Endocytic Trafficking of Sphingomyelin Depends on Its Acyl Chain Length[□]

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To study the principles of endocytic lipid trafficking, we introduced pyrene sphingomyelins (PyrSMs) with varying acyl chain lengths and domain partitioning properties into human fibroblasts or HeLa cells. We found that a long-chain, ordered-domain preferring PyrSM was targeted Hrs and Tsg101 dependently to late endosomal compartments and recycled to the plasma membrane in an NPC1- and cholesterol-dependent manner. A short-chain, disordered domain preferring PyrSM recycled more effectively, by using Hrs-, Tsg101- and NPC1-independent routing that was insensitive to cholesterol loading. Similar chain length-dependent recycling was observed for unlabeled sphingomyelins (SMs). The findings 1) establish acyl chain length as an important determinant in the endocytic trafficking of SMs, 2) implicate ESCRT complex proteins and NPC1 in the endocytic recycling of ordered domain lipids to the plasma membrane, and 3) introduce long-chain PyrSM as the first fluorescent lipid tracing this pathway.

INTRODUCTION

Sphingomyelin (SM) is the most abundant sphingolipid and one of the major phospholipids in the mammalian cell plasma membrane (PM). The acyl chain profile of SM differs from that of most other phospholipids, being enriched in saturated and either relatively short or very long chains. For example, in human primary fibroblasts SMs with a 16:0 or 24:0 acyl chain constitute ~70% of the total (Blom *et al.*, 2001). The physiological significance of such bipartite acyl chain length distribution is not clear. SM is enriched in the PM, and it has a relatively long half-life suggesting that upon endocytosis, it is efficiently recycled to the PM, with only a small fraction targeted to lysosomes for degradation (Koval and Pagano, 1990; van Meer and Holthuis, 2000).

SM has high affinity for cholesterol in model membranes, and it has been proposed to partition into liquid-ordered domains, termed lipid rafts in cells (Simons and Ikonen, 1997; Brown and London, 1998; Simons and Vaz, 2004). Endocytic organelles have been suggested to sort lipids

based on their domain association. By using dialkylindocarbocyanine (DiI) probes, Mukherjee and Maxfield found that probes with a propensity to partition into domains of different fluidity were differentially sorted in endosomes. DiI analogs with short or unsaturated hydrocarbon chains that preferred disordered domains were targeted to the endocytic recycling compartment, whereas those with long and saturated chains preferring more ordered domains entered late endosomes (Mukherjee *et al.*, 1999; Hao *et al.*, 2004). Whether differential endocytic sorting based on domain partitioning applies to naturally occurring lipids has not been investigated. Moreover, the endosomal proteins involved have not been identified and the effect of such differential sorting on lipid degradation has not been studied.

There is limited information on the mechanisms of SM internalization from the PM and subsequent intracellular targeting. Most of the data are based on studies with fluorescently labeled SMs. *N*-(*N*-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl])sphingosylphosphorylcholine (C₆-NBD-SM) incorporated into the PM was endocytosed and mainly sorted for recycling to the PM, whereas some was transported to lysosomes (Koval and Pagano, 1990). *N*-[5-(5,7-dimethyl Bodipy)-1-pentanoyl]-D-erythro-sphingosylphosphorylcholine (C₅-DMB-SM; BODIPY₅-SM), in contrast, predominantly labeled the Golgi apparatus (Puri *et al.*, 2001). This Golgi labeling may reflect vesicular movement of BODIPY-SM from endosomes to the Golgi and/or hydrolysis of the probe to the corresponding ceramide, which has a high affinity for the Golgi (Pagano *et al.*, 1999). However, neither C₆-NBD-SM nor BODIPY₅-SM partition into ordered domains in model membranes (Wang and Silvius, 2000), and the trafficking pattern of C₆-NBD-SM is very similar to that of the short chain DiI analogs (Hao *et al.*, 2004).

In this work, we studied whether the acyl chain length and differential domain partitioning play a role in the endocytic trafficking of SMs. We used pyrene-labeled SMs (PyrSMs) and unlabeled SMs with acyl chain lengths similar to naturally occurring SMs. Pyrene is a polycyclic fluorescent moiety that is more hydrophobic than NBD or BODIPY.

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Abbreviations used: BMP, bis(monoacylglycero)phosphate; CD, cyclodextrin; DRM, detergent-resistant membrane; ESCRT, endosomal sorting complex required for transport; ESI-MS, electrospray ionization mass spectrometry; Hrs, hepatocyte growth factor receptor substrate; MVB, multivesicular body; NPA, Niemann-Pick type A; NPC, Niemann-Pick type C; PM, plasma membrane; PyrSM, pyrenylacylsphingomyelin; SM, sphingomyelin; SMase, sphingomyelinase; TNP-LPE trinitrophenyl-lysophosphatidylethanolamine; Tsg, tumor susceptibility gene.

Therefore, pyrene does not distort the conformation of the labeled chain, i.e., the depth of the pyrene moiety in the bilayer is determined by the length of the labeled acyl chain (Eklund *et al.*, 1992; Sassaroli *et al.*, 1995). Pyrene lipids mimic the behavior of their natural counterparts also in several other aspects (Somerharju, 2002). We have recently shown that PyrSMs with long acyl chains partition preferentially into ordered domains in model membranes, whereas short-chain PyrSMs prefer disordered domains (Koivusalo *et al.*, 2004). We therefore chose a short-chain Pyr₄SM and a long-chain Pyr₁₂SM (and Pyr₁₄SM) to study SM trafficking in living cells.

SMs were introduced into the PM of primary fibroblasts or HeLa cells and their distribution analyzed after various chase times. Two methods were used to quantify the fraction of the probes in the PM: fluorescence quenching and compositional analysis of PM-derived vesicles. We found a systematic difference in the PM recycling of both fluorescently labeled and unlabeled SMs, with the short-chain SMs becoming more efficiently recycled compared with the long-chain SMs. Furthermore, cholesterol loading or depletion of proteins involved in multivesicular body formation or late endosomal trafficking altered the recycling and/or lysosomal degradation of the probes in a chain length-dependent manner. We discuss the potential mode of action of these proteins and the relevance of the findings with respect to domain-based lipid sorting in endosomes.

MATERIALS AND METHODS

Materials

Glycerophospholipids and commercially available sphingolipids were from Avanti Polar Lipids (Alabaster, AL). NBD₆-ceramide, BODIPY₁₂-SM, and pyrene-labeled fatty acids were from Invitrogen (Carlsbad, CA), unmodified fatty acids (15:0, 17:0, 21:0, 23:0, 25:0) were from Larodan (Malmö, Sweden), γ -cyclodextrin (γ -CD) was from Cyclodextrin Technologies Development (High Springs, FL), Lipofectamine 2000 and DiI-low-density lipoprotein (LDL) were from Invitrogen, and other chemicals were from Sigma-Aldrich (St. Louis, MO). PyrSMs and other SMs were synthesized from sphingophosphorylcholine and a fatty acid, and they were purified as described previously (Ahmad *et al.*, 1985; Koivusalo *et al.*, 2004). Trinitrophenyl-lysophosphatidylethanolamine (TNP-LPE 12:0) was synthesized from 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphatidylethanolamine and 2,4,6-trinitrobenzenesulfonic acid as described previously (Tanhuanpaa *et al.*, 2000). All lipids were >98% pure, and they were stored in chloroform/methanol (9:1) (C/M 9:1, vol/vol) below -20°C .

Cell Culture and Transfections

Control human skin fibroblasts F92-99, Niemann-Pick type A (NPA) fibroblasts CC-95-62, and HeLa cells were cultured as described previously (Holttä-Vuori *et al.*, 2000, 2005). The Rab5Q79L construct (a kind gift from Marino Zerial, Max-Planck Institute, Dresden, Germany) was transfected into HeLa cells for 24 h using Lipofectamine 2000. RNA interference (RNAi) oligonucleotides against hepatocyte growth factor receptor substrate (Hrs), tumor susceptibility gene 101 (Tsg101), Niemann-Pick C1 (NPC1), and the control RNAi (GL2) were as described previously (Holttä-Vuori *et al.*, 2005; Ganley and Pfeffer, 2006; Razi and Futter, 2006), and they were transfected into HeLa cells with Lipofectamine 2000. At 48 or 72 h (Hrs + Tsg101 or NPC1, respectively) after transfection, the cells were retransfected for another 48 or 72 h, followed by labeling with PyrSM.

Labeling of Cells with PyrSM for Microscopy and Metabolic Studies

PyrSMs were introduced into cells by using their γ -CD complexes. Lipid in C/M 9:1 was first evaporated under nitrogen and further dried under vacuum. One hundred mM γ -CD in PBS was added, and the mixture was probe-sonicated (Soniprep 150; Sanyo Gallenkamp, Leicestershire, United Kingdom) for 3×2 min at room temperature. Then, 80–90% confluent cell monolayers on glass-bottomed dishes (MatTek, Ashland, MA) were labeled for 5 min at 37°C in serum-free medium. To obtain similar levels of cellular incorporation, the final lipid/ γ -CD concentrations were 3.3 μM /3.3 mM for Pyr₄SM, 6.7 μM /6.7 mM for Pyr₁₂SM, and 13.4 μM /6.7 mM for Pyr₁₄SM. After the pulse, cells were washed with phosphate-buffered saline (PBS) and chased in serum-free medium at 37°C . Imaging was done within 2 h after labeling to ensure that at least 75–80% of the pyrene fluorescence derived

from the parental PyrSM. Control experiments indicated that the differential γ -CD concentrations used did not account for the observed differential distributions of the Pyr_nSMs (data not shown).

Labeling of Cells with PyrSM Species for Electrospray Ionization-Mass Spectrometry (ESI-MS) Analysis

PyrSMs were introduced into cells from donor vesicles (PyrSM/1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine [POPC]/1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphate [POPA]/di23:0-PC 40/40/5/2 nmol per Ø 6-cm dish) with the aid of γ -CD (Tanhuanpaa and Somerharju, 1999). The molar ratio of Pyr₄SM/Pyr₁₂SM/Pyr₁₄SM was 1:2:2 to obtain similar levels of incorporation of the probes. Vesicles were generated from dried lipids in PBS by probe sonication for 4×2 min at 50°C . Then, 80–90% confluent cell monolayers were incubated with donor vesicles (26.7 μM PyrSM) and γ -CD (6.7 mM) for 1 h at 37°C in serum-free medium, washed with PBS, and chased in serum-free medium. The amounts of PyrSMs incorporated into cells using this method were similar to those obtained with direct transfer from γ -CD complexes (see above). This protocol was used for ESI-MS analyses because it allowed the assessment of possible vesicle attachment to the cell surface or dish by determination of the nontransferable di23:0-PC marker. ESI-MS analysis showed that the amount of di23:0-PC was <0.5% of total cellular phosphatidylcholine (PC), indicating an insignificant amount of adhering vesicles.

Labeling of Cells with Unmodified SM Species

We introduced 15:0-SM and 21:0-SM into cells from donor vesicles (SM/cholesterol/POPA/di23:0-PC, 25:25:5:2 nmol per Ø 6-cm dish) by using methyl- β -cyclodextrin (m β CD) as a carrier. m β CD was used instead of γ -CD because the size of its hydrophobic cavity is more compatible with SMs containing unlabeled acyl chains. Cholesterol was included in the vesicles to maintain the cellular cholesterol level (loss <10%; data not shown). The labeling protocol was essentially the same as for PyrSMs. The molar ratio of 15:0-SM/21:0-SM was 1:2 to obtain a similar degree of incorporation of both lipids. Cells were incubated with donor vesicles (16.7 μM SM) and m β CD (10.7 mM) for 1 h at 37°C .

Acid SMase In Vitro Assay

The specificity of fibroblast acid SMase toward SM species was determined essentially as described previously (Lusa *et al.*, 1996; Liu and Hannun, 1999). Fibroblast extracts were prepared freshly before use by sonication of cell pellet in lysis buffer (50 mM Na-acetate, 1 mM EDTA, 4 mM Na-taurocholate, pH 5.0, and a protease inhibitor cocktail of chymostatin, leupeptin, antipain, and pepstatin A at 25 $\mu\text{g}/\text{ml}$ each) for 5×15 s on ice. Undispersed material was pelleted ($10\,000 \times g$ at 4°C for 5 min), and the supernatant was used to assay SM hydrolysis. A dried lipid mixture (SM/POPC/POPA, 9:8:1, mol/mol/mol) was dispersed in assay buffer (250 mM Na-acetate 1 mM EDTA, and 4 mM Na taurocholate, pH 5.0) by sonication in water bath at 50°C for 15 min. This micellar substrate (9 nmol of total SM) was incubated with the cell-free fibroblast extract (15 μg of protein) at 37°C for 4 h, the reaction stopped by addition of C/M 1:1, the lipids were extracted and analyzed with ESI-MS.

Isolation of PM vesicles

PM vesicles were isolated after induction of cell surface blebbing with formaldehyde (Scott *et al.*, 1979; Holowka and Baird, 1983). Fibroblast or HeLa cell monolayers were incubated for 20–30 min at 37°C in blebbing buffer (10 mM HEPES, 0.15 M NaCl, and 2 mM CaCl₂, pH 7.4) containing 25 mM formaldehyde and 2 mM dithiothreitol. The suspension of vesicles was collected and centrifuged at $130 \times g$ for 5 min to remove detached cells. Lipids from vesicles and cells were extracted and analyzed with ESI-MS.

Lipid Analysis by ESI-MS

Lipids were extracted according to Folch *et al.* (1957), and the SM species were quantified after removal of glycerophospholipids with alkaline hydrolysis. For quantification of PC, SM, and ceramide (cer) lipid extracts were spiked with the following mixture of internal standards dissolved in C/M 1:2: di14:1-PC, di20:1-PC, di22:1-PC, 17:0-SM, 23:0-SM, 25:0-SM, 14:0-cer, 17:0-cer, and 20:0-cer. The concentrations of phospholipid and ceramide standards were determined as described previously (Bartlett and Lewis, 1970; Naoi *et al.*, 1974). Extracts were evaporated under nitrogen and dissolved in C/M 1:2 containing 10 mM ammonium acetate. ESI-MS analysis was carried out with a Quattro Microtriple quadrupole mass spectrometer (Micromass, Manchester, United Kingdom). All the lipids were analyzed in the positive ion mode by scanning for the precursors of m/z 184 (PC and SM) or m/z 264 (ceramide) (Hermansson *et al.*, 2005). Data analysis was performed with the LIMS software (Haimi *et al.*, 2006).

Live Cell Fluorescence Microscopy

Cells on glass-bottomed dishes were imaged at 37°C (unless otherwise stated) in serum-free CO₂-independent minimum essential medium (I-MEM; Invitrogen) containing oxygen depletion reagents to minimize pyrene photobleaching (Tanhuanpaa and Somerharju, 1999). Imaging was carried out with a TILLPhotonics imaging system (TILLPhotonics, Gräfelfing, Germany)

equipped with the Polychrome IV light source, an Olympus IX70 cooled charge-coupled device camera (Olympus, Melville, NY), TILLVision 4.0 software (TILLPhotonics), and a Solent Scientific (Segensworth, United Kingdom) temperature-controlled chamber. Excitation light source was set at 345 nm for pyrene, 480 nm for NBD, and 550 nm for DiI-LDL. Emission filters were 405 nm (40-nm bandpass), 480 nm (60-nm bandpass), 525 nm (30-nm bandpass), and 630 nm (60-nm bandpass) for pyrene monomer, pyrene excimer, NBD and DiI-LDL, respectively. In all experiments PyrSMs were imaged at the monomer wavelength. We confirmed that imaging at the excimer wavelength gave identical results (data not shown). UApo 40 $\times$ /numerical aperture (NA) 1.15 water or UApo/340 40 $\times$ /NA 1.35 oil immersion objective was used. To selectively quench pyrene fluorescence at the plasma membrane, 1 ml of 30 μ M TNP-LPE in PBS was added to the dish containing 2 ml of I-MEM (Tanhuanpaa and Somerharju, 1999). To calculate the fraction of fluorescence inside the cells and in the PM, the same cell was imaged before and after TNP-LPE addition and the latter image subtracted from the former. Both images were corrected for glass/medium background fluorescence before subtraction. Imaging of unlabeled cells showed that the contribution of cellular autofluorescence was negligible with the exposure times used. Image analysis was performed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Other Methods

PyrSM degradation was analyzed by high-performance liquid chromatography (HPLC) according to (Silversand and Haux, 1997). Detergent-resistant membranes (DRMs) were isolated from cells by flotation at 4°C in an Opti-Prep gradient in the presence of 1% Triton X-100 as described previously (Harder *et al.*, 1998). To increase the resolution of the gradient in the low-density fractions, an otherwise similar gradient but with less OptiPrep was used (steps of 0, 12, 15, 18, 21, and 24% OptiPrep). To analyze DRM association, fluorescent lipids from OptiPrep fractions were analyzed by HPLC as described above. To analyze endogenous SM species from OptiPrep fractions by ESI-MS, Triton X-100 was first removed from lipid extracts with a reversed-phase HPLC cartridge (OASIS HLB, particle size 2.5 μ m, 2.1 $\times$ 20 mm; Waters, Toronto, ON, Canada). Lipids were trapped to the column in methanol:H₂O 2:1 and eluted with C/M 1:1. To label endosomes with DiI-LDL, fibroblasts were starved overnight in medium containing 5% lipoprotein-deficient serum, followed by DiI-LDL (20–30 μ g/ml) incubation at 37°C for 10–30 min. The quenching efficiency of PyrSM by TNP-LPE in liposomes was determined as described previously (Koivusalo *et al.*, 2004). Cholesterol loading of cells was performed for 1 h at 37°C using cholesterol/m β CD complex as described in Blom *et al.* (2001). Cellular protein was determined as described previously (Lowry *et al.*, 1951) and cholesterol concentration was determined with the Amplex Red kit (Invitrogen). Western blot analysis, and

labeling of cellular unesterified cholesterol for microscopy were carried out as described previously (Holtta-Vuori *et al.*, 2002, 2005). Statistical significance of differences was analyzed by Student's *t* test.

RESULTS

Introduction of SM Species into Cells

PyrSMs with different acyl chain lengths, i.e., Pyr₄SM, Pyr₁₂SM, or Pyr₁₄SM, were introduced into the PM of human fibroblasts by using γ -CD as a carrier at 37°C. Adequate labeling was not obtained at lower temperatures (data not shown). The length of the pyrene moiety is $\sim$ 5.5 in methylene units (Sassaroli *et al.*, 1995). Thus, the length of Pyr₄, Pyr₁₂, or Pyr₁₄-fatty acid is roughly 10, 18, or 20 methylene units, respectively (Figure 1). PyrSM species with a labeled chain longer than 14 carbons could not be incorporated into cells in adequate amounts due to the limited ability of CDs to mediate the intermembrane translocation of very hydrophobic lipids (Tanhuanpaa and Somerharju, 1999). We also introduced short- and long-chain unlabeled SMs, i.e., 15:0-SM and 21:0-SM, into fibroblasts from vesicles by using m β CD as a carrier (Figure 1). These SM species are not naturally present in human fibroblasts, and they can thus be readily distinguished from the endogenous species by mass spectrometry. The labeling conditions were adjusted so that the amounts of short-chain and long-chain SM species introduced into the cells were similar. The amounts of PyrSMs and unlabeled SMs incorporated corresponded to $\sim$ 10–20% of total cellular SM (Figure 2A).

Figure 1. Structures of the SMs studied.

Figure 2. Incorporation of SMs into human fibroblasts. (A) ESI-MS analysis of exogenous SM species in cells ($n = 6$ –10). (B) Association of SMs with DRMs as analyzed by flotation in an OptiPrep gradient in the presence of 1% Triton X-100. DRM association of only two major endogenous fibroblast SMs is shown but the result was similar for the other endogenous SM species ($n = 1$ –4). (C) Fraction of unlabeled SMs in DRMs using a modified gradient with increased resolution. Values for exogenously introduced 21:0-SM were normalized to those of 15:0-SM, and values for endogenous 24:0-SM to those of 16:0-SM in the same cells ($n = 3$; * $p < 0.05$). Error bars are SEM.

Association of SMs with Detergent-resistant Membranes

We next studied the domain partitioning of SMs in cells by analyzing their association with low-density membranes resistant to Triton X-100 extraction at 4°C (DRMs). Approximately 60% of the major saturated SM species endogenously present in fibroblasts, i.e., 16:0 and 24:0, were resistant to Triton X-100 extraction (Figure 2B). In comparison, ~35% of Pyr₁₂SM or Pyr₁₄SM was recovered in DRMs, but only ~10 and ~3% of Pyr₄SM and BODIPY₁₂-SM, respectively (Figure 2B). These results are in line with the preferential association of Pyr₁₂SM and Pyr₁₄SM with ordered domains and Pyr₄SM and BODIPY₁₂-SM with disordered domains in model membranes (Koivusalo *et al.*, 2004; Shaw *et al.*, 2006). To further dissect the DRM affinity of the unlabeled SMs, a modified OptiPrep gradient with increased resolution in the low-density region was used. Using this protocol, we found that 21:0-SM partitioned more avidly into DRMs than 15:0-SM. The endogenous 24:0-SM and 16:0-SM showed a similar tendency, with higher DRM affinity for the long-chain species (Figure 2C).

Distribution of PyrSMs in Human Fibroblasts

The prominent cell surface staining immediately after the labeling indicated that PyrSMs were first incorporated into the PM (Figure 4A). Within a few minutes, PyrSMs were also found in punctate intracellular structures, some of which represent early endosomes as judged from partial colocalization with DiI-LDL, which had been internalized for 10 min (Figure 3A). This agrees with the partial overlap of BODIPY₅-SM and DiI-LDL stainings observed in previous studies (Puri *et al.*, 2001). At later times, some of the PyrSMs were also found in late endosomal compartments (Figure 9A). BODIPY-labeled sphingolipids predominantly localize to the Golgi apparatus in human fibroblasts (Pagano *et al.*,

1999; Puri *et al.*, 1999). To investigate whether this is the case for PyrSMs, we colabeled fibroblasts with the Golgi marker NBD-ceramide (Pagano and Martin, 1994). At 1–1.5 h after labeling, PyrSMs did not stain the Golgi as prominently as NBD-ceramide (Figure 3B).

To determine the fraction of PyrSM inside the cells, we used selective quenching of PM fluorescence by TNP-LPE. This lipid rapidly incorporates into the PM, and it selectively and completely quenches pyrene fluorescence therein. By imaging the cells before and after TNP-LPE addition, the fluorescence in the PM versus internal membranes can be quantified as described previously (Tanhuanpää *et al.*, 2000) (Figure 4). We found that immediately after a 5-min labeling, the fraction of Pyr₄SM, Pyr₁₂SM, and Pyr₁₄SM fluorescence inside the cells was 15–20% (Figure 4, A and C). This reinforces the idea that the endocytosis of all PyrSMs was rapid, with no significant differences in their rate of internalization. After a 30-min chase, ~17% of Pyr₄SM and ~21% Pyr₁₂SM or Pyr₁₄SM fluorescence was found inside the cells, suggesting a slight preference of the longer chain analogues for internal membranes (Figure 4C). However, at 2 h of chase there was a significant difference in the fraction of intracellular fluorescence between PyrSMs: ~25% of Pyr₄SM was intracellular compared with 45% of Pyr₁₂SM or Pyr₁₄SM (Figure 4, B and C). This is unlikely to result from differential quenching of the short- and long-chain probes, because they were similarly quenched in liposomes (Supplemental Figure 1).

Distribution of PyrSMs and Unlabeled SMs in PM Vesicles

As an alternative approach to determine the distribution of short- and long-chain SMs between the PM and intracellular membranes of fibroblasts, we isolated PM-derived vesicles. This method yields 1- to 10-μm-diameter sealed vesicles enriched in PM markers, and with a lipid composition characteristic for the PM (Scott *et al.*, 1979; Holowka and Baird, 1983; Fridriksson *et al.*, 1999). In line with this, the PM vesicles isolated from human fibroblasts showed an approximately fourfold enrichment of SM, approximately sixfold enrichment of cholesterol, and ~1.5-fold enrichment of saturated PC species compared with the rest of the cell (Supplemental Figure 2).

PM vesicles were isolated from fibroblasts that had been labeled with Pyr₄SM, Pyr₁₂SM, and Pyr₁₄SM and chased for 2 h. The amount of PyrSMs in vesicles and remaining cellular material was determined by ESI-MS. We found that Pyr₁₂SM and Pyr₁₄SM were depleted in the PM vesicles compared with the short-chain Pyr₄SM (Figure 5, A and B). These results agree well with those of the PM fluorescence-quenching assay (Figure 4). We then investigated whether the exogenous unlabeled SMs, 15:0-SM and 21:0-SM, exhibit a similar chain-length dependent distribution in PM vesicles. After 2 h of chase, 21:0-SM showed a moderate yet significant depletion from the PM vesicles compared with 15:0-SM (Figure 5, C and D). The effect became more pronounced with increasing the chase time to 2–3 d (Figure 5, C and D). These results indicate that among the SMs introduced into cells, the short-chain SMs became enriched in the PM fraction relative to the long-chain SMs.

Distribution of SM Species between PM and Endomembranes in HeLa Cells

To investigate whether the observed chain length-dependent differences in PyrSM distribution are observed in cells other than primary human fibroblasts, we studied HeLa cells. First, the fraction of short- and long-chain PyrSMs in

Figure 3. Partial colocalization of PyrSM with DiI-LDL and NBD-ceramide in human fibroblasts. (A) Localization of Pyr₄SM, Pyr₁₂SM, or Pyr₁₄SM, and DiI-LDL chased into early endosomes. Fibroblasts were labeled with PyrSM for 5 min at 37°C, chased for 50 min at 37°C, and labeled with DiI-LDL for 10 min at 37°C before imaging. Arrows indicate colocalization of DiI and pyrene fluorescence. (B) Localization of PyrSM and NBD-ceramide. Fibroblasts were labeled with PyrSM, chased for 30 min at 37°C, labeled with NBD-ceramide for 30 min on ice, and then chased for 30 min at 37°C. PyrSM was imaged at room temperature (RT) after TNP-LPE quenching of PM fluorescence. Arrows indicate the Golgi region. Bar, 10 μm.

Figure 4. Assessment of the PM versus intracellular pool of PyrSM fluorescence in human fibroblasts. (A) Images of Pyr₄SM, Pyr₁₂SM, and Pyr₁₄SM fluorescence after a 5-min pulse and 3-min chase at 37°C before and after quenching of PM fluorescence with TNP-LPE. (B) Images of Pyr₄SM, Pyr₁₂SM, and Pyr₁₄SM fluorescence after 5-min pulse and 2-h chase at 37°C before and after quenching with TNP-LPE. Images $\pm$ TNP-LPE within each lipid acquired with the same exposure time and displayed with the same intensity scale. The +TNP-LPE image also shown with intensity multiplied by the factor indicated. (C) Quantification of the fraction of PyrSM fluorescence inside cells at different chase times in wild-type (WT) or NPA fibroblasts (n = 30–56 cells). Error bars are SEM. **p < 0.025, ***p < 0.001. Bar, 10 μ m.

the PM was assessed by the TNP-LPE quenching assay. Similarly to fibroblasts, Pyr₄SM fluorescence was more efficiently quenched than that of Pyr₁₂SM (Figure 6, A and B). Of note, in HeLa cells the difference in the cellular distribution between short- and long-chain PyrSMs was evident already at ~10–30 min of chase. Second, PM vesicles were isolated, and the amounts of PyrSMs and unlabeled 15:0-SM and 21:0-SM in cells and vesicles were quantified by ESI-MS at 30 min to 2 h of chase. We found that Pyr₄SM was approximately twofold enriched in the isolated PM vesicles

compared with Pyr₁₂SM (Figure 6C). Analogously, 15:0-SM was enriched in the vesicles relative to 21:0-SM (Figure 6C). These data indicate that also in HeLa cells exogenous short-chain SMs were enriched in the PM relative to the long-chain SMs.

To address the early endomembrane targeting of PyrSMs, we transfected HeLa cells with a constitutively active mutant of Rab5 (Rab5Q79L-GFP), which stimulates early endosome fusion and results in their enlargement (Stenmark *et al.*, 1994). The enlarged Rab5-positive structures were prominently labeled by both PyrSMs, indicating internalization into early endosomes (Figure 7A). However, no change in the PM versus intracellular distribution of PyrSMs was observed upon Rab5Q79L-GFP expression (Figure 7B).

Depletion of Hrs and Tsg101 Inhibits Endomembrane Enrichment of the Long-Chain SM

We next examined whether interfering with the formation of multivesicular bodies (MVBs) and sorting to late endosomes would affect PyrSM distribution. Recycling receptors typically remain in the limiting membrane of MVBs from where they are returned to the plasma membrane, whereas lysosomally targeted proteins are sorted into internal vesicles of MVBs. Components of the machinery required for cargo sorting into MVBs include the endosomal sorting complex required for transport (ESCRT) protein complexes, which act sequentially to select proteins for retention within the internal vesicles (Hurley and Emr, 2006; Slagsvold *et al.*, 2006). The ESCRT-0 complex protein Hrs was shown to be involved in the accumulation of internal vesicles within MVBs and the ESCRT-I protein Tsg101 in the formation of stable vacuolar domains within early endosomes that develop into MVBs (Razi and Futter, 2006). We therefore analyzed whether depletion of Hrs and/or Tsg101 affects PyrSM sorting in HeLa cells. Introduction of small interfering RNAs (siRNA) against Hrs or Tsg101 resulted in at least 90% reduction of the proteins in 4 d compared with control RNAi (GL2)-transfected cells (Supplemental Figure 3). The PM fluorescence quenching assay showed that in cells depleted of Hrs and Tsg101, the preferential endomembrane localization of the long-chain PyrSM relative to the short-chain PyrSM was lost (Figure 8, A and B). Compositional analysis of PM vesicles from Hrs + Tsg101-depleted cells corroborated

Figure 5. ESI-MS analysis of the distribution of PyrSMs and unlabeled SMs between PM vesicles and cells. Pyr₄SM, Pyr₁₂SM, and Pyr₁₄SM association with PM vesicles isolated from fibroblasts after 1-h labeling and 2-h chase is expressed as percentage of total PyrSM in PM vesicles (A) and long-chain/short-chain PyrSM ratio in PM vesicles and cells (B) (n = 6). Distribution of unlabeled SM species between cells and vesicles isolated after 1 h labeling and 2-h or 2- to 3-d chase is expressed as percentage of SM in PM vesicles (C) and long-chain/short-chain SM ratio in PM vesicles and cells (D) (n = 9–22). Error bars are SEM. **p < 0.01, ***p < 0.001.

Figure 6. TNP-LPE quenching and ESI-MS analysis of SM distribution between PM and internal membranes in HeLa cells. (A) Images of Pyr₄SM and Pyr₁₂SM fluorescence after a 5-min pulse and 10-min chase before and after quenching of PM fluorescence with TNP-LPE. Images $\pm$ TNP-LPE with each lipid acquired with the same exposure time, +TNP-LPE images shown with intensities multiplied by a factor of 2.5. Bar, 10 μ m. (B) Quantification of the fraction of Pyr₄SM and Pyr₁₂SM fluorescence inside the cells by TNP-LPE quenching after a 5-min pulse and 5- to 20-min chase ($n = 24$). (C) ESI-MS analysis of short-chain (Pyr₄SM/15:0-SM) versus long-chain (Pyr₁₂SM/21:0-SM) SM distribution between cells and PM vesicles. Vesicles from PyrSM-labeled cells were isolated after 30 min labeling + 30 min chase ($n = 3-4$) and from cells labeled with unmodified SMs after 30-min labeling and 30-min to 2 h chase ($n = 6$). Data normalized to the value of short-chain SM. Error bars are SEM. * $p < 0.05$, ** $p < 0.01$.

rated these results: preferential PM enrichment of the short-chain Pyr₄SM in control RNAi-treated cells but no chain length difference in the incorporation of PyrSMs into PM vesicles in Hrs + Tsg101 RNAi-treated cells (data not shown). Hrs depletion alone had no effect and the effect of

Figure 7. PyrSM internalization in cells expressing Rab5Q79L-GFP. (A) Images of Pyr₄SM and Pyr₁₄SM fluorescence after 5-min pulse and 7-min chase in HeLa cells transfected with Rab5Q79L-GFP. Bar, 10 μ m. (B) Quantification of the fraction of Pyr₄SM and Pyr₁₄SM fluorescence inside cells by TNP-LPE quenching after a 2-min pulse and 2- to 3-min chase ($n = 21-37$ cells). Error bars are SEM.

Tsg101 depletion alone produced a small but nonsignificant difference in PyrSM distribution (data not shown).

Depletion of NPC1 or Cholesterol Loading Increases Endomembrane Enrichment of the Long-Chain SM

SM accumulates in the late endocytic compartments in sphingolipid storage diseases, such as Niemann-Pick disease (Liscum, 2000). Niemann-Pick types A and B result from mutations in the acid sphingomyelinase gene, and most of Niemann-Pick type C (NPC) cases result from mutations in the NPC1 gene. The precise function of NPC1 is unknown, but it is involved in late endosomal cholesterol-sphingolipid trafficking (Ikonen and Holtta-Vuori, 2004). We therefore studied whether the depletion of NPC1 affects PyrSM sorting in HeLa cells. NPC1 levels were efficiently reduced after 6 d of RNAi treatment (Supplemental Figure 3). The PM fluorescence-quenching assay showed that in NPC1-depleted cells, the intracellular sequestration of the long-chain PyrSM relative to the short-chain PyrSM was increased (Figure 8, A and B). In contrast, no change was found in the cellular distribution of the short-chain PyrSM (Figure 8, A and B). No differences were found in the incorporation of the short- or long-chain PyrSMs into cells treated with control, NPC1 or Hrs + Tsg101 siRNAs.

Because cholesterol loading has been shown to result in sphingolipid accumulation in late endocytic organelles (Puri *et al.*, 1999), we analyzed whether cholesterol loading affects PyrSM sorting. Cholesterol was loaded to primary fibroblasts from a m β CD complex for 1 h, resulting in an approximately twofold increase in cellular cholesterol levels (Blom *et al.*, 2001). Thereafter, the cells were labeled with PyrSMs as described above. Cholesterol loading enhanced the cellular incorporation of PyrSMs by $\sim 30\%$ for both the short- and long-chain species. The PM fluorescence quenching assay revealed that cholesterol loading significantly increased the intracellular fraction of the long-chain PyrSM but not that of the short-chain PyrSM (Figure 8C).

Lysosomal Degradation of PyrSMs

The partial colocalization of PyrSMs with DiI-LDL chased for 2 h (Figure 9A) suggested that some of the probes reached late endocytic organelles. Given the preferential

Figure 8. Effect of Hrs + Tsg101 depletion, NPC1 depletion or cholesterol loading on PyrSM distribution between the PM and internal membranes. (A) Images of Pyr₄SM and Pyr₁₂SM fluorescence after quenching of PM fluorescence with TNP-LPE in HeLa cells transfected with control (GL2), Hrs + Tsg101 siRNA, or NPC1 siRNA. Cells were imaged after a 5-min pulse and 5- to 30-min chase. Same display range in all images. Bar, 10 μ m. (B) Quantification of the fraction of Pyr₄SM and Pyr₁₂SM fluorescence inside the cells by TNP-LPE quenching (n = 54–126 cells). Error bars are SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (C) Effect of cholesterol loading on the fraction of Pyr₄SM and Pyr₁₂SM fluorescence inside fibroblasts as quantified by TNP-LPE quenching. Control (ctr) or cholesterol-loaded (chol-load) fibroblasts were labeled with PyrSM and chased for 3-min to 2 h (n = 30–39). Error bars are SEM. ***p < 0.001.

endomembrane localization of the exogenous long-chain SMs, we considered that they may be preferentially sorted for lysosomal degradation. SM is hydrolyzed by lysosomal acid sphingomyelinase (SMase) and in principle, degradation can be used to monitor the delivery of SMs to the hydrolytic compartments. However, the interpretation is complicated by the potential acyl chain selectivity of acid SMase and contribution of neutral SMases (Levade *et al.*, 1999). To address the first issue, we determined the rate of hydrolysis of short- and long-chain PyrSMs by acid SMase *in vitro*. The results indicated that acid SMase preferred Pyr₁₂SM as substrate (Supplemental Figure 4). Instead, Pyr₁₄SM was degraded only slightly more efficiently than Pyr₄SM and was chosen as the long-chain PyrSM to study lysosomal targeting in cells.

The degradation of Pyr₄SM and Pyr₁₄SM in fibroblasts was monitored by HPLC analysis of pyrene ceramide (PyrCer), which was the only degradation product detected, in accordance with earlier reports (Levade *et al.*, 1991a,b). We found that at 1–2 h of chase, fourfold more of Pyr₁₄SM was hydrolyzed compared with Pyr₄SM (Figure 9B). Similar results were obtained when the disappearance of PyrSM was monitored by ESI-MS (data not shown). Because acid SMase only slightly preferred Pyr₁₄SM over Pyr₄SM in the *in vitro* assay, these data suggest that Pyr₁₄SM is targeted to the hydrolytic organelles more efficiently than Pyr₄SM. To investigate the involvement of acid SMase, Pyr₄SM and Pyr₁₄SM were introduced into NPA fibroblasts lacking acid SMase activity. Both analogues were degraded only marginally and to a similar extent in NPA cells (Figure 9B). Finally, to test if the differential distribution of the short- and long-chain PyrSMs is observed in the absence of acid SMase

activity, we analyzed their distribution in NPA cells. The results showed that also in this case, the long-chain PyrSM was more intracellular (Figure 4C). Together, these data strongly suggest that acid SMase was the enzyme mainly responsible for the more rapid hydrolysis of Pyr₁₄SM in human fibroblasts and that there was a chain length-dependent difference in the efficiency of PyrSM reaching the degradative compartment.

We then analyzed the effect of Hrs + Tsg101 depletion on the degradation of PyrSMs. The degradation of both the long- and short-chain PyrSM was decreased, but the effect was stronger for the long-chain species. The hydrolysis of Pyr₁₄SM was reduced approximately fourfold and that of Pyr₄SM only approximately twofold (Figure 9C). This agrees with the enhanced PM retrieval of the long-chain PyrSM in Hrs + Tsg101-depleted cells. Instead, NPC1 depletion did not alter PyrSM degradation despite the intracellular sequestration of the long-chain PyrSM (Figure 9C). The long-chain SM might be targeted from late endosomes to other endomembranes, such as the Golgi, although no evident redistribution of the long-chain PyrSM to the Golgi was observed in NPC1 RNAi cells (Figure 8A). Alternatively, the unaltered degradation of the long-chain SM may reflect increased shunting of the lipid to the degradative compartments in combination with posttranslational inhibition of acid SMase in NPC cells (Reagan *et al.*, 2000).

DISCUSSION

In this work, we introduced pyrene-labeled and unlabeled SMs into the PM, and we addressed the question whether

Figure 9. Degradation of PyrSM in human fibroblasts and in Hrs + Tsg101- or NPC1-depleted HeLa cells. (A) Partial colocalization of Pyr₄SM or Pyr₁₄SM with DiI-LDL chased into late endosomes in human fibroblasts. Cells were first labeled with DiI-LDL for 30 min and then with PyrSM for 5 min and chased for 1 to 1.5 h at 37°C. PyrSM was imaged after TNP-LPE quenching of PM fluorescence at RT. Arrows indicate colocalization of DiI and pyrene fluorescence. (B) Pyr₄SM and Pyr₁₄SM hydrolysis in control and NPA fibroblasts after 1–2 h of chase ($n = 10$ for control and 5 h for NPA cells). (C) Hydrolysis of Pyr₄SM and Pyr₁₄SM after a 30-min labeling and 2-h chase in HeLa cells transfected with control (GL2), Hrs + Tsg101, or NPC1 RNAi ($n = 3$ –7). Error bars are SEM. *** $p < 0.001$.

the acyl chain length plays a role the endocytic routing of SM.

We found that the initial internalization of PyrSMs from the PM was rapid and occurred with closely similar kinetics for all PyrSM species. Soon after internalization, all PyrSMs entered early endosomes as judged by colocalization with DiI-LDL, which had been internalized for 10 min. Moreover, the Rab5Q79L-GFP-containing compartments were PyrSM positive immediately after labeling, indicating rapid uptake of both the short- and long-chain species into the enlarged

Figure 10. Schematic model of SM recycling. Both long- and short-chain SMs internalized from the PM enter early endosomes (EE). Short-chain SMs are recycled from the limiting EE membrane to the PM via tubular intermediates. This route may involve the endocytic recycling compartment (not shown). Long-chain SMs are sorted to the inward invaginating membranes of EE that form intraendosomal vesicles of MVBs, from where they enter late endosomes (LE). Long-chain SMs may be recycled from LE to the PM or be targeted to lysosomes (LYS) for degradation by acid SMase. Hrs and Tsg101 are involved in the incorporation of the long-chain SMs into MVBs, whereas NPC1 may be involved in their recycling from late endosomes to the PM. All membranes consist of mixtures of ordered and disordered domains (not shown), but membranes of the EE recycling tubules are more disordered than those of the inward invaginating membranes of EE and internal vesicles of MVB and LE. Both differ from intralysosomal, hydrolytic membranes. Sorting efficiency is not absolute, i.e., some long-chain SM is recycled from EE, whereas some short-chain SM enters LE (not shown).

early endosomes. In contrast, our results provide several lines of evidence to suggest that SMs differing only in their acyl chain length are differentially recycled to the PM.

We found that after a short (30 min–2 h) chase, a smaller fraction of the long-chain PyrSM was returned to the PM compared with the short-chain PyrSM. A similar difference was observed between unlabeled short- and long-chain SMs, indicating that the difference was not induced by the fluorescent modification. The involvement of specific proteins was then studied by using RNAi. Depletion of Hrs and Tsg101 selectively enhanced the PM recycling of the long-chain SM. In contrast, depletion of NPC1 inhibited the recycling of the long-chain SM, and a similar effect was observed upon cholesterol loading. A possible interpretation is that the long-chain SM was preferentially targeted Hrs and Tsg101 dependently toward late endosomal compartments and recycled to the plasma membrane in an NPC1- and cholesterol-dependent manner, whereas the short-chain SM recycled more effectively, by using Hrs-, Tsg101-, and NPC1-independent routing that was insensitive to cholesterol loading (see Figure 10 for a schematic model). This model is based on the available information on the localization and sites of action of Hrs, Tsg101, and NPC1. However, it should be pointed out that the assays used do not identify the precise sites or mechanisms of SM recycling. Therefore, other scenarios not necessarily involving differential recycling pathways for the short- and long-chain SMs could also be envisaged. Moreover, the routing of the short- and long-chain SMs probably overlaps in part, as suggested by the

moderate differences in biochemical analyses and relatively similar subcellular distributions of the fluorescent SMs (also when comparing the extent of colocalization e.g., to early, recycling and late endosomal Rab GTPases; our unpublished data).

Hrs and Tsg101 are key components of the MVB sorting machinery on the limiting membrane of early endosomes, and they are involved in MVB formation and inward vesiculation (Razi and Futter, 2006). When inward vesiculation and MVB formation were impaired (Hrs + Tsg101 knock-down), the relative endomembrane preference of the long-chain SM was lost. This, together with the ordered domain partitioning of the long-chain PyrSM supports the idea that the invaginating membranes in early endosomes and the internal membranes of MVBs are rich in ordered domains (Figure 10). Moreover, cholesterol loading resulted in increased intracellular retention of the long-chain SM, but it did not affect the PM recycling of the short-chain species. This further supports the involvement of ordered domains in targeting lipids to the late endosomal route. In contrast, the recycling tubules emanating from early endosomes are likely to be more disordered and to attract short-chain SMs. This is in line with studies showing that when thin tubules are pulled from liposomes with coexisting disordered and ordered domains, the disordered domains are incorporated into the tubules (Roux *et al.*, 2005).

The mechanisms mediating inward budding of the endosome membrane and assigning particular lipids into the endovesicles of MVBs are not well understood. Previous studies have implicated bis(monacylglycero)phosphate (BMP, also known as lysobisphosphatidic acid) and the Alix protein in the invagination process (Matsuo *et al.*, 2004). We envisage that components of the ESCRT complexes, possibly together with BMP and Alix, may increase the curvature of the limiting membrane locally and thus drive lipid sorting. Long-chain SMs could enrich in the highly curved invaginating domains because in the presence of cholesterol, their physical properties are more compatible with a high membrane curvature. Notably, studies in model membranes indicate that membranes consisting of cholesterol and 24:0-SM have a lower bending modulus (i.e., bend more easily) than those consisting of cholesterol and 16:0-SM (Li *et al.*, 2003).

Inclusion into the intraluminal vesicles of MVBs is a mechanism to ferry protein and lipid cargo to lysosomes for degradation as MVBs fuse with the late endosomal hydrolytic organelles (Hurley and Emr, 2006). Accordingly, in Hrs + Tsg101-depleted cells we observed a striking inhibition in the degradation of the long-chain PyrSM. To our knowledge, these data provide the first evidence that ESCRT proteins are involved in the delivery of lipids for degradation. Earlier studies indicate that sphingolipid degradation takes place in intraendosomal/lysosomal membranes that contain high amounts of BMP and low amounts of cholesterol (Kolter and Sandhoff, 2005). This membrane composition favoring hydrolysis may, in part, be generated by the removal of cholesterol from the late endocytic organelles by NPC1 (see below). Cholesterol loading should thus slow down sphingolipid degradation by increasing the cholesterol content in the endosomal membranes. This may explain why SM degradation was not enhanced in NPC1-depleted cells.

Our findings also provide insights regarding the function of NPC1. The NPC defect is associated with mistrafficking of a variety of membrane-associated molecules to the endo/lysosomal storage organelles, but its relationship to the loss of NPC1 function is not clear. BODIPY-lactosylceramide and BODIPY-SM that are normally targeted to the Golgi, accumulated in the endo/lysosomal compartment in NPC cells

(Puri *et al.*, 1999; Puri *et al.*, 2001). The NPC defect also affects early and recycling compartments, as indicated by cholesterol loading of early endosomes (Choudhury *et al.*, 2004) and defective transferrin receptor recycling (Pipalia *et al.*, 2007) in NPC cells. Moreover, DiIC₁₂ and DiIC₁₆ dyes, which in normal cells were differentially routed to the endocytic recycling compartment and late endosomes, respectively (Mukherjee *et al.*, 1999), were both directed to late endocytic compartments in NPC cells (Pipalia *et al.*, 2007). In contrast to the DiI probes, we found that the PM recycling of the short-chain SM was not affected in NPC1 depleted cells, whereas that of the long-chain species was impaired. Thus, the present data provide the first indication for a selective, chain length dependent sphingolipid-recycling defect in cells lacking NPC1 function.

We have previously shown that NPC1 is involved in recycling cholesterol to the PM and in reducing DRMs in late endosomal organelles (Lusa *et al.*, 2001). This agrees with the enrichment of cholesterol in the internal membranes of early and late endosomes and depletion in the internal membranes of lysosomes (Mobius *et al.*, 2003). It is also compatible with the present data, because NPC1 might regulate the recycling of cholesterol-sphingolipid-rich intraluminal contents from late endosomes to the PM (Figure 10). NPC1 could be involved, e.g., in the generation of tubules emanating from the limiting membrane or at an earlier step, in the back-fusion of intraluminal vesicles with the limiting late endosomal membrane (van der Goot and Gruenberg, 2006). Cholesterol removal from the late endocytic organelles should serve to generate a cholesterol-poor intralysosomal milieu favoring sphingolipid digestion (Kolter and Sandhoff, 2005). How individual sphingolipid molecules are destined for recycling versus degradation remains to be addressed.

In summary, this work provides evidence for differential endocytic trafficking of SMs depending on their acyl chain length: short-chain SMs recycle to the PM more effectively and independently of NPC1, whereas long-chain SMs are preferentially routed to the late endocytic pathway and recycle in an NPC1- and cholesterol-dependent manner. Different domain preferences of the short- and long-chain SMs are considered to contribute to their differential sorting. The affinity of the long-chain PyrSMs for DRMs, their slow degradation compared with other fluorescent lipids, e.g., BODIPY-SM, and potential for probing sphingolipid recycling from late endocytic compartments to the PM, make long-chain PyrSMs attractive tools for further studies on sphingolipid trafficking and the role of sterol-sphingolipid domains in cells.

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