport to the Golgi. In control experiments, we used BODIPY-ceramide as a vital stain for the Golgi apparatus (Lipsky and Pagano, 1985; Pagano et al., 1991) and showed that the morphology of this organelle was not significantly affected in chlorpromazine-treated NP-C cells (Fig. 6 a, inset). Thus, the absence of SM targeting to the Golgi complex with chlorpromazine was not due to disruption of this organelle. Identical results to those in Fig. 6 a were also obtained using GM₂ gangliosidosis and Niemann pick type A (NP-A) fibroblasts; other SLSD cell types have not been tested.

In parallel experiments, we also studied the trafficking of BODIPY-SM and -LacCer in normal fibroblasts after overloading the cells with cholesterol (Fig. 6 b). In control fibroblasts with normal levels of cholesterol, both the SM and LacCer analogues were targeted primarily to the Golgi apparatus, although some punctate cytoplasmic fluorescence was also seen. In contrast, when cells were grown under conditions which elevated cellular cholesterol (Puri et al., 1999), Golgi

Figure 6. Intracellular targeting of endocytosed fluorescent SL analogues depends on the initial mechanism of internalization.

(a) Effects of pathway-specific inhibitors on BODIPY-LacCer and -SM targeting in SLSD cells. NP-C fibroblasts were pretreated (or Untreated) with chlorpromazine or $m\beta$ -CD to block clathrin-dependent or -independent endocytosis, respectively, and then labeled (pulse for 30 min at 10°C; chase for 30 min at 37°C) with the indicated BODIPY-SL. Note that both the endosomes/lysosomes and the Golgi complex (G) were labeled by the SM analogue and that targeting to these compartments was differentially affected by chlorpromazine and $m\beta$ -CD. Inset shows that the Golgi morphology of a different cell, visualized using BODIPY-ceramide, was unaffected by chlorpromazine treatment. (b) Effect of excess LDL-cholesterol (XS-Chol) and chlorpromazine treatment on the internalization of BODIPY-SM versus -LacCer in normal human skin fibroblasts. Cells with normal (control) or excess cholesterol were pulse-labeled as above with BODIPY-SM or -LacCer. Note the absence of Golgi complex labeling in the sample treated with the SM analogue with chlorpromazine. Bars, 10 μ m.



targeting of LacCer was eliminated and almost all the lipid was targeted to punctate cytoplasmic structures. In the case of SM, overloading with cholesterol did not eliminate Golgi targeting, but the punctate cytoplasmic staining was much more prominent than in control cells (Fig. 6 b, XS-Chol). Chlorpromazine had no effect on LacCer targeting in cholesterol-overloaded cells, whereas for the SM analogue, punctate cytoplasmic labeling without any obvious Golgi fluorescence was now observed (Fig. 6 b, XS-Chol + chlorpromazine). In control experiments with normal cells, chlorpromazine did not affect the Golgi targeting of BODIPY-SM or -LacCer internalized through the clathrin-independent pathway (data not shown). These results show that SL uptake through the clathrin pathway gave rise to Golgi labeling in all cell models tested, whereas SLs which enter by the clathrin-independent route are subject to cholesterol-modulated intracellular targeting in both normal and SLSD fibroblasts.

Finally, we studied the internalization and targeting of endogenous PM SLs in control and SLSD fibroblasts using fluorescently labeled SL-binding toxins. For these experiments we used CtxB, which binds to GM₁ ganglioside and is internalized via caveolae or a caveolar-like mechanism (Orlandi and Fishman, 1998), and StxB, which binds globosides and

is internalized by clathrin-mediated endocytosis (Sandvig and van Deurs, 1994; Johannes and Goud, 1998). As shown in Fig. 7, CtxB was targeted to the Golgi apparatus of normal cells, but to punctate structures in GM₁ gangliosidosis fibroblasts. Importantly, we found that cholesterol depletion of GM₁ gangliosidosis fibroblasts restored the targeting of CtxB to the Golgi complex of these cells (Fig. 7), similar to the effect of cholesterol depletion on BODIPY-LacCer targeting in SLSD cells (Puri et al., 1999). In contrast, StxB was transported to the Golgi complex of both normal and GM₁ gangliosidosis cells regardless of the level of cellular cholesterol. Similar results were obtained using these toxins in GM₂ gangliosidosis, NP-A, and NP-C fibroblasts (data not shown).

Discussion

In this study we used a series of fluorescent SL analogues, as well as SL binding toxins, to study the internalization and subsequent intracellular targeting of SLs from the PM of human skin fibroblasts. We found that two different GSL analogues, LacCer and globoside, were internalized nearly exclusively through a clathrin-independent, caveolar-

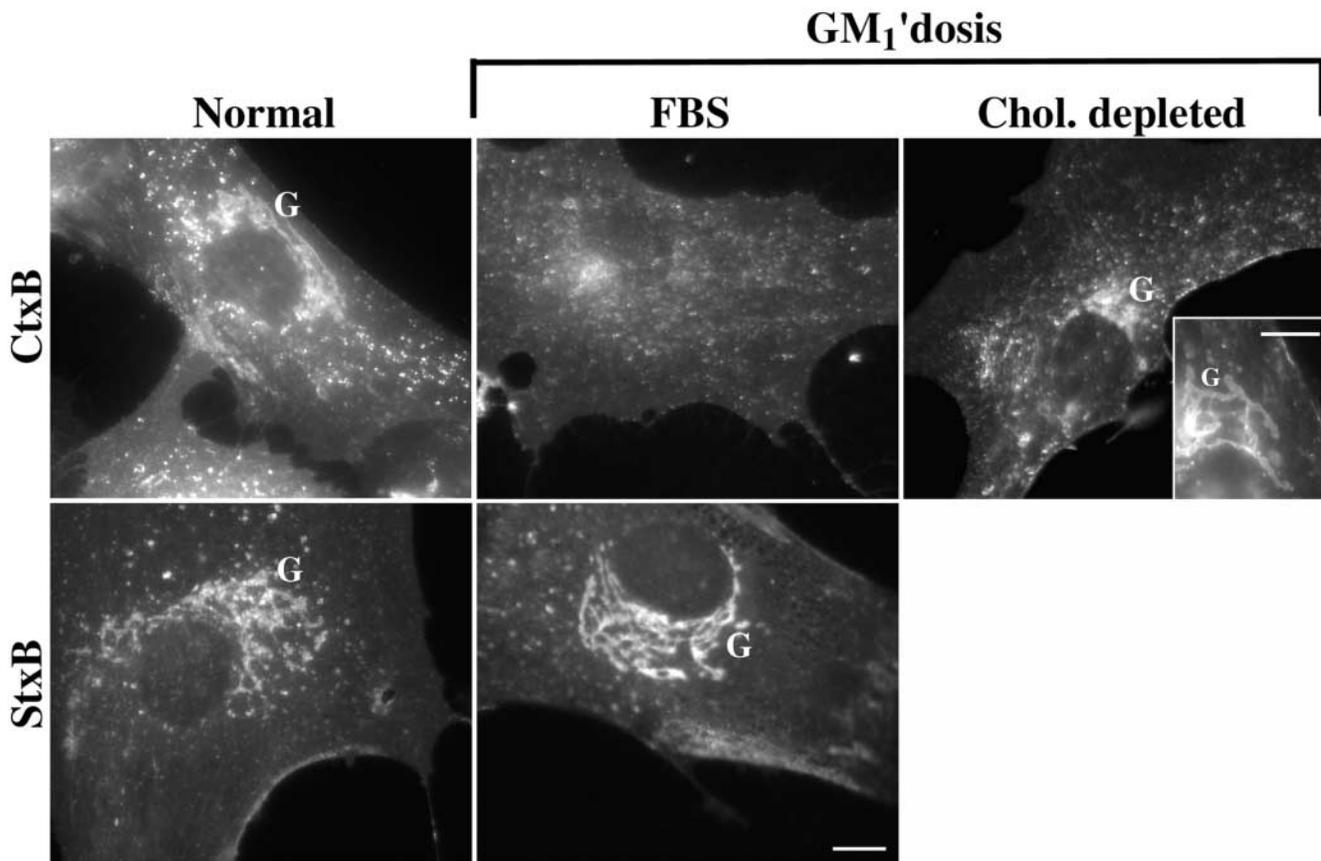


Figure 7. Internalization of fluorescent CtxB and StxB in normal and GM₁ gangliosidosis fibroblasts. Cells were incubated with FITC-labeled CtxB or StxB for 30 min at 10°C, washed, and further incubated for 2–3 h (CtxB) or 1 h (StxB) at 37°C. Note that StxB was targeted to the Golgi (G) in both cell types, whereas CtxB labeled the Golgi of normal cells and punctate cytoplasmic structures in GM₁ gangliosidosis cells. Furthermore, cholesterol depletion of GM₁ gangliosidosis cells induced CtxB targeting to the Golgi complex (inset shows the Golgi region from another cell in the same experiment). Bar, 10 μm.

like pathway, whereas another SL, SM, was internalized approximately equally by both clathrin-dependent and -independent mechanisms. These findings are the first direct demonstration of differential SL sorting into early endosomes in living cells and provide cell biologists with a “vital marker” for endocytic vesicles derived from caveolar-like internalization. A second important finding in the current study is that the endocytic mechanism for SL internalization determines the subsequent pathway by which the lipids are targeted to the Golgi complex. Although it is already known that different membrane proteins can reach the Golgi complex by different routes, we believe this is the first demonstration of multiple Golgi targeting pathways for lipids. Finally, we show that elevated cellular cholesterol specifically perturbs Golgi targeting of SLs internalized through the clathrin-independent pathway, but has no effect on the targeting of lipids which enter via the coated pit pathway. This result demonstrates that cholesterol plays an important role in the intracellular targeting of lipids after their entry via the clathrin-independent route. Importantly, several of the results in the present study were confirmed using fluorescently labeled toxins to study the internalization and intracellular targeting of endogenous SLs from the PM. These experiments provide strong evidence that endogenous SLs internalized via the clathrin-dependent or -independent pathways follow the

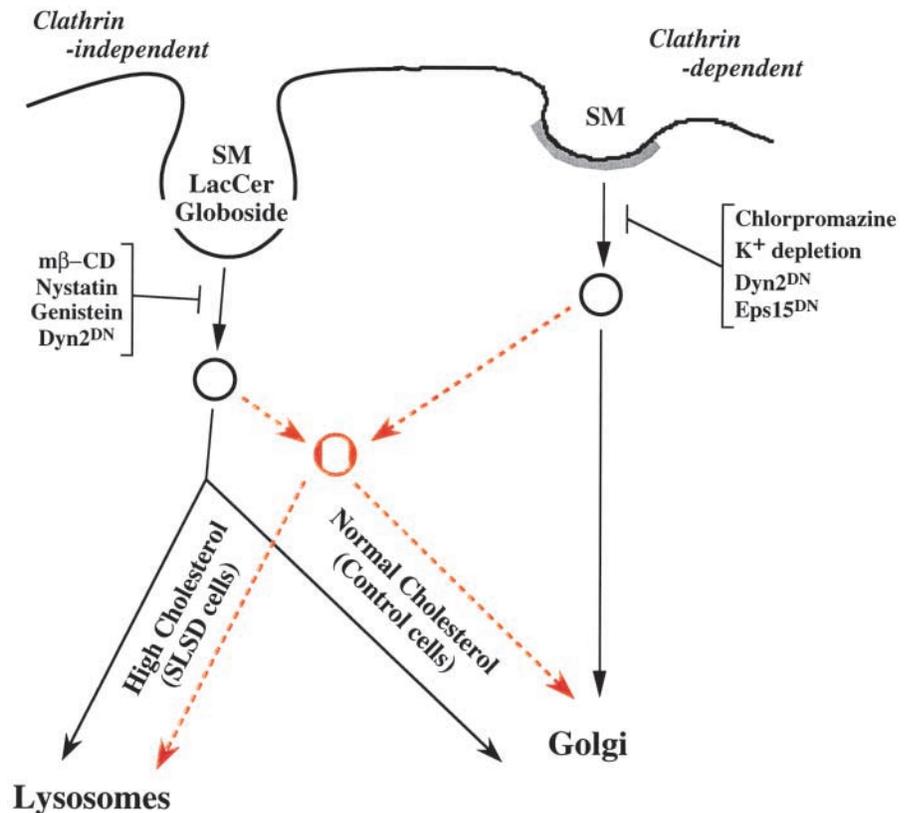
same intracellular transport pathways as the fluorescent SLs in both normal and SLSD fibroblasts.

LacCer and globoside, but not SM, are internalized almost exclusively by a clathrin-independent mechanism

Our results show that BODIPY-LacCer and -globoside were internalized from the PM of human skin fibroblasts exclusively by a clathrin-independent mechanism. Several lines of evidence strongly suggest that this clathrin-independent mechanism involved internalization through caveolae. First, we showed that inhibitors of caveolar-like endocytosis (e.g., of CtxB) completely blocked internalization of LacCer, whereas inhibitors of clathrin-dependent endocytosis had no significant effect on LacCer internalization. In contrast, uptake of SM was partially inhibited by both classes of inhibitors. To rule out nonspecific effects of the drug treatments, we demonstrated similar results using multiple inhibitors, which inhibit endocytosis by different mechanisms. Second, we demonstrated that the internalization of LacCer and SM were both inhibited by expression of a dominant negative dynamin mutant. Inhibition of internalization of both lipids is consistent with our interpretation of the endocytic mechanisms involved in SL uptake, since dynamin is reported to

Figure 8. **Model for internalization and targeting of SLs in normal and SLSL fibroblasts.**

LacCer and globoside were internalized nearly exclusively by a clathrin-independent mechanism, whereas SM was endocytosed approximately equally well by clathrin-independent and clathrin-dependent processes. SL internalization through the clathrin-dependent pathway was blocked by expression of dominant negative (DN) constructs of Dyn2 or Eps15, or by potassium depletion or chlorpromazine treatments. SL internalization through the clathrin-independent pathway was blocked by expression of a dominant negative Dyn2 or by treatment of cells with m β -CD, nystatin, or genistein. Lipids initially internalized via clathrin-dependent or -independent mechanisms were subsequently transported to the lysosomes or Golgi apparatus. Only the pathway taken by SLs internalized by the clathrin-independent mechanism was modulated by cellular cholesterol. Thus, in normal fibroblasts, LacCer was targeted to the Golgi region, whereas in normal fibroblasts with excess cholesterol (or in SLSL cells where cholesterol homeostasis is perturbed), the lipid was targeted to late endosomes and lysosomes. Lipids internalized by the two pathways may never intermix (solid black lines), or may intermix at some point along the endocytic pathway (dashed red lines) and then subsequently segregate into discrete pathways which have different sensitivities to intracellular cholesterol.



be essential for both clathrin-dependent and caveolar uptake. Expression of an Eps15 dominant negative mutant affected only the internalization of SM, consistent with our results showing partial inhibition of SM, but not LacCer, uptake by inhibitors of the clathrin pathway.

To provide further characterization of SL uptake through the clathrin-independent pathway, we showed extensive colocalization of BODIPY-LacCer with Rh-CtxB and of Rh-CtxB with caveolin-1-GFP (Figs. 4 and 5). These experiments suggest that (a) CtxB marks the caveolar compartment in human skin fibroblasts, as shown previously in other cell types (Parton, 1994), and (b) most of the intracellular LacCer analogue was present in a caveolin-1-positive compartment after 5 min of internalization. However, we note that transfection of human skin fibroblasts with an NH₂-terminal GFP construct of caveolin-1 had no effect on internalization of the SL analogues in the present study (data not shown) although this construct blocked internalization of SV-40 via the caveolar pathway in CV-1 cells (Pelkmans et al., 2001). In contrast to the LacCer analogue, we found partial overlap of SM with both Rh-CtxB and DiI-LDL in colocalization studies (Fig. 5). This result fully supports our conclusion that SM is endocytosed approximately equally by both pathways.

Finally, we note that the initial pathways of internalization for the SL analogues were the same in SLSL cells as in normal fibroblasts, since the uptake of LacCer and globoside analogues was nearly completely inhibited by m β -CD in both normal (Table I) and SLSL cells (Fig. 6 a), whereas

SM uptake was only partially inhibited by the different classes of inhibitors (Table I; Fig. 6) or by expression of dominant negative Eps15 (data not shown).

Initial mechanisms of SL internalization determine the utilization of two distinct Golgi targeting pathways

Our studies have further defined the pathways by which PM SLs are internalized and transported to the Golgi apparatus versus the late endosomes and lysosomes (Fig. 8). When SLSL cells were pulse labeled with BODIPY-SM, the fluorescent lipid entered cells by a combination of clathrin-dependent and -independent internalization mechanisms. These dual pathways of uptake gave rise to both punctate cytoplasmic labeling and to Golgi labeling as seen in Fig. 6 a. The "Golgi component" of this fluorescence could be eliminated by blocking uptake through the clathrin pathway (e.g., by chlorpromazine treatment or by expression of dominant negative Eps15), whereas the "punctate cytoplasmic" component could be eliminated by inhibition of the clathrin-independent internalization (i.e., by m β -CD treatment) (Fig. 6 a, right versus middle panels for SM).

We showed previously that the Golgi complex targeting of BODIPY-LacCer is disrupted in SLSL cells due to elevated levels of intracellular cholesterol, and that a similar effect occurs in normal fibroblasts overloaded with LDL-cholesterol (Puri et al., 1999). Since LacCer is internalized almost exclusively by clathrin-independent endocytosis, we conclude that the intracellular Golgi complex targeting initiated by in-

ternalization through the clathrin-independent pathway is modulated by intracellular cholesterol levels. The specificity of this modulation by cholesterol was clearly demonstrated by our studies of BODIPY-SM transport in SLSD cells and in cholesterol-overloaded normal cells, where we showed that the Golgi targeting of SM internalized by the clathrin-independent mechanism was disrupted, whereas the Golgi complex targeting of SM endocytosed via clathrin-coated pits was unaffected (Fig. 6). In addition, control experiments showed that the extent of endocytosis of LacCer and SM by the clathrin-dependent versus clathrin-independent mechanisms was not significantly altered in normal cells grown in high concentrations of LDL (to elevate cellular cholesterol) or in SLSD cells (data not shown). Therefore, the changes in SL targeting induced by modulating intracellular cholesterol levels were not due to effects on the initial mode of internalization, but rather to specific cholesterol-modulated alterations in the intracellular transport of lipids at some point after clathrin-independent internalization.

Together, these results demonstrate that the initial pathways of SL internalization define two discrete Golgi targeting pathways as outlined in Fig. 8. We are currently further characterizing these two pathways and have recently found that SLs internalized by the clathrin-independent, caveolar-like pathway reach a late endosomal compartment by a rab7-dependent step before being transported to the Golgi apparatus, whereas SLs internalized via the clathrin-mediated pathway are targeted to the Golgi apparatus independent of rab7 (unpublished data).

In summary, our studies provide convincing evidence for the existence of two independent pathways for internalization and Golgi targeting of PM SLs; however, many important questions remain concerning these transport pathways. (a) The mechanism for sorting of SL analogues into clathrin-coated pits versus caveolar-like PM specializations is unknown. Although we were able to observe differential sorting and enrichment of LacCer and SM analogues in endocytic vesicles derived from different PM specializations within the first 30–40 s of endocytosis (Fig. 2), we were unable to visualize a heterogeneous distribution of these analogues at the PM before internalization (Fig. 1). The absence of such heterogeneities, comparable in size to early endosomes, suggests that any PM “microdomains” were either too small to be visualized (Kenworthy et al., 2000; Pralle et al., 2000) by our current methods or an alternative mechanism for sorting of PM SLs needs to be considered. (b) The itinerary for the internalized SLs and the determinants for Golgi complex targeting need to be identified. The demonstration that only the targeting of SLs internalized via the caveolar-like pathway was perturbed by cholesterol suggests that SLs internalized by different mechanisms may be transported through two different sets of endosomal compartments, only one of which is sensitive to intracellular cholesterol. Further studies are required to define these intracellular compartments and to determine the site and mechanism by which cholesterol modulates targeting. The existence of two endosomal pathways for Golgi complex targeting of SLs also raises the question of where along the endosomal pathway do components of vesicles derived from the clathrin-dependent and -independent pathways intermix

(Tran et al., 1987) with one another. As depicted in Fig. 8, it is possible that SLs internalized by the two pathways are always in different endosomal vesicles and never intermix, or alternatively intermix at some point along the endocytic pathway, and then subsequently segregate into discrete pathways with unique characteristics with respect to cholesterol modulation. (c) The functional significance of the two PM-to-Golgi complex pathways needs to be determined. Presumably, the delivery of SLs to the Golgi complex by two independent routes represents part of the overall recycling pathway for PM SLs. The possible internalization of LacCer via caveolae and the modulation of LacCer targeting to the Golgi complex by cellular cholesterol suggest a novel role for sterol in regulating lipid recycling. These data also raise the possibility that (some) cholesterol may be transported to the Golgi complex (in normal cells) or retargeted to endosomes/lysosomes (in SLSD cells) via the same pathway that we have identified for the LacCer and globoside analogues.

Materials and methods

Cells and cell culture

Normal and SLSD skin fibroblasts were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository and cultured as described (Martin and Pagano, 1994; Chen et al., 1999; Puri et al., 1999).

Fluorescent lipids, toxins, and other reagents

BODIPY-SM, BODIPY-Ceramide, and LysoTracker Red were from Molecular Probes. BODIPY-LacCer was synthesized and purified as described (Martin and Pagano, 1994); BODIPY-globoside was synthesized in an analogous manner using lyso-ceramide trihexoside (Matreya, Inc.). Complexes of the BODIPY-SLs with defatted bovine serum albumin (DF-BSA) were prepared as described (Martin and Pagano, 1994) and diluted in HMEM (10 mM HEPES-buffered minimal essential medium, pH 7.4). Rh-labeled CtxB was from List Biological Laboratories, Inc. FITC-labeled StxB was a gift from Dr. David Haslam (Washington University School Medicine, St. Louis, MO). Dil-LDL was from Intracel. All other reagents were from Sigma-Aldrich unless otherwise noted.

Incubation of cells with BODIPY lipids

Cell cultures were washed with ice cold HMEM, transferred to a water bath at 10°C, and then incubated with varying amounts of BODIPY-SL/DF-BSA for 30 min to label the PM (see Results). The cells were then washed with cold HMEM and warmed to 37°C for various times to induce endocytosis. After this incubation, the medium was replaced with ice cold HMEM without glucose containing the metabolic inhibitors, 5 mM Na₃N₃, 50 mM 2-deoxyglucose, and 10 μM CCCP (HMEM-G+I), and the culture dishes were transferred to a 10°C bath. Fluorescent lipid present at the cell surface was then removed by “back exchange” (6 × 10 min incubations with 5% DF-BSA in HMEM-G+I at 10°C [Chen et al., 1998]). The cells were then observed under the fluorescence microscope using a temperature-controlled stage maintained at 4°C.

Inhibitor treatments

Human skin fibroblasts grown on glass coverslips were treated with inhibitors of endocytosis as follows. (a) Chlorpromazine (Gustavsson et al., 1999; Okamoto et al., 2000): samples were incubated with 6 μg/ml of the drug for 30 min at 37°C and then pulse labeled with fluorescent lipid in the presence of chlorpromazine. (b) Potassium depletion (Larkin et al., 1983; Hansen et al., 1993): samples were rinsed with K⁺-free buffer (140 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/ml D-glucose, pH 7.4) and then rinsed in hypotonic buffer (K⁺-free buffer diluted 1:1 with distilled water) for 5 min. Cells were then quickly washed three times in K⁺-free buffer and incubated for 20 min at 37°C in K⁺-free buffer. Samples were then incubated with fluorescent lipids as above, except that all solutions were K⁺-free. Control experiments were carried out in an identical manner, except that all solutions contained 10 mM KCl. (c) mβ-CD (Hansen et al., 2000; Okamoto et al., 2000): cells were pretreated with 10 mM mβ-CD in serum-free EMEM for 30 min at 37°C to deplete PM cholesterol before incubation with fluorescent lipid. In separate experiments, cel-

lular cholesterol was quantified (Slotte and Bierman, 1988) and found to be ~25% depleted by m β -CD treatment. (d) Nystatin (Rothberg et al., 1992): samples were incubated with 25 μ g/ml nystatin in serum-free EMEM for 30 min at 37°C, washed with HMEM containing 25 μ g/ml nystatin, and then pulse labeled with the fluorescent lipids in the presence of nystatin. (e) Genistein (Aoki et al., 1999; Chen and Norkin, 1999; Liu and Anderson, 1999): samples were pretreated with 200 μ M genistein for 2 h in HMEM, washed, and pulse labeled with the fluorescent lipids as above except with 200 μ M genistein present throughout. No significant difference in PM loading of the fluorescent SL analogues was observed in inhibitor-treated versus -untreated cells. For each inhibitor treatment, cell viability was >90%; quantitative lipid analyses (Chen et al., 1998) also showed that the degradation of the SM and LacCer analogues was <10%.

Transfection studies

The following GFP-constructs in mammalian expression vectors were gifts as indicated: Eps15 (D3 Δ 2 [control] and the EH21 mutant E Δ 95/295 [dominant negative Eps15]) were from Drs. A. Benmerah and A. Dautry-Varsat (Institut Pasteur, Paris, France); dynamin 2 (Dyn2ab [control] and GFP-Dyn2ab K44A [dominant negative]) were from Dr. M. McNiven (Mayo Clinic); NH₂- and COOH-terminal GFP-constructs of caveolin-1 were from Drs. L. Pelkmans and A. Helenius (Swiss Federal Institute of Technology) and Dr. R.G.W. Anderson (University of Texas Southwestern, Dallas, TX). Cells were treated with TransIT-LT1 transfection reagent (Mirus Corporation) and 2 μ g/ml DNA using the manufacturer's protocol. After a 4–6-h treatment, the cells were washed and subsequently cultured for 18–24 h in EMEM containing 10% FBS before treatment with the fluorescent lipids as above. Transfected cells were detected by GFP-fluorescence and the effect on lipid internalization was evaluated from observations in the red region of the spectrum where BODIPY-lipid, but not GFP-fluorescence, could be visualized.

Microscopy and colocalization studies

Conventional fluorescence microscopy was performed with an fluorescence microscope (IX70; Olympus) equipped with filter packs which allowed the specimens to be excited at 450–490 nm and viewed at "green" (λ_{em} = 520–560 nm), "red" (λ_{em} = 590 nm), or "green + red" (λ_{em} \geq 520 nm) wavelengths (Pagano et al., 1991). Rh-CT and Dil-LDL-labeled specimens were observed under the fluorescence microscope using optics appropriate for these fluorophores (λ_{ex} = 540/25 nm; λ_{em} = 620/60 nm). In any given experiment, all photomicrographs were exposed and printed identically. Confocal microscopy was carried out using a ZEISS model 510 instrument using a 100 \times (1.4 NA) objective. Quantitative image analysis was performed using the "Metamorph" image processing program (Universal Imaging Corp.) as described (Chen et al., 1997; Pagano et al., 2000).

For colocalization studies using BODIPY-SLs, cells were incubated for 30 min at 10°C with the BODIPY-lipid (see above) in the presence of 0.2 μ M Rh-CtxB or 0.5 μ g/ml Dil-LDL (Molecular Probes, Inc.) and washed. The samples were then incubated for 30 s at 37°C and back exchanged. (For Dil-LDL treated specimens, the samples were then washed with HMEM-G+I and "acid stripped" to remove any Dil-LDL remaining at the cell surface [Hopkins and Trowbridge, 1983]). Samples were then chased at 37°C and observed under the fluorescence microscope. In control experiments using cells in which only Rh-CtxB or Dil-LDL was present, no fluorescence was detected with the "green" or "red" microscope filters under the exposure conditions used for BODIPY-fluorescence.

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