Lipid Recycling between the Plasma Membrane and Intracellular Compartments: Transport and Metabolism of Fluorescent Sphingomyelin Analogues in Cultured Fibroblasts

Michael Koval and Richard E. Pagano
Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210-3301

Abstract. We examined the metabolism and intracellular transport of the \textit{d-erythro} and \textit{l-threo} stereoisomers of a fluorescent analogue of sphingomyelin, $N$-\{6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl\}-sphingosylphosphorylcholine (C$_6$-NBD-SM), in Chinese hamster ovary (CHO-K1) fibroblast monolayers. C$_6$-NBD-SM was integrated into the plasma membrane bilayer by transfer of C$_6$-NBD-SM monomers from liposomes to cells at 7°C. The cells were washed, and within 10–15 min of being warmed to 37°C, C$_6$-NBD-SM was internalized from the plasma membrane to a perinuclear location that colocalized with the centriole and was distinct from the lysosomes and the Golgi apparatus. This perinuclear region was also labeled by internalized rhodamine-conjugated transferrin. C$_6$-NBD-SM endocytosis was not inhibited when the microtubules were disrupted with nocodazole; rather, the fluorescent lipid was distributed in vesicles throughout the cell periphery instead of being internalized to the perinuclear region of the cell. The metabolism of C$_6$-NBD-SM to other fluorescent sphingolipids at 37°C and its effect on C$_6$-NBD-SM transport was also examined.

To study plasma membrane lipid recycling, C$_6$-NBD-SM was first inserted into the plasma membrane of CHO-K1 cells and then allowed to be internalized by the cells at 37°C. Any C$_6$-NBD-SM remaining at the plasma membrane was then removed by incubation with nonfluorescent liposomes at 7°C, leaving cells containing only internalized fluorescent lipid. The return of C$_6$-NBD-SM to the plasma membrane from intracellular compartments upon further 37°C incubation was then observed. The half-time for a complete round C$_6$-NBD-SM recycling between the plasma membrane and intracellular compartments was ~40 min. Pretreatment of cells with either monensin or nocodazole did not inhibit C$_6$-NBD-SM recycling.

Recycling of plasma membrane receptors is a process involving membrane vesicle budding, fusion/fission, and transport (10, 38). Since intracellular vesicles are involved in all known steps of the recycling process, there should also be considerable lipid transport in conjunction with protein recycling. However, no direct evidence for plasma membrane lipid recycling between intracellular compartments and the cell surface is available.

Previous work in our laboratory used fluorescent acyl chain-labeled glycerolipids as probes for studying endocytosis in cultured cells (32, 37). However, these probes are not practical for studies of lipid recycling because such studies require prolonged incubations at 37°C during which the fluorescent glycerolipids are extensively hydrolyzed by cellular phospholipases, releasing fluorescent fatty acid (37). Since sphingomyelin (SM)$^1$ is a major lipid constituent of the plasma membrane that is highly resistant to acyl chain hydrolysis (1), we have used a fluorescent analog of SM, \textit{N}-\{6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl\}-sphingosylphosphorylcholine (C$_6$-NBD-SM), to circumvent this problem in the present study. Using Chinese hamster ovary fibroblast cell line (CHO-K1) cells, we examined the internalization of this fluorescent lipid from the plasma membrane to intracellular compartments, and its subsequent return to the plasma membrane ("recycling") with time. We also studied the effect of SM stereochemistry on its metabolism and intracellular transport using stereoisomers of C$_6$-NBD-SM corresponding to the natural \textit{d-erythro} or non-natural \textit{l-threo} forms of SM. Finally, the patterns of intracellular labeling obtained with C$_6$-NBD-SM were compared to those of a recycling protein to learn whether the same compartments are involved in lipid and protein recycling.

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$^1$Abbreviations used in this paper: Cer, ceramide; CHO-K1, Chinese hamster ovary fibroblast cell line; DOPC, dioleoylphosphatidylcholine; GlcCer, glucosylceramide; HCMF, 10 mM HEPES-buffered calcium and magnesium-free Puck's saline, pH 7.4; HMEM, HEPES-buffered MEM; LUVET, large unilamellar vesicle by extrusion techniques; C$_6$-NBD, \textit{N}-\{6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl\}; C$_6$-NBD-Cer, \textit{N}(C$_6$-NBD)-sphingosine; C$_6$-NBD-GlcCer, \textit{N}(C$_6$-NBD)-glucosylphosphingosine; C$_6$-NBD-SM, \textit{N}(C$_6$-NBD)-sphingosylphosphorylcholine; Rh, rhodamine; Rh-Tf, rhodamine-conjugated transferrin; SM, sphingomyelin; SRh, sulforhodamine; SUV, small unilamellar vesicle.
Materials and Methods

Materials

Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). C6-NBD-aminoethoxyc acid, sulfur-horadmine chloride and sulfur-horadmine dextran, 10 KD (SRB-dextran) were purchased from Molecular Probes Inc. (Eugene, OR). Sodium cacodylate was from Electron Microscopy Sciences (Fort Washington, PA). Triphenylphosphine was from Aldrich Chemical Co. (Milwaukee, WI). All organic solvents were purchased from Burdick & Jackson Laboratories Inc. (Muskegon, MI). Rhodamine(Rh)-conjugated second antibodies were from Organon Teknika-Cappel (West Chester, PA). Rh-conjugated Ricinus communis agglutinin 120 was purchased from Vector Laboratories, Inc. (Burlingame, CA). Unless otherwise stated, all other materials were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Monolayer cultures of CHO-K1 fibroblasts (CCL 61; American Type Culture Collection, Rockville, MD) were grown in MEM Alpha medium (No. 410-2000; Gibco Laboratories, Grand Island, NY) supplemented with 5% FBS in a water-saturated atmosphere of 5% CO2 in air. Cells were grown for 48-72 h on No. 1 thickness, 25-mm acid-washed glass cover slips to 20% confluency for microscopy, or on 60-mm plastic tissue culture dishes to 80% confluency for biochemical analysis.

Lipid Synthesis and Analysis

C6-NBD-SM was synthesized from C6-NBD-fatty acid and sphingosylphosphorylcholine by oxidation-reduction condensation with triphenylphosphine and 2-2'-dipyridyl disulfide (19, 24). TLC of the reaction mixture on silica gel 60 plates (E. Merck, Darmstadt, FRG) developed in CHCl3/CH3OH/28% NH4OH/H2O (72:48:2:9, vol/vol/vol/vol) resolved two products, C6-NBD-SMI (Rf = 0.31) and C6-NBD-SM2 (Rf = 0.36), both having the expected molecular mass of 740.9 D as determined by mass spectrometry.

Samples of each C6-NBD-SM isomer were hydrolyzed to C6-NBD-ceramide (Cer) using sphingomyelinase (human placenta) in vitro as previously described (25). Thus, we identified C6-NBD-SM1 as N-(C6-NBD)-o-erythro-sphingosine (C6-NBD-Cer) (43) and C6-NBD-SM2 as N-(C6-NBD)-L-threo-sphingosine (C6-NBD-Cer; 33) were synthesized and purified as previously described. Concentrations of lipid stock solutions were determined by phosphorus measurement (35) or by reference to known concentrations of fluorescent standards.

Lipid Vesicles

Small unilamellar vesicles (SUV) were formed by ethanol injection (20) as follows. C6-NBD-SM and DOPC (typically 2:3; mol/mol) were mixed in chloroform/methanol (2:1), dried first under nitrogen, then in vacuo, and dissolved in ethanol (2.8 mM total lipid concentration). This ethanol solution was injected into 2 ml HCMF to obtain a final DOPC concentration of 10-40 ug/ml. SUV were prepared as large unilamellar vesicles by extrusion (LUVET) as follows (14).

The percentage of C6-NBD-SM removed by the back-exchange process % (C6-NBD-SM)rem was calculated using the equation

\[%(\text{C}_6\text{-NBD-SM})_{\text{rem}} = \left(1 - \frac{(\text{C}_6\text{-NBD-SM})_{\text{ax}}}{(\text{C}_6\text{-NBD-SM})_{\text{tot}}} \right) \times 100, \]

where the amount of C6-NBD-SM was determined as pmole fluorescent lipid located at the plasma membrane during the second 37°C incubation. After each 15-min period of reincubation at 37°C, the medium was replaced with the same type of prewarmed medium.

Analysis of Fluorescent Lipid Metabolism

Monolayer cultures of CHO-K1 fibroblasts (CCL 61; American Type Culture Collection, Rockville, MD) were grown in MEM Alpha medium (No. 410-2000; Gibco Laboratories, Grand Island, NY) supplemented with 5% FBS in a water-saturated atmosphere of 5% CO2 in air. Cells were grown for 48-72 h on No. 1 thickness, 25-mm acid-washed glass cover slips to 20% confluency for microscopy, or on 60-mm plastic tissue culture dishes to 80% confluency for biochemical analysis.

Incubation of Lipid Vesicles with Cells

Monolayer cultures were cooled to 7°C for 5 min, washed twice with HMEM and then incubated with vesicles containing fluorescent lipid, typically using 25 uM C6-NBD-SM/DOPC (2:3; mol/mol) SUV in HMEM at 7°C for 30 min (standard conditions). Incubations were stopped by washing the cells three times with cold HMEM. In most experiments, the cultures were subsequently warmed to 37°C by adding prewarmed HMEM to the cells and incubating at 37°C in a water-saturated incubator.

To remove C6-NBD-SM associated with the plasma membrane, the cells were back-exchanged (37, 40) by incubating at 7°C with back-exchange medium (DOPC LUVETs in HMEM; see above). The back-exchange medium was replaced every 5 min with fresh 7°C back-exchange medium for a total of six treatments.

After back-exchange, cultures sometimes were further incubated at 37°C with either prewarmed HMEM alone or prewarmed HMEM containing back-exchange medium (to remove any C6-NBD-lipid transported to the plasma membrane during the second 37°C incubation). After each 15-min period of reincubation at 37°C, the medium was replaced with the same type of prewarmed medium.

Calculation of Results

The percentage of C6-NBD-SM removed by the back-exchange process % (C6-NBD-SM)rem was calculated using the equation

\[%(\text{C}_6\text{-NBD-SM})_{\text{rem}} = \left[1 - \frac{(\text{C}_6\text{-NBD-SM})_{\text{ax}}}{(\text{C}_6\text{-NBD-SM})_{\text{tot}}} \right] \times 100, \]

where the amount of C6-NBD-SM was determined as pmole fluorescent lipid located at the plasma membrane during the second 37°C incubation. After each 15-min period of reincubation at 37°C, the medium was replaced with the same type of prewarmed medium.

The amount of C6-NBD-SM or N-Srh-diolysylphosphatidylethanolamine in HMEM present were determined by reference to standard curves.

To examine C6-NBD-SM metabolism, the cell pellet was resuspended in 900 uL HMEM. A 100-uL aliquot of this suspension was assayed for DNA content, and the lipids were extracted from the remaining 800 uL using the procedure of Bligh and Dyer (3) using 0.9% NaCl and 10 mM HCl in the aqueous phase. Lipid extracts were chromatographed on silica gel 60 thin-layer plates using CHCl3/CH3OH/28% NH4OH/H2O (72:48:2:9, v/v/v/v) as the developing solvent. TLC plates were analyzed quantitatively as follows. A Newvicon camera (Dage-MTI Inc., Michigan City, IN) was used to obtain a video image of a TLC plate illuminated by UV light. The video image was then digitized using an IP-512 image processing system (Imaging Technology, Inc., Woburn, MA). Regions of the digitized image corresponding to areas of the plate containing a single C6-NBD-lipid species were identified by the operator as regions to be quantified. Blank areas adjacent to selected regions were used to determine the amount of background signal resulting from the TLC plate. The amount of NBD fluorescence was then calculated as the difference between the total intensity within each region and the background intensity. Absolute amounts of the C6-NBD-lipid species were determined by reference to known amounts of fluorescent lipid chromatographed and analyzed under the same conditions.

The percentage of C6-NBD-SM removed by the back-exchange process % (C6-NBD-SM)rem was calculated using the equation

\[%(\text{C}_6\text{-NBD-SM})_{\text{rem}} = \left[1 - \frac{(\text{C}_6\text{-NBD-SM})_{\text{ax}}}{(\text{C}_6\text{-NBD-SM})_{\text{tot}}} \right] \times 100, \]

where the amount of C6-NBD-SM was determined as pmole fluorescent lipid located at the plasma membrane during the second 37°C incubation. After each 15-min period of reincubation at 37°C, the medium was replaced with the same type of prewarmed medium.

The percentage of C6-NBD-SM removed by the back-exchange process % (C6-NBD-SM)rem was calculated using the equation

\[%(\text{C}_6\text{-NBD-SM})_{\text{rem}} = \left[1 - \frac{(\text{C}_6\text{-NBD-SM})_{\text{ax}}}{(\text{C}_6\text{-NBD-SM})_{\text{tot}}} \right] \times 100, \]

where the amount of C6-NBD-SM was determined as pmole fluorescent lipid located at the plasma membrane during the second 37°C incubation. After each 15-min period of reincubation at 37°C, the medium was replaced with the same type of prewarmed medium.
Results. The amount of C₆-NBD-SM that was not metabolized was determined by:

\[
(\text{nonmetabolized C₆-NBD-SM}) = (\text{total C₆-NBD-SM}) - (\text{newly synthesized C₆-NBD-SM}).
\]

Values in Eqs. 2 and 3 were determined as pmole C₆-NBD-SM/µg DNA.

Microscopy and Rh-Transferrin Labeling Procedures

A microscope (model IM-35, Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics was used. Filter combinations eliminated cross-over between NBD and rhodamine fluorescence channels.

Rhodamine-conjugated transferrin (Rh-Tf) was kindly provided by Drs. T. McGraw and F. Maxfield (Columbia University). For colocalization studies, cells were first labeled with C₆-NBD-SM for 30 min at 7°C, washed, and then incubated at 37°C for 30 min in HMEM containing 20 µg/ml Rh-Tf. The cells were then treated with back-exchange medium at 7°C and photographed sequentially using optics appropriate for NBD and rhodamine fluorescence.

Lysosome Labeling

Lysosomes were visualized by indirect immunofluorescence using an antibody to a 95-kD lysosomal glycoprotein (anti-lgp95), kindly provided by Drs. S. Schmid and I. Mellman (Yale University). Cells were first labeled with C₆-NBD-SM for 30 min at 7°C and then warmed for 30 min at 37°C, followed by treatment with back-exchange medium at 7°C. All remaining steps were performed at room temperature. The cells were fixed (22) using 3% paraformaldehyde-0.02% glutaraldehyde in PBS for 15 min, washed, and then photographed using optics appropriate for NBD fluorescence. The samples were then treated with 0.2 M glycine in H₂O for 5 min, followed by a 10-s treatment with 100% methanol at -20°C to render the cells permeable to antibodies. The cells were then washed with PBS containing 0.2% gelatin, and incubated with anti-lgp95 at a 1:100 dilution in PBS-gelatin for 30 min. The cells were washed, incubated with rhodamine-conjugated rabbit anti-mouse IgG at 1:200 dilution in PBS-gelatin for 30 min, followed by treatment with back-exchange medium at 7°C. All remaining steps were performed at room temperature. The cells were fixed using 3% paraformaldehyde-0.02% glutaraldehyde in PBS for 15 min, washed, and then photographed using optics appropriate for rhodamine fluorescence.

Alternatively, cells were labeled with C₆-NBD-SM for 30 min at 7°C and then warmed to 37°C for 1 h in HMEM containing 2 mg/ml SRH-dextran (10 kD), followed by a 1-h incubation at 37°C in HMEM alone (6, 39). The cells were treated with back-exchange medium at 7°C and then photographed using optics appropriate for both NBD and rhodamine fluorescence.

Centriole Labeling

Centrioles were visualized by indirect immunofluorescence using autoantibody 5051 (4), kindly provided by Drs. T. Mitchison and M. Kirschner (University of California at San Francisco). Cells were labeled with C₆-NBD-SM for 30 min at 7°C and then warmed for 15 min at 37°C. All remaining steps were performed at room temperature. The cells were fixed using 1.6% paraformaldehyde in fixing buffer (0.1 M sodium cacodylate/0.1 M sucrose) for 15 min and photographed using optics appropriate for NBD fluorescence. The samples were then treated with 0.1% Triton X-100 in fixing buffer for 3 min, washed with HCMF, and incubated with autoantibody 5051 at 1:100 dilution in HCMF for 30 min. The cells were washed, incubated with Rh-conjugated goat anti-human IgG at 1:200 dilution in HCMF for 30 min, and then washed with HCMF. Cells previously photographed were relocated and photographed using optics appropriate for Rh fluorescence.

Golgi Complex Labeling

Cells were labeled with 25 µM C₆-NBD-Cer/DOPC (2:3; mol/mol) SUV in HMEM at 7°C for 30 min, washed and then incubated for 30 min at 37°C in HMEM containing 5% FBS, conditions that have been used to label the Golgi apparatus (26). All remaining steps were performed at room temperature. The cells were fixed using 3% paraformaldehyde-0.02% glutaraldehyde in PBS for 15 min, washed, and then photographed using optics appropriate for NBD fluorescence. The samples were then treated with 0.2 M glycine in H₂O for 5 min, followed by a 10-s treatment with 100% methanol at -20°C to render the cells permeable to proteins. The cells were then washed with PBS containing 0.2% gelatin and incubated with Rh-conjugated Ricinus communis agglutinin 120 at 100 µg/ml in PBS-gelatin for 30 min to label the Golgi apparatus (46). The cells were washed with PBS-gelatin and cells previously photographed were relocated and photographed using optics appropriate for Rh fluorescence.

Miscellaneous Procedures

Nocodazole. Cells were preincubated with nocodazole (10 µg/ml) for 90 min at 37°C by adding a 1000× nocodazole stock solution (in DMSO) to the culture medium. All incubation solutions contained 10 µg/ml nocodazole.

Monensin. Cells were preincubated with monensin (10 µM) for 90 min at 37°C by adding a 1000× stock solution (in ethanol) into the culture medium. All incubation solutions contained 10 µM monensin.

Energy depletion was performed by incubating cells at 7°C with SUV containing fluorescent lipid in the presence of 5 mM sodium azide and 50 mM 2-deoxyglucose for 30 min, followed by 37°C incubation in HMEM containing 5 mM sodium azide and 50 mM 2-deoxyglucose for 30 min (37).

Results

C₆-NBD-SM Labeling and Internalization

The amount of spontaneous transfer of C₆-NBD-SM from SUV to CHO-K1 monolayers at 7°C was determined as a function of time, SUV concentration, or mole fraction of C₆-NBD-SM in the SUV (Fig. 1). The amount of each C₆-NBD-SM isomer incorporated into CHO-K1 cells incubated with 25 µM C₆-NBD-SM/DOPC (2:3; mol/mol) SUV in HMEM for 30 min at 37°C (standard conditions) is given in Table I. In some experiments, the SUV used for labeling also contained 2 mole % N-SRh-DOP, a nonexchangeable fluorescent lipid marker (37, 40, 41) that provided a measure of SUV nonspecifically adsorbed to the cells. From these experiments, we calculate that >96% of the cell-associated C₆-NBD-SM fluorescence was the result of transfer of fluorescent SM to the plasma membrane of cells treated under standard incubation conditions.

Cells labeled with SUV containing C₆-NBD-SM at 7°C...
Table I. Insertion of C₆-NBD-SM into the Plasma Membrane

| SM isomer          | pmol/μg cell DNA | pmol/nmol cell phospholipid |
|--------------------|------------------|-----------------------------|
| d-erythro-C₆-NBD-SM| 36.3 ± 6.0 (n = 6)| 22.9                        |
| L-threo-C₆-NBD-SM  | 26.7 ± 3.0 (n = 9)| 16.8                        |

Cells were incubated with each C₆-NBD-SM isomer under standard labeling conditions, and the amount of specific incorporation of C₆-NBD-SM was measured. CHO-K1 cells contained 19.9 ± 1.5 (n = 3) μg DNA/10⁶ cells and 31.6 ± 2.8 (n = 3) nmol phospholipid/10⁶ cells.

showed intense plasma membrane fluorescence (Fig. 2a). As long as labeled cells were kept at 7°C, at least 95.5% of the cell-associated fluorescent lipid could be removed by back-exchange. When cells labeled at 7°C were warmed to 37°C, internalization of the fluorescent lipid was observed (Fig. 2, b–d). With increasing time at 37°C, there was an accumulation of fluorescent lipid in intracellular vesicles, and, by 10 min, most of these vesicles had been transported to a central, perinuclear region of the cell. For 37°C incubations of 30 min or less, internalized d-erythro and L-threo-C₆-NBD-SM showed the same labeling pattern (compare Fig. 2d to Fig. 3a). Incubation of cells in the presence of 5 mM sodium azide and 50 mM 2-deoxyglucose inhibited endocytosis of C₆-NBD-SM from the plasma membrane (not shown), supporting the notion that C₆-NBD-SM internalization is energy-dependent.

When high concentrations of C₆-NBD-SM are incorporated into membranes, self-quenching of the probe can occur (27, 29, 37). To determine if self-quenching of C₆-NBD-SM was occurring, cells were labeled with C₆-NBD-SM at 7°C, washed, incubated at 37°C for varying amounts of time, washed, and then the amount of fluorescence in the presence or absence of 2% Triton X-100 was determined. The ratio of fluorescence in the presence or absence of Triton X-100 for C₆-NBD-SM-labeled cells that were incubated at 37°C for 0, 30, and 60 min was 1.57 ± 0.07, 1.57 ± 0.08, and 1.58 ± 0.01, respectively (n = 3 in each case), suggesting that neither the plasma membrane nor intracellular compartments contained C₆-NBD-SM at self-quenching concentrations. Also, preliminary results using low light-level digital imaging microscopy have shown that CHO-K1 cells labeled with 100-fold less C₆-NBD-SM (equivalent to 0.02% of the total

Figure 2. Appearance of C₆-NBD-SM labeling and internalization in CHO-K1 cells. (a) CHO-K1 cells were labeled with d-erythro-C₆-NBD-SM/DOPC (2:3, mole/mole) SUV at 25 μM total lipid concentration for 30 min at 7°C, washed, and photographed. Cells labeled as in a were washed; incubated at 37°C in HMEM for (b) 5 min, (c) 10 min, or (d) 30 min; treated with back-exchange medium at 7°C to remove any C₆-NBD-SM remaining at the plasma membrane; washed, and then photographed. Bar, 10 μm.

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Figure 3. Comparison of endocytosed C₆-NBD-SM with lysosomal markers. (a and b) Cells were labeled with SUV containing l-threo-C₆-NBD-SM for 30 min at 7°C, washed, and incubated in HMEM for 30 min at 37°C. The cells were then treated with back-exchange medium at 7°C to remove any C₆-NBD-SM remaining at the plasma membrane, washed, fixed and photographed for C₆-NBD-lipid fluorescence (a). The cells were subsequently rendered permeable, treated with an antilyosomal antibody (anti-lgp95), and then labeled with Rh-conjugated rabbit anti-mouse IgG. The cells were washed and the field previously photographed for C₆-NBD-lipid fluorescence was rephotographed for Rh fluorescence (b). (c and d) Cells were labeled with SUV containing d-erythro-C₆-NBD-SM in HMEM for 30 min at 7°C, washed, and incubated for 1 h at 37°C in HMEM containing 2 mg/ml SRh-dextran. The cells were then washed and further incubated for 1 h at 37°C in HMEM alone, treated with back-exchange medium at 7°C to remove C₆-NBD-SM remaining at the plasma membrane, washed, and photographed for either (c) C₆-NBD-lipid or (d) SRh-dextran fluorescence. This procedure does not require relocation of labeled cells and thus minimizes the possibility of a change in the plane of focus during photography. Note the absence of NBD and SRh fluorescence colocalization (arrowheads). Bar, 10 μm.

cell phospholipid) endocytose fluorescent SM to the perinuclear region of the cell (not shown), suggesting that the addition of larger amounts of C₆-NBD-SM to the plasma membrane was not affecting the internalization process.

Characterization of Compartments Labeled by Endocytosed C₆-NBD-SM

To determine whether internalized C₆-NBD-SM was transported to the lysosomes, cells were first labeled with fluorescent SM under standard conditions and incubated for 30 min at 37°C, followed by back-exchange. The cells were then subsequently labeled with anti-lgp95, which recognizes a 95-kD lysosomal glycoprotein (Fig. 3, a and b). Note the lack of lysosomes in the central region of the cell, in contrast to the intracellular vesicles labeled by internalized C₆-NBD-lipid.

Also, it appeared that very little fluorescent lipid accumulated in the lysosomes under these conditions as indicated by a lack of probe colocalization. In a complementary experiment (Fig. 3, c and d), cells were labeled with C₆-NBD-SM under standard conditions and then incubated at 37°C for 1 h in the presence of the fluid phase marker SRh-dextran, followed by a 1-h chase at 37°C in HMEM alone to insure that the SRh-dextran was transported to the lysosomes (39). Even after a 2-h incubation at 37°C, little, if any, C₆-NBD-lipid appeared to have accumulated in the lysosomes.

Cells containing internalized C₆-NBD-SM were also subsequently labeled with an antibody that labels pericentriolar material (4). Endocytic vesicles containing fluorescent lipid aggregated around the centriole in C₆-NBD-SM-labeled CHO-K1 cells (Fig. 4, a and b). Since the centriole acts as
Figure 4. Colocalization of endocytosed C₆-NBD-SM with the centriole and the effect of microtubule disruption on C₆-NBD-SM internalization. In (a and b), cells were labeled with SUV containing L-threo-C₆-NBD-SM for 30 min at 7°C, washed, and incubated in HMEM for 15 min at 37°C. The cells were then treated with back-exchange medium at 7°C to remove any C₆-NBD-SM remaining at the plasma membrane, washed, fixed and photographed for C₆-NBD-lipid fluorescence (a). Note that the plane of focus selected does not enable the visualization of fluorescently labeled peripheral endosomes. The cells were subsequently rendered permeable, treated with antibody 5051, which recognizes pericentriolar material, and then labeled with Rh-conjugated goat anti-human IgG. The cells were washed and the field previously photographed for C₆-NBD-lipid fluorescence was rephotographed for Rh fluorescence (b). (c) Cells were preincubated with 10 µg/ml nocodazole for 90 min at 37°C in culture medium, washed, and labeled with SUV containing L-threo-C₆-NBD-SM in HMEM with 10 µg/ml nocodazole. The cells were washed, incubated with back-exchange medium containing 10 µg/ml nocodazole at 7°C to remove any C₆-NBD-SM remaining at the plasma membrane, washed, and photographed. Bar, 10 µm.

Figure 5. Metabolism of O-erythro and L-threo-C₆-NBD-SM. Cells were labeled with either (a) O-erythro- or (b) L-threo-C₆-NBD-SM in HMEM for 30 min at 7°C, washed and incubated for the indicated times at 37°C in HMEM. The cell-associated lipids were extracted, separated by TLC, and the fluorescent metabolites were measured and expressed as a percentage of total cell-associated C₆-NBD-lipid. (●) C₆-NBD-SM; (▲) C₆-NBD-GlcCer; (●) C₆-NBD-Cer. Data points are the mean of triplicate measurements.

Quantitation of Metabolism and Endocytosis of C₆-NBD-SM

Cells labeled with either O-erythro or L-threo-C₆-NBD-SM at 7°C under standard conditions and then incubated at 37°C metabolized C₆-NBD-SM to other fluorescent sphingolipids (Fig. 5, a and b). Both C₆-NBD-SM isomers showed partial hydrolysis to the corresponding C₆-NBD-Cer isomer, but only O-erythro-C₆-NBD-SM-labeled cells produced C₆-NBD-GlcCer (from C₆-NBD-Cer) during the 37°C incubation, probably because of the stereospecificity of the conversion of C₆-NBD-Cer to C₆-NBD-GlcCer (33). No other fluorescent lipid species, including C₆-NBD-fatty acid, were produced during 37°C incubations of up to 6 h in cells labeled with either fluorescent SM isomer. In addition, a small amount (1.7 ± 0.7%; n = 3) of O-erythro-C₆-NBD-SM was hydrolyzed to C₆-NBD-Cer during the 30 min incubation at 7°C.

CHO-K1 cells were labeled directly with O-erythro-C₆-NBD-Cer and the conversion to C₆-NBD-SM and C₆-NBD-
GlcCer during 37°C incubation was examined under a wide range of conditions. Cells were labeled at 7°C for 30 min with C₆-NBD-Cer/DOPC (2:3, mol/mol) SUV (a) at concentrations ranging from 3 to 50 µM total lipid, washed, and then incubated for 30 min at 37°C, or (b) at 25 µM total lipid, washed, and then incubated at 37°C for times ranging from 5 min to 2 h. In all cases both C₆-NBD-SM and C₆-NBD-GlcCer were synthesized from fluorescent Cer. The ratio (C₆-NBD-SM produced/C₆-NBD-GlcCer produced) was 1.41 ± 0.33 (n = 26). D-erythro-C₆-NBD-Cer metabolism was not affected when cells were treated with either 10 µg/ml nocodazole or 10 µM monensin.

The intracellular distribution of exogenously supplied D-erythro-C₆-NBD-Cer was examined in CHO-K1 cells. Cells labeled with SUV containing fluorescent Cer at 7°C for 30 min followed by 37°C incubation for 30 min showed prominent labeling largely around the perimeter of the nucleus (Fig. 6 a). To confirm that C₆-NBD-Cer labeled the Golgi apparatus in CHO cells, as previously shown in other cell types (26), the intracellular distributions of C₆-NBD-Cer and a lectin that preferentially labels the Golgi apparatus (Rh-Ricinus communis agglutinin 120; 34, 46) were compared. As seen in Fig. 6 (a and b) Rh-RCA and C₆-NBD-Cer fluorescence extensively colocalized, although subtle differences, possibly because of a slight change in the plane of focus, were observed. In contrast, cells labeled with C₆-NBD-SM under standard conditions and then incubated at 37°C for 30 min largely contained intracellular fluorescent lipid in a central, perinuclear region of the cell with little labeling around the perimeter of the nucleus (Fig. 6 c). However, with increasing incubation time at 37°C, C₆-NBD-SM–labeled cells showed increasing amounts of labeling around the perimeter of the nucleus in addition to labeling of the central perinuclear region (Fig. 6 d). These data suggest that, during 37°C incubations of fluorescent SM–labeled cells, C₆-NBD-Cer produced by the hydrolysis of C₆-NBD-SM was transported to the Golgi apparatus.

To determine whether some of the cell-associated C₆-NBD-Cer labeling of the Golgi apparatus. (a and b) CHO-K1 cells were labeled with SUV containing D-erythro-C₆-NBD-Cer in HMEM for 30 min at 7°C, washed, incubated for 30 min at 37°C in HMEM containing 5% FBS, fixed, and then photographed for C6-NBD-lipid fluorescence (a). The cells were then rendered permeable, and treated with Rh-conjugated Ricinus communis agglutinin 120, which preferentially labels the Golgi apparatus (34, 46). The cells were washed, and the field, previously photographed for C6-NBD-lipid fluorescence, was rephotographed for Rh fluorescence (b). Note labeling around the perimeter of the nucleus (arrowheads). (c and d) Cells were labeled with SUV containing D-erythro-C₆-NBD-SM at 7°C, washed, incubated for 30 min (c) or 2 h (d) at 37°C in HMEM, followed by treatment with back-exchange medium at 7°C and then photographed. Note the appearance of fluorescence around the perimeter of the nucleus with increasing time at 37°C (arrowheads) in addition to labeling of the central perinuclear region. Bar, 10 µm.

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Cells were either untreated (control) or were preincubated with 10 μM monensin or 10 μg/ml nocodazole in culture medium for 90 min at 37°C. All solutions used for treated cells contained either 10 μM monensin or 10 μg/ml nocodazole. Cells were labeled with SUV containing α-erythro-C6-NBD-SM in HMEM for 30 min at 7°C, washed, incubated in HMEM at 37°C for 2 h, and then either harvested immediately or treated with back-exchange medium at 7°C to remove any fluorescent lipid from intracellular compartments to the plasma membrane. The cell-associated lipids were extracted and analyzed by TLC as described in the text. The amount of back-exchangeable C6-NBD-lipid was calculated using Eq. 1 (see Materials and Methods) and all values are expressed as percentage of total cell-associated C6-NBD-lipid. The amounts of newly synthesized C6-NBD-SM and nonmetabolized C6-NBD-SM were calculated by Eq. 2 and 3, respectively (see Materials and Methods).

Table II. Effect of Nocodazole and Monensin on C6-NBD-SM Endocytosis

| Treatment          | C6-NBD-SM internalized | C6-NBD-GlCer synthesized |
|--------------------|-------------------------|--------------------------|
|                    | pmol/μg DNA             | Percent of cell-associated C6-NBD-lipid | pmol/μg DNA | Percent of cell-associated C6-NBD-lipid |
| Control            | 5.24 ± 0.60             | 28.1 ± 3.2               | 0.33 ± 0.05 | 1.7 ± 0.3 |
| 10 μM monensin     | 6.20 ± 2.10             | 33.3 ± 11.3              | 0.53 ± 0.11 | 2.8 ± 0.6 |
| 10 μg/ml nocodazole| 3.76 ± 0.39             | 37.9 ± 3.9               | 0.40 ± 0.04 | 4.0 ± 0.4 |

Cells were either untreated (control) or were preincubated with 10 μM monensin or 10 μg/ml nocodazole in culture medium for 90 min at 37°C. All solutions used for treated cells contained either 10 μM monensin or 10 μg/ml nocodazole. Cells were labeled with SUV containing α-erythro-C6-NBD-SM in HMEM for 30 min at 7°C, washed, incubated in HMEM at 37°C for 2 h, and then either harvested immediately or treated with back-exchange medium at 7°C. The cell-associated lipids were extracted, and both the amount of endocytosed, nonmetabolized C6-NBD-SM and of newly synthesized C6-NBD-GlCer were determined as described in the legend to Fig. 7. Data are the mean of triplicate measurements ± SD.
Recycling of intracellular C₆-NBD-SM to the plasma membrane. (a) Cells were labeled with SUV containing D-erythro-C₆-NBD-SM in HMEM for 30 min at 7°C, washed, incubated at 37°C for 30 min in HMEM, and treated with back-exchange medium at 7°C resulting in cells containing only internalized C₆-NBD-lipid. (b) Cells treated as in a were further incubated at 37°C in HMEM for 30 min, washed, and photographed. The plasma membrane (arrowheads) showed the reappearance of fluorescent lipid labeling. (c) Cells treated as in b were then treated with back-exchange medium at 7°C to remove any C₆-NBD-lipid returned to the plasma membrane, washed, and photographed. Note peripheral endosomes containing reinternalized fluorescent lipid. (d) Cells treated as in a were further incubated for 30 min at 37°C in back-exchange medium that continuously removed any C₆-NBD-lipid being transported to the plasma membrane from intracellular compartments during the 37°C incubation. The cells were then washed and photographed. Note the lack of fluorescently labeled peripheral vesicles in d versus c. In some cases, perinuclear labeling was not visible for each cell in the field because of the plane of focus selected. Bar, 10 μm.

Quantitative analysis of cells containing only internalized D-erythro-C₆-NBD-SM that were further incubated for 30 min determined that 3.03 ± 0.22 (n = 3) pmole of nonmetabolized C₆-NBD-SM/μg DNA was recycled back to the plasma membrane, while the combined amount of newly synthesized C₆-NBD-SM and C₆-NBD-GlcCer resulted in only 0.46 ± 0.31 (n = 3) pmole/μg DNA. Thus, >86% of the plasma membrane fluorescence observed in Fig. 8 b was the result of the recycling of nonmetabolized C₆-NBD-SM.

The time course of nonmetabolized D-erythro-C₆-NBD-SM return to the plasma membrane from intracellular compartments was also determined (Fig. 9). C₆-NBD-SM was transported to the plasma membrane from intracellular compartments with a half-time of ~15–20 min. Combined with a half-time of ~15–20 min for C₆-NBD-SM internalization (Fig. 7), this gives a half-time for one complete round of recycling of ~30–40 min.

The effects of 10 μg/ml monensin on C₆-NBD-SM recycling were also examined (Table III). Similar amounts of transport of nonmetabolized, intracellular C₆-NBD-SM to the plasma membrane occurred in monensin-treated and control cells. However, transport of newly synthesized C₆-NBD-SM to the plasma membrane was abolished, consistent with previous results (25).

In spite of the altered pattern of C₆-NBD-SM internalization (Fig. 4 c), nocodazole-treated cells exhibited plasma membrane recycling of nonmetabolized C₆-NBD-SM (Table III). Neither the return of nonmetabolized C₆-NBD-SM to the plasma membrane nor the transport of newly synthesized C₆-NBD-SM were inhibited by nocodazole. In fact, nocodazole appeared to increase the amount of newly synthesized C₆-NBD-SM transport to the plasma membrane.

Since C₆-NBD-SM was capable of recycling, we wanted to compare the distribution of internalized C₆-NBD-SM.
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In this study, we have examined the transport of the D-erythro and L-threo stereoisomers of a fluorescent analogue of sphingomyelin, C6-NBD-SM, as probes for plasma membrane lipid transport in CHO-K1 cells. Most of the intracellular C6-NBD-SM was transported along the recycling pathway illustrated in Fig. 11, pathway I. Both isomers of C6-NBD-SM labeled only the plasma membrane when incubated with cells at 7°C. Cells labeled with C6-NBD-SM at 7°C and then incubated at 37°C endocytosed fluorescent SM to a perinuclear region of the cell that colocalized with the centriole. When cells containing only internalized C6-NBD-lipid were further incubated at 37°C, C6-NBD-SM was returned to the plasma membrane. Thus, C6-NBD-SM recycles between the plasma membrane and intracellular compartments. Both D-erythro and L-threo-C6-NBD-SM were capable of being recycled.

In addition to the endocytosis of C6-NBD-SM, some hydrolysis of both C6-NBD-SM isomers to the corresponding C6-NBD-Cer isomer was observed upon 37°C incubation. C6-NBD-Cer can spontaneously undergo transbilayer movement (31), in contrast to C6-NBD-SM which has a highly polar head group restricting it to one leaflet of a bilayer (17).

Figure 11 depicts the partial hydrolysis of C6-NBD-SM occurring at the plasma membrane. This is consistent with our observation that some hydrolysis of D-erythro-C6-NBD-SM occurred at 7°C, in the absence of endocytosis. Neutral sphingomyelinas have been found in plasma membrane-enriched fractions from rat liver (16), neuroblastoma cell cultures (5), and human renal proximal tubule cell cultures (9). It is possible that C6-NBD-SM was also hydrolyzed by lysosomal (acid) sphingomyelinas (1, 7, 18); however, C6-NBD-SM did not accumulate in the lysosomes, probably because of the sorting of endocytosed C6-NBD-SM from the degradation pathway to a recycling pathway. Also, increasing lysosomal pH with NH4Cl, which should reduce lysosomal sphingomyelinas activity, did not inhibit C6-NBD-SM hydrolysis (data not shown). Examination of C6-NBD-SM transport in Niemann-Pick fibroblasts, which are

Discussion

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Table III. Effect of Nocodazole and Monensin on C6-NBD-SM Recycling

| Treatment             | Nonmetabolized C6-NBD-SM recycled | New C6-NBD-SM transported | Percent nonmetabolized C6-NBD-SM recycled | Percent new C6-NBD-SM transported |
|-----------------------|-----------------------------------|---------------------------|------------------------------------------|----------------------------------|
| Control               | 43.3 ± 4.2                        | 4.0 ± 2.8                 | 68.7 ± 9.8                                | 10.8 ± 1.0                      |
| 10 μM monensin        | 56.1 ± 19.1                       | 0*                        | 76.0 ± 25.9                               | 0*                              |
| 10 μg/ml nocodazole   | 46.8 ± 5.9                        | 9.9 ± 1.8                 | 60.8 ± 7.7                                | 43.0 ± 7.8                      |

Cells were either untreated (control) or preincubated with 10 μM monensin or 10 μg/ml nocodazole in culture medium for 90 min at 37°C. All solutions used for treated cells contained either 10 μM monensin or 10 μg/ml nocodazole. Cells were labeled with SUV containing D-erythro-C6-NBD-SM in HMEM for 30 min at 7°C, washed, incubated at 37°C in HMEM for 30 min, and then treated with back-exchange medium to remove any C6-NBD-SM remaining at the plasma membrane. The cells were then further incubated at 37°C for 30 min in either HMEM or back-exchange medium and washed. The cell-associated lipids were extracted and both the amount of nonmetabolized C6-NBD-SM recycled to the plasma membrane and of newly synthesized C6-NBD-SM transported to the plasma membrane were determined as described in the legend to Fig. 9. Data are the mean of triplicate measurements ± SD, expressed as percent total cell-associated C6-NBD-SM.

* Not detectable.
Figure 10. Colocalization of internalized C₆-NBD-SM and Rh-Tf. Cells were labeled with SUV containing 5-erythro-C₆-NBD-SM at 7°C, washed, and incubated at 37°C for 30 min in HMEM that contained 20 μg/ml Rh-Tf. The cells were treated with back-exchange medium at 7°C to remove any C₆-NBD-SM remaining at the plasma membrane and then photographed for either C₆-NBD-lipid (a) or Rh-Tf (b) fluorescence. Bar, 10 μm.
The internalization of an analogue of phosphatidylcholine is internalized from the plasma membrane and transported to the region of the Golgi apparatus in Chinese hamster V79 fibroblasts (37). In CHO-K1 cells, we found that the Golgi apparatus and perinuclear endosomal compartments are often associated with the centriole, particularly when migrating fibroblasts are examined (36). Previous work from our laboratory indicates that a fluorescent Cer produced spontaneously moves to the Golgi apparatus where it is metabolized to newly synthesized C6-NBD-SM and, in the case of α-erythro-C6-NBD-Cer, to C6-NBD-GlcCer. The metabolites are then transported from the Golgi apparatus to the plasma membrane by a process that is inhibited by monensin treatment.

Figure 11. Model of C6-NBD-SM transport and recycling. Thick lines represent portions of the bilayer containing fluorescent lipid. Pathway I, nonmetabolized C6-NBD-SM inserted into the outer leaflet of the plasma membrane (PM) is endocytosed to intracellular vesicles in the region of the centriole. The internalized C6-NBD-SM is subsequently transported to intracellular compartments back to the plasma membrane, resulting in a plasma membrane lipid recycling pathway. Pathway II, a small amount of C6-NBD-SM is hydrolyzed to C6-NBD-Cer at the plasma membrane. The fluorescent Cer produced spontaneously moves to the Golgi apparatus where it is metabolized to newly synthesized C6-NBD-SM and, in the case of α-erythro-C6-NBD-Cer, to C6-NBD-GlcCer. The metabolites are then transported from the Golgi apparatus to the plasma membrane by a process that is inhibited by monensin treatment.

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