Accumulation of Glycosphingolipids in Niemann-Pick C Disease Disrupts Endosomal Transport

Received for publication, October 22, 2003, and in revised form, April 8, 2004
Published, JBC Papers in Press, April 12, 2004, DOI 10.1074/jbc.M311591200

Danielle te Vrugt†, Emyr Lloyd-Evans§, Robert Jan Veldman¶, David C. A. Neville, Raymond A. Dwek, Frances M. Platt, Wim J. van Blitterswijk‖, and Dan J. Silence¶¶

From the Glycobiology Institute, Department of Biochemistry, South Parks Road, University of Oxford, Oxford OX1 3QU, United Kingdom and ¶¶ Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Glycosphingolipids are endocytosed and targeted to the Golgi apparatus but are mistargeted to lysosomes in sphingolipid storage disorders. Substrate reduction therapy utilizes imino sugars to inhibit glucosylceramide synthase and potentially abrogate the effects of storage. Niemann-Pick type C (NPC) disease is a disorder of intracellular transport where glycosphingolipids (GSLs) and cholesterol accumulate in endosomal compartments. The mechanisms of altered intracellular trafficking are not known but may involve the mistargeting and disrupted function of proteins associated with GSL membrane microdomains. Membrane microdomains were isolated by Triton X-100 and sucrose density gradient ultracentrifugation. High pressure liquid chromatography and mass spectrometric analysis of NPC1-/- mouse brain revealed large increases in GSL. Sphingosine was also found to be a component of membrane microdomains, and in NPC liver and spleen, large increases in cholesterol and sphingosine were found. GSL and cholesterol levels were increased in mutant NPC1-null Chinese hamster ovary cells as well as U18666A and progesterone induced NPC cell culture models. However, inhibition of GSL synthesis in NPC cells with N-butyldeoxygalactonojirimycin led to marked decreases in GSL but only small decreases in cholesterol levels. Both annexin 2 and 6, membrane-associated proteins that are important in endocytic trafficking, show distorted distributions in NPC cells. Altered BODIPY lactosylceramide targeting, decreased endocytic uptake of a fluid phase marker, and mistargeting of annexin 2 (phenotypes associated with NPC) are reversed by inhibition of GSL synthesis. It is suggested that accumulating GSL is part of a mislocalized membrane microdomain and is responsible for the deficit in endocytic trafficking found in NPC disease.

Increasing evidence suggests that the membranes of eukaryotic cells are not homogeneously fluid. They contain membrane microdomains, often referred to as lipid rafts and/or caveolae, enriched in glycosphingolipids (GSLs), specific proteins, and cholesterol (1–3). Being operationally defined, i.e., by detergent insolubility and low buoyant density, the natural (in situ) existence of such membrane microdomains has been and continues to be controversial (4). However, they have recently been visualized by using electron microscopy as small structures covering as much as 35% of the cell surface (5). Although membrane microdomains on the cell surface tend to be transient (6), relatively stable microdomains have been visualized in the endocytic pathway (7) where they are proposed to play a role in protein and lipid sorting (8). Many GSL storage diseases, including Niemann-Pick type C (NPC), are often characterized by enlarged liver and spleen as well as severe neurodegeneration. In these diseases, cholesterol and GSLs accumulate in the endocytic pathway because of reduced breakdown. Hence, they may be a useful tool for determining the relative importance of GSLs in endocytic transport. Fibroblasts from patients suffering from GSL storage diseases do show altered endocytic sorting (9), and it has been suggested that the accumulation of endocytic membrane microdomains may be the basis for altered endocytic sorting (10). It is possible that accumulating membrane microdomains in storage bodies mislocalizes microdomain proteins involved in signaling and transport, precluding normal function (10, 11). However, the relative importance of GSLs and proteins in membrane microdomain formation is still not known (12, 13).

Annexins 2 and 6 are lipid-binding proteins that are widely involved in facilitating intracellular transport, including endocytic trafficking, and are reported to associate with membrane microdomains (14–23). Proteins involved in both early and late endocytic transport have been shown already to be inhibited in NPC disease. Annexin 2 normally localizes to early endosomes but has been shown to be mislocalized because of lipid accumulation in late endosomes in NPC disease (15). Rab 7, a protein involved in endocytic transport from late endosomes, is also compromised as a result of lipid storage (24, 25).

In this study, we have quantified the levels of GSLs, sphingosine, and cholesterol that occur in lipid rafts (the detergent-
insoluble fraction) isolated from various organs of a mouse model of Niemann-Pick C (NPC1) disease by HPLC and mass spectrometry. Raft-associated GSLs increased in all tissues. Cholesterol and sphingosine increased in non-neuronal tissues. GSL-lowering therapy, using imino sugar inhibitors of GSL synthesis, is effective in the treatment of a mouse model of NPC disease (26). In NPC cell culture models, inhibition of GSL synthesis ablated the accumulation of GSLs but had little effect on cholesterol levels. However, phenotypes associated with NPC, such as annexin 2 mislocalization, associated decrease of uptake of fluid phase markers (15), and altered BODIPY LacCer trafficking (9) were all reversed by inhibition of GSL synthesis. At least under conditions of intracellular storage, the accumulation of GSLs appears intimately linked to the perturbation of endocytic transport.

**EXPERIMENTAL PROCEDURES**

HPLC-grade acetonitrile, methanol, chloroform, and butanol were from VWR Scientific (Poole, UK). Acetone was from Fisher. Tissue culture media and supplements were from Invitrogen. o-Phthaldialdehyde and progesterone were from Sigma. Tetrabutylammonium bromide was from Fluka (Zwijndrecht, Netherlands). C:17-sphingosine 1-phosphate was from Avanti Polar Lipids (Alabaster, AL). U18666A was from Affiniti Research Chemicals (Exeter, UK), and NB-DGJ was from Thermo Fisher Scientific (Poole, UK). Annexin 2 and 6 antibodies were purchased from TD Labs (Oxford, UK), and secondary antibodies were from Molecular Probes (Leiden, Netherlands). Cell Culture—Bovine aortic endothelial cells, human umbilical vein endothelial cells, and EC-RF24 endothelial cells were grown to confluency in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were dissociated with trypsin/EDTA and were plated at a density of 50,000 cells/cm² in an atmosphere of air/CO₂ (19:1) at 37 °C in a humidified incubator in 10-cm dishes. CT43 and RA25 Chinese hamster ovary (CHO) cells were a gift of TY Chang, Dartmouth Medical School. The CT43 cell line is a cholesterol trafficking mutant defective in the NPC1 protein. The RA25 cell line is the parental cell line and has a normal NPC1 allele. Both sets of cells contain a gain of function mutation in SCAP, resulting in resistance to sterol-dependent transcriptional regulation. CHO cells seeded in Ham’s F-12 Glutamax-I medium, supplemented with 10% fetal calf serum and 100 units/ml of penicillin/streptomycin, were grown as monolayers at 37 °C with 5% CO₂. RAW 264.1 and J774.A1 mouse macrophages were obtained from the ECACC (Porton Down, UK) and maintained at a density of 5 × 10⁵ cells/ml in RPMI supplemented with 10% FCS, 50 units/ml penicillin/streptomycin, and 10% fetal calf serum.

**Animals—npc1<sup>−/−</sup>** spontaneous mutant mice on the BALB/c background (27) (denominated as NPC1<sup>−/−</sup> mice in this paper) were maintained by brother-sister mating of heterozygous animals. At weaning (21 days), tail tips were removed for DNA preparation. PCR was used to determine genotypes at the npc1 locus using the following primer pairs: IMR928 and IMR929, expected fragment size, 173 bp; annealing temperature was 55 or 57 °C; IMR928 5’ GTG TAG CTC ATC TGC CAT CG 3’ and IMR929 5’ TCT CAC AGC CAC AAG CTT CC 3’. IMR927 and IMP258F, expected fragment size, 475 bp. Annealing temperature was 53 °C; IMR927 5’ TGA GCC CAA GCA TAA CTT CC 3’ and IMP258F 5’ GGT GCT GGA CAG CCA AGT A 3’. NPC1<sup>−/−</sup> mice developed disease symptoms between 6 and 7 weeks of age, and tissues were harvested at a 10 weeks of age.

**Membrane Microdomain Isolation—**Membrane microdomains were purified by the method of Lisanti et al. (28). Briefly, tissues were homogenized by 10 strokes of a Dounce homogenizer in 0.5% (w/v) Triton X-100 in 25 mM Mes-buffered saline (pH 6.5) and incubated for 30 min on ice. The solution made up to 40% sucrose and transferred to a Beckman Ultraclear 14 × 89-mm tube on ice with a discontinuous gradient of 5 ml of 30% sucrose and 3 ml of 5% sucrose layered on top. Samples were ultracentrifuged in an SW41 rotor at 100,000 × g for 16 h at 4 °C, and 2-ml fractions were manually collected.

**Purification of GSLs for HPLC and Mass Spectrometry—**GSLs were extracted by addition of 3.2 volumes of CHCl₃/Methanol (1:2) for 10 min at room temperature followed by the addition of 1 volume of CHCl₃ and 1 volume of H₂O (29). This rapid extraction procedure gives similar recoveries of GSLs to other commonly used methods of lipid extraction.

**Fig. 1.** Separation of brain GSLs from NPC1<sup>−/−</sup> and NPC1<sup>−/−</sup> mice by HPLC. Top trace, cerebral GSLs from normal NPC1<sup>−/−</sup> mice. Bottom trace, cerebral GSLs from NPC1<sup>−/−</sup> mice. Representative traces of GSLs after digestion with ceramide glycanase and derivatization of the released oligosaccharides with anthranilic acid before separation by HPLC. Apart from mass spectrometry of intact GSLs, the identity of some GSLs was confirmed by (a) removal of sialic acid residues by trifluoroacetic acid and (b) mass spectrometry of the same samples both before and after derivatization (not shown).

**Fig. 2.** Sphingosine and dihydrospingosine but not phytosphingosine associate with membrane microdomains. Endothelial cells (bovine aortic endothelial cells, human umbilical vein endothelial cells, and EC-RF24) were harvested and resuspended in 0.5% Triton X-100, homogenized, and placed at the bottom of a discontinuous sucrose gradient. After centrifugation, fractions were collected, and sphingoid bases were extracted and derivatized before separation and quantification by HPLC.
were dried under nitrogen, 0.5 ml of water, 1 ml of 1-butanol; 20°C for 1 hour. The fluorescence was monitored for 120 min at a flow rate of 1.2 ml/min was maintained, and the fluorescence was monitored for 120 min. 

Gangliosides were eluted with 6 ml of CHCl3/MeOH (1:3), and the enzyme digest followed by 80 ml of 99:1 CH3CN/H2O, 0.5 ml of 97:3 CH3CN/H2O, and the derivatized oligosaccharides were eluted two times with 0.6 ml of water into screw-cap vials. 100 μl of incubation buffer (1 mg/ml sodium cholate in 50 mM sodium acetate (pH 5.0)). After vigorous vortexing and spinning in a benchtop picofuge, 10 μl of 50 milliunits/100 μl of concentrated H2SO4, the plate was centrifuged at 3000 rpm for 5 min and then one time with 100 μl of CHCl3/MeOH (1:2:2) and transferred to a 1.5-ml tube. The samples were dried down in a SpeedVac and resuspended in 20 μl of MeOH. Matrix-assisted laser desorption ionization-mass spectrometry was performed as described previously (34).

Quantitation of Cholesterol—Cholesterol was quantitated by using a method similar to that of Franey and Amador (35). Samples were resuspended in 50 μl of EtOH and transferred to a microtitre plate along with cholesterol standards (1 mg/ml in ethanol) in duplicate and dried overnight at 80°C. After the addition of 75 μl of 3 mM FeCl3-6H2O in glacial acetic acid and 50 μl of concentrated H2SO4, the plate was incubated for 10 min in the dark and the absorbance read at 570 nm. 

Extraction and Quantitation of Sphingoid Bases by HPLC—Sphingoid bases were extracted and quantified by the method of Merrill et al. (36). Tissues or membrane microdomain fractions were extracted with butanol containing 1% v/v concentrated HCl and dried under nitrogen. Samples were saponified by the addition of 1 ml of chloroform and 1 ml of 0.2 M NaOH in methanol and incubated overnight at 37°C. Lipids were dried under nitrogen, 0.5 ml of water, 1 ml of 1-butanol; 20 μl of concentrated HCl was added, and the upper phase was removed. This procedure was repeated several times, and the combined extract was dried under nitrogen. Sphingoid bases were derivatized with sodium cyanoborohydride (45 mg/ml) to each digest and incubated for 18 h, 10 μl of water was added to each enzyme digest followed by 80 μl of anthraquinonic acid (30 mg/ml) and sodium cyanoborohydride (45 mg/ml) to each digest and incubated for 1 h at 80°C. Derivatized oligosaccharides were purified on DPA-6S (Supelco, Ballafonte, PA), and columns were pre-equilibrated two times with 1 ml of CH3CN. 1 ml of 97:3 CH3CN/H2O was added to each sample and vortexed prior to loading. Columns were washed four times with 1 ml of CH3CN/H2O, 0.5 ml of 97:3 CH3CN/H2O, and the derivatized oligosaccharides were eluted two times with 0.6 ml of water into screw-cap Eppendorf and stored at 4°C in the dark until ready for normal phase-HPLC using a Waters Alliance 2695 separations module, an autosampler vial. 100 μl of the derivatized lipids were injected onto a Waters 5 μm C18 8 × 100 mm column, pore size 100 Å (Waters, Milford, MA). Samples were eluted isocratically with filtered and degassed methanol, 10 mM K3HPO4 (pH 7.4), 0.5 μM tetrabutylammonium dihydrogen phosphate (85:16:1, v/v) as the mobile phase. A flow rate of 1.2 ml/min was maintained, and the fluorescence was monitored for 120 min at λex 340 nm and λem 455 nm.

Quantitation of GSL Derivatives by HPLC—GSLs were analyzed according to Neville et al. (37). Briefly dried lipid extracts were resuspended in 100 μl of incubation buffer (1 mg/ml sodium chloride in 50 mM sodium acetate (pH 5.0)). After vigorous vortexing and spinning in a benchtop picofuge, 10 μl of 50 milliunits/100 μl of ceramide glycanase (EC 3.2.1.123, Calbiochem) in incubation buffer was added to cleave the glycans. Glucosylceramide is partially digested because of the specificity of the glycanase (37). After 18 h, 10 μl of water was added to each enzyme digest followed by 80 μl of anthraquinonic acid (30 mg/ml) and sodium cyanoborohydride (45 mg/ml) to each digest and incubated for 1 h at 80°C. Derivatized oligosaccharides were purified on DPA-6S (Supelco, Ballafonte, PA), and columns were pre-equilibrated two times with 1 ml of CH3CN. 1 ml of 97:3 CH3CN/H2O was added to each sample and vortexed prior to loading. Columns were washed four times with 1 ml of CH3CN/H2O, 0.5 ml of 97:3 CH3CN/H2O, and the derivatized oligosaccharides were eluted two times with 0.6 ml of water into screw-cap Eppendorf and stored at 4°C in the dark until ready for normal phase-HPLC using a Waters Alliance 2695 separations module, an in-line Waters 474 fluorescence detector separation and a 4.6 × 250 mm TSK gel-Amide 80 column (Anachem, Luton, UK).

Immunocytochemistry—J774 and CHO cells were seeded at a density of 5000 cells/cover slip (22 mm) and allowed to adhere overnight. Coverslips were washed twice in PBS prior to fixation with 3.7% paraformaldehyde at room temperature for 10 min. Coverslips were blocked with 1% bovine serum albumin containing 0.1% Tween 20 for 1 h. Monolayers were incubated with 2.5 μg/ml primary mouse monoclonal antibodies specific for annexin 2 or 6 in blocking solution for 45 min, followed by three washes in PBS (5 min each wash), and incubation with 5 μg/ml Alexa-Fluor 488 (annexin 2) or 564 (annexin 6)-conjugated rabbit anti-mouse secondary antibody for 30 min. Samples were washed three times in PBS (5 min each wash) followed by mounting in Vectashield. Fluorescent cells were observed using a Zeiss Axioplan 2 fluorescence microscope. Images were collected using a charge-coupled device camera.

Cholesterol Staining—Monolayers on coverslips were washed three times with PBS and fixed in 3.7% paraformaldehyde for 15 min followed by quenching in medium. Cells were incubated with 125 μg/ml filipin (a cytochemical probe specific for cholesterol) for 30 min at 37°C followed by three washes with PBS and mounting in Vectashield.

Fluid Phase Endocytosis—Cellular uptake of HRP was performed as described (15, 38). RAW, J774, RA25, and CT43 cells were treated with 2 μg/ml U18666A (U) or 20 μg/ml progesterone (Prog) for 18 h. 50% confluent monolayers were grown on coverslips and incubated in the presence of 13 μM BODIPY LacCer and pulse-chased as described under “Experimental Procedures.” Scale bar indicates 5 μm.

RESULTS

Structural Confirmation and Quantitation of GSLs by HPLC—The changes in total GSL levels between NPC1+/− and NPC1−/− macrophages or NPC1-containing RA25 cells were treated with 2 μg/ml U18666A (U) or 20 μg/ml progesterone (Prog) for 18 h. 50% confluent monolayers were grown on coverslips and incubated in the presence of 13 μM BODIPY LacCer and pulse-chased as described under “Experimental Procedures.” Scale bar indicates 5 μm.
Cerebella, cerebra, liver, and spleen were dissected from normal and NPC1−/− mice and homogenized. The equivalent of 5 mg of tissue was incubated with Triton X-100 before separation of the floating detergent-insoluble and -soluble portions by ultracentrifugation. GSLs from both portions were extracted and purified before digestion with ceramide glycanase before derivatization and quantitation by HPLC. Data are from 3 to 9 experiments and are expressed as pmol/g protein ± S.E. Data in parentheses indicate the concentration in the homogenate (pmol/g protein S.E. < 20%). Total raft protein varied from 210 ± 40 μg for NPC1−/− cerebrum to 140 ± 10 μg for NPC1−/− (n = 9).

|               | LacCer | GA2  | GM3a | GM2a | GM1b | GD1a | GD1b | GT1b | GQ1b | Gb3 | Gb4 | GA1 | GalNAc |
|---------------|--------|------|------|------|------|------|------|------|------|-----|-----|-----|--------|
| Cerebrum      |        |      |      |      |      |      |      |      |      |     |     |     |         |
| NPC1−/−       | 0.03 ± 0.01 | --   | 0.1 ± 0.04 | 0.07 ± 0.02 | 2 ± 0.5 | 3 ± 0.7 | 1 ± 0.2 | 1 ± 0.3 | 0.1 ± 0.03 | -- | -- | -- |        |
| NPC1−/−       | 0.5 ± 0.2 | 0.3 ± 0.1 | 2 ± 0.4 | 3 ± 0.6 | 3 ± 1 | 10 ± 2 | 2 ± 1 | 3 ± 0.8 | 0.2 ± 0.07 | -- | -- | -- |        |
| Liver         | 1.5 ± 0.3 | 1 ± 0.4 | 1.3 ± 0.3 | 18 ± 3 | -- | -- | -- | -- | -- | -- | -- | -- |        |
| NPC1−/−       | 5 ± 1   | 3 ± 0.2 | 7 ± 1 | 23 ± 1 | -- | -- | -- | -- | -- | -- | -- | -- |        |
| Spleen        | 19 ± 0.2 | 0.1 ± 0.02 | 0.4 ± 0.07 | -- | 0.5 ± 0.01 | -- | -- | -- | 4 ± 1.6 | 3 ± 0.6 | 0.3 ± 0.05 | 2 ± 0.3 |        |
| NPC1−/−       | 2 ± 0.2 | (0.2) | (0.05) | (0.09) | (0.6) | (0.8) | (0.15) | (0.2) | (0.3) |        |        |        |        |        |

a N-Glycolyl versions were found in peripheral tissues.
b GM1a was found in the brain but in spleen GM1b predominated. --, below detection limit (<0.01 pmol/μg).
NPC1−/− mice were initially analyzed by matrix-assisted laser desorption ionization-mass spectrometry (34). No large differences in fatty acid composition could be detected between normal and NPC1−/− brain, liver, and spleen tissues (not shown). GSLs were extracted and digested by ceramide glycocyanase (37) followed by derivatization, separation, and quantitation of the resultant oligosaccharides by HPLC (Fig. 1). For instance, normal mouse brain GSLs were found to be similar to those reported previously with GM1a, GD1a, GD1b, GT1b, and GQ1b predominating and lower levels of LacCer, GA2, GM3, and GM2 (Fig. 1) (39). However, in brains from NPC1−/− mice, GSLs increased, including GM2, GA2, GM3, and LacCer in agreement with studies employing thin layer chromatography (40, 41).

Glycosphingolipids in NPC1−/− Mice Accumulate in Membrane Microdomains—Because GSLs are thought to be important components of membrane microdomains, we investigated whether they accumulated in microdomains in the NPC mouse tissues. All the GSLs were found to be highly enriched in membrane microdomains (Table I). Membrane microdomains from the cerebrum, cerebellum, liver, and spleen were purified by the Triton X-100 detergent procedure using sucrose gradients. In the brains of NPC1−/− mice GSLs increased in agreement with the mass spectrometric and HPLC analysis of the total levels (Fig. 1). The fold increases were broadly similar to that found in total GSL levels in human NPC patients (42). Similar increases were seen in cerebellum (Table I). Table I also shows the levels of membrane microdomain GSLs in NPC1−/− liver and spleen. In liver, the N-acylglycolyl derivative of GM2 predominated with smaller quantities of neutral lipids. In membrane microdomains isolated from NPC1−/− liver, large increases in GcGM3, LacCer, and GA2 were detected. In NPC1−/− spleen, levels of membrane microdomain LacCer, GA2, GA1, GM3, Gb3, GalNacGA1, and GM1b increased.

Cholesterol in NPC1−/− Mice Accumulates in Membrane Microdomains—During the last 20 years NPC has been viewed as a cholesterol storage disease. We determined whether the elevated cholesterol that occurred in NPC was enriched in membrane microdomains. Up to 90% of the cholesterol was found in membrane microdomains (the detergent-insoluble low density fraction) in both normal and NPC1−/− tissues (Table II). Table II shows that membrane microdomain cholesterol levels varied in control mice between different tissues, and large increases (4–10-fold) in cholesterol could be detected in NPC1−/− liver and spleen, similar to that found in human patients (43). Only a minor increase in cholesterol can be detected in brain.

Sphingosine Preferentially Resides in Membrane Microdomains—Although the occurrence of sphingoid bases in membrane microdomains has not been reported previously, several of the enzymes that were involved in their production, such as acid sphingomyelinase and ceramidase as well as their precursors sphingomyelin and ceramide, have been localized to lipid rafts/caveolae (44–46). We initially looked in a variety of known caveolae-rich endothelial cell lines to determine whether sphingoid bases were located in membrane microdomains. An HPLC technique was used to separate different sphingoid bases following derivatization with o-phthalaldehyde (36). Clear separation of sphingosine from sphingosine 1-phosphate, dihydroxyphosphosine, and phytosphingosine could be obtained. Other unidentified peaks are possibly either lysophosphatidylglycerol and/or lysophosphatidylethanolamine and/or lyso forms of complex glycolipids (data not shown).

Fig. 2 shows the distribution of sphingosine, dihydroxyphosphosine (sphinganine), and phytosphingosine over the density gradient fractions from three different endothelial cell lines. Sphingosine and dihydroxyphosphosine were enriched in the floating membrane microdomain fraction. In contrast, phytosphingosine, which has an extra hydroxyl group, is not en-

| Tissue genotype | GSL | Cholesterol | Sphingosine |
|----------------|-----|-------------|-------------|
| Brain | pmol | n mol | pmol |
| +/+ | 7 ± 2 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| −/− | 24 ± 5 | 2 ± 0.4 | 1.6 ± 0.2 |
| Liver | pmol | n mol | pmol |
| +/+ | 23 ± 3 | 0.4 ± 0.04 | 0.7 ± 0.1 |
| −/− | 40 ± 2 | 1.6 ± 0.3 | 8.7 ± 1 |
| Spleen | pmol | n mol | pmol |
| +/+ | 30 ± 6 | 0.7 ± 0.1 | 1.2 ± 0.2 |
| −/− | 109 ± 20 | 2.5 ± 0.4 | 12 ± 1 |

Fig. 4. Effect of GSL depletion on the intracellular accumulation of HRP in an NPC macrophage culture model. RAW and J774 macrophages were treated with 2 μg/ml U18666A (P) or 20 μg/ml progesterone (P) and the indicated concentrations of NB-DGJ (an inhibitor of GSL synthesis) for 18 h and then incubated with 3 mg/ml HRP. The amounts of HRP that accumulated in cells were quantitated after 2 h of incubation. The results are expressed as % control ± S.E. (n = 3). RAW controls were 1.5 ± 0.3 millimoles/μg and J774 controls were 5.8 ± 0.2 millimoles/μg protein.
riched in the membrane microdomain fraction and therefore serves as a control to show the specificity of the localization of sphingosine and sphinganine to rafts/caveolae.

Membrane Microdomain Association of Sphingosine in NPC1<sup>−/−</sup> Mice—Because sphingosine was localized to rafts/caveolae in endothelial cell lines, we determined whether it localized to membrane microdomains in NPC disease; large increases in sphingosine have been reported in the liver and spleen of NPC patients as well as mouse models of NPC disease (47, 48). Table II shows large increases in microdomain sphingosine in liver and spleen of NPC<sup>−/−</sup> mice. Modest changes were detected in brain microdomains, similar to whole tissue (48). Low levels of microdomain dihydro sphingosine also accumulated in NPC1<sup>−/−</sup> tissues (not shown); sphingosine 1-phosphate levels were too small to be detected reliably. Overall, the results clearly show that a significant proportion of the sphingosine, cholesterol, and GSL that accumulates in NPC disease was located in membrane microdomains.

NPC Cell Cultures Show Increases in Both GSL and Cholesterol—Although increases in cholesterol in NPC cell culture models are well documented (49–51), the study of GSL is not as extensive. We determined whether increases in GSL occur in NPC cell culture models, and we compared this to the increases in cholesterol (Table III). By using U18666A and progesterone treatment to induce an NPC phenotype (52, 53), total GSL levels increased (Table III). LacCer, GA2, GM3, GM2, GD1a, and GM1a were detected in RAW macrophages, and all increased to similar degrees. Increases in GSLs (LacCer, GM3, GcGM3, and GM1b) were observed when NPC1-containing RA25 cells were compared with NPC1 null CT43 cells (Table III). Cholesterol levels were larger but showed similar fold increases to GSL levels. However, administration of NB-DGJ decreased GSL levels without significantly affecting cholesterol levels (Table III). To decrease cholesterol levels further sphingomyelin may also have to be depleted.

Altered Endocytic Trafficking in an NPC Cell Culture Model Is Corrected by GSL-lowering Drugs—When normal RA25 CHO cells were pulse-labeled with BODIPY-LacCer, the fluorescent lipid was targeted primarily to the Golgi complex (Fig. 3A) (9). In NPC1-null CT43 cells, fluorescence accumulates in punctate endocytic structures, reminiscent of previous results in NPC patient fibroblasts (24). Because GSL-lowering therapy was effective in a mouse model of NPC disease (26), we investigated whether GSL depletion can also reverse altered endocytic trafficking. Administration of NB-DGJ, an inhibitor of GSL synthesis, for 5 days leads to a reversal of altered trafficking and perinuclear localization is restored (Fig. 3A); shorter incubations were not as effective (not shown). These results suggest that NPC is similar to other GSL storage disorders; altered BODIPY LacCer sorting in a Gaucher cell culture model is also reversed by GSL-lowering drugs (54).

Altered GSL Trafficking Occurs in U18666A and Progesterone-induced NPC Cell Culture Models—Because clear increases in GSL levels were seen in U18666A and progesterone-treated cells, we determined whether GSL trafficking was also affected. Fig. 3B shows that BODIPY LacCer is targeted to a perinuclear region in RAW macrophages (54). Treatment of both the RAW macrophages or NPC1 containing RA25 cells with U18666A or progesterone alters BODIPY LacCer trafficking to punctate endocytic structures.

Decreased Fluid-phase Uptake Is Reversed by the Inhibition of GSL Synthesis in NPC Cell Culture Models—Annexins are of particular interest because they are thought to be important in endocytic trafficking and are associated with membrane microdomains (16, 21, 55, 56). NPC cell culture models show altered annexin 2 localization and a decrease in the intracellular accumulation of the fluid phase marker HRP (15). The intracellular accumulation of cholesterol is involved in this process (15), so do GSLs also play a role? Similar to previous results in U18666A-treated baby hamster kidney cells (15), when RAW or J774 macrophages were treated with U18666A the amount of intra-

![Figure 5. Time course of the effects of GSL depletion on the intracellular accumulation of HRP in an NPC CHO cell culture model.](image)

![Figure 6. GSL depletion reverses altered annexin 2 distribution.](image)

**Table III**

| Cell model                  | GSL | Cholesterol |
|-----------------------------|-----|-------------|
| RAW                         | 0.5 ± 0.1 | 23 ± 1 |
| RAW + U18666A               | 1.8 ± 0.5 | 65 ± 6 |
| RAW + NB-DGJ                | 0.5 ± 0.1 | 28 ± 2 |
| RAW + U18666A + NB-DGJ      | 0.7 ± 0.2 | 58 ± 6 |
| RAW + progesterone          | 1 ± 0.2   | 36 ± 2 |
| RAW + progesterone + NB-DGJ | 0.4 ± 0.1 | 32 ± 1 |
| RA25                        | 0.19 ± 0.01 | 33 ± 2 |
| RA25 + NB-DGJ               | 0.14 ± 0.02 | 30 ± 2 |
| CT43                        | 0.36 ± 0.02 | 61 ± 4 |
| CT43 + NB-DGJ               | 0.22 ± 0.03 | 56 ± 4 |

*Fig. 5. Time course of the effects of GSL depletion on the intracellular accumulation of HRP in an NPC CHO cell culture model. NPC1 containing RA25 cells and CT43 null cells were incubated with 50 μM NB-DGJ for varying times. Cell monolayers were then incubated with 3 mg/ml HRP. The amounts of HRP that accumulated in cells were quantitated after 2 h of incubation, mg HRP/mg protein ± S.E. (n = 3).*
cellular accumulation of HRP was found to be reduced (Fig. 4). Simultaneous treatment with NB-DGJ led to a reversal in HRP uptake (Fig. 4). These results suggest that the level of GSL can influence this process. GSL depletion with NB-DGJ also stimulated the uptake of HRP by about 100% in both macrophage cell lines (not shown) suggesting that GSLs may play a role in endocytic trafficking in some cell types, as has been reported previously (54). Comparison of RA25 cells with NPC1-null CT43 cells showed a large decrease in the amount of HRP uptake (Fig. 5), similar to what was reported in U18666A-treated cells. In contrast to macrophages, treatment of NPC1 containing RA25 cells with NB-DGJ did not significantly affect the level of HRP uptake (Fig. 5). When NPC1-null CT43 cells were treated with NB-DGJ, a time-dependent increase in the levels of HRP uptake was observed; complete reversal took 10 days (Fig. 5). We next investigated whether the changes in fluid phase uptake correlated with annexin 2 distribution. In untreated RA25 cells annexin 2 was found to be distributed throughout the cytoplasm with a punctate peripheral distribution (Fig. 6), consistent with previous studies showing that annexin 2 partly localizes to early endosomes (57). No large changes were seen following administration with NB-DGJ in RA25 cells. A prominent decrease in peripheral staining was observed in CT43 cells (Fig. 6) consistent with redistribution to late endosomes, similar to other NPC cell culture models (15). Treatment of CT43 cells with NB-DGJ for 5 days caused a marked reversal in the altered annexin 2 distribution (Fig. 6). These results suggest that GSL levels and annexin 2 distribution are interlinked, at least under conditions of GSL storage. It is also possible that inhibition of GSL synthesis decreases the levels of downstream catabolites such as sphingosine. However, addition of ceramide analogues to NPC cell culture models does not restore the altered BODIPY LacCer trafficking (not shown). One further possibility was that inhibition of GSL synthesis decreases the levels of downstream catabolites such as sphingosine. However, addition of a ceramidase inhibitor (58) also fails to restore altered BODIPY LacCer targeting in NPC cells (not shown).

**DISCUSSION**

In this study, we found that NPC disease is associated with increased levels of GSLs, cholesterol, and sphingosine in membrane microdomains. Although our study uses detergent insolubility as an operational definition of membrane microdomains, a good correlation between detergent resistance and the mobility of lipid probes visualized by single molecule microscopy has been reported (6). A previous study (51) reported evidence that the level of membrane microdomains is increased in an NPC cell culture model; however, relatively small changes in the detergent insolubility of cholesterol were detected. Given that the present data disrupted endocytic trafficking may only engage a small percentage of the total cholesterol pool. This is consistent with the yeast NPC model where altered localization of sphingolipids occurred without a large increase in ergosterol levels (61).

Our data suggest that the accumulation of stored lipids is linked to mislocalization of membrane microdomain-associated proteins such as annexin 2 and 6 (16–18, 20, 21, 56). Recently, using BODIPY LacCer, GSL membrane microdomains have

---

**Annexin 6 Shows an Altered Localization in NPC Cells—**

NPC cells show changes in late endosomal transport (59, 60) as well as changes in early endosomal transport. We looked at annexin 6 because it has been implicated in late endosomal transport and cholesterol trafficking (22, 23). In RA25 CHO cells, we localized cholesterol by using filipin to the perinuclear region (Fig. 7A). However, in NPC1-null CT43 cells and in RA25 cells treated with U18666A (22), a more punctate cholesterol-filipin distribution is seen throughout the cell (Fig. 7A). When a similar experiment was performed by using an antibody to annexin 6, RA25 cells show a perinuclear distribution (Fig. 7B), whereas CT43 (NPC1<sup>+/−</sup>/H11002/H11002) and U18666A-treated cells show a punctate distribution throughout the cell. Similar changes have been shown for annexin 6 overexpressing CHO cells treated with U18666A (22). Hence, annexin 6 appears to be relocalized to punctate endocytic structures in NPC cells in a similar fashion to that previously observed for annexin 2 (15).

**FIG. 7.** NPC cells show an altered distribution of cholesterol (filipin) and annexin 6 to punctate endocytic structures. NPC-containing RA25 cells, NPC1-null CT43 cells, and U18666A (2 µg/ml for 18 h)-treated RA25 cells were fixed and stained with filipin (A) or anti-annexin 6 (B) as described under “Experimental Procedures.” Scale bar 5 µm.
been visualized in the early endocytic pathway, even in normal cells (7). Both early and recycling endosomes as well as the trans-Golgi network are enriched in membrane lipids that are expected to form membrane microdomains (62–64). In contrast, late endosomal and lysosomal membranes are normally unlikely to accumulate microdomains because of the activity of sphingolipid hydrolases (10). In NPC cells, however, the situation may be different because the GSL and sphingosine microdomain content is very high (65). Although the precise localization of these membrane microdomains is a subject for future study, GSLs have been shown to accumulate in endocytic compartments (51, 66).

Apart from the accumulation of microdomains, the mislocalization of GSL may have specific effects on cell physiology. Particular GSLs have also been found to be important in the transport of toxins and viruses (67, 68), and it has been suggested that GSLs are employed for the trafficking of endogenous proteins, perhaps with specific GSL-binding domains (69, 70). Intracellular transport from the plasma membrane to the endoplasmic reticulum by these specific GSL-dependent pathways is also reliant on their ability to form membrane microdomains (71, 72). GSL-dependent trafficking may be particularly sensitive to disruption in NPC and other GSL storage diseases (68). It is still unclear why particular GSLs predominate in NPC, as possibly specific GSL recycling pathways are affected (73). Calcium mobilization is also necessary for endosomal fusion (74), and defective calcium transport may form the basis for related storage disorders such as mucolipidoses type IV (75, 76). Calcium transport has been shown to be influenced by GSLs and their lyso derivatives (77) and is defective in a mouse model of Sandhoff disease, a phenotype corrected by GSL depletion (78). It should also be noted that GSLs have also been implicated in signaling (79, 80), and their accumulation may well disrupt the function of specific signaling processes in NPC either directly or by altering plasma membrane phospholipid saturation (65, 81, 82). The accumulation of GSLs may disrupt cell physiology by more than one mechanism, in a cell type-specific fashion. It has been reported that NPC can be protective against viral infection (83). However, given the severe phenotypes that NPC and most other sphingolipid storage diseases present in the homozygote, identification of heterozygote specific fashion. It has been reported that NPC can be protective against viral infection (83). However, given the severe phenotypes that NPC and most other sphingolipid storage diseases present in the homozygote, identification of heterozygote

It is suggested that the intracellular accumulation of GSL is causally linked to changes in endocytosis in NPC disease, through changes in annexin distribution. How this relates to the disease pathology has not been addressed in this study, and the success in treating NPC1−/− mice with imino sugar inhibitors of GSL synthesis (26) suggests GSLs are important. The current observation of large increases in microdomain components in NPC disease, and presumably other GSL storage diseases, indicates that these diseases may be good models for the role of membrane microdomains in intracellular trafficking.

**REFERENCES**

1. Tillack, T. W., Aliotta, M., Moran, R. E., and Young, W. W., Jr. (1983) J. Cell Biol. 97, 5791–5801.
2. Okada, Y., Mugnai, G., Bremer, E. G., and Hakomori, S. (1984) Exp. Cell Res. 155, 448–456.
3. Simons, K., and van Meer, G. (1988) Biochemistry 27, 6197–6202.
4. Muro, S. (2003) Cell 115, 377–388.
5. Prior, I. A., Muncke, J. P., Parton, R. G., and Hancock, J. F. (2003) J. Cell Biol. 160, 165–170.
6. Schütz, G. J., Kada, G., Pastushenko, V. P., and Schindler, H. (2000) EMBO J. 19, 892–901.
7. Sharma, D. K., Choudhury, A. Singh, R. D., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2003) J. Biol. Chem. 278, 7564–7572.
8. Grunewald, J. (2001) Nat. Rev. Mol. Cell. Biol. 2, 741–750.
9. Chen, C. S., Patterson, M. C., Wheatley, C. L., O'Brien, J. F., and Pagano, R. E. (1999) Lancet 354, 901–905.
10. Simons, K., and Gruenberg, J. (2000) Trends Cell Biol. 10, 459–462.
11. Nix, M., and Stoffel, W. (2000) Cell Death Differ. 7, 413–424.
12. Ostermeyer, A. G., Beckrich, B. T., Ivarson, K. A., Grove, K. E., and Brown, D. A. (1999) J. Biol. Chem. 274, 34459–34466.
13. Lai, E. C. (2003) J. Cell Biol. 162, 305–370.
14. Moss, S. E. (1997) Trends Cell Biol. 7, 87–89.
15. Mayran, N., Parton, R. G., and Gruenberg, J. (2003) EMBO J. 22, 3243–3253.
16. Sargiacomo, M., Sudol, M., Tang, Z. L., and Lisanti, M. P. (1993) J. Biol. Chem. 268, 789–807.
17. Oliferenko, S., Paiha, K., Harder, T., Gerke, V., Schwarzler, C., Schwarz, H., Beug, H., Gunthert, U., and Huber, L. (1999) J. Cell Biol. 146, 843–854.
18. Harder, T., and Varki, A. (1994) Biochim. Biophys. Acta 1223, 375–382.
19. Corvera, S., DiBonaventura, C., and Shpeter, H. S. (2000) J. Cell Biol. 151, 1341–1347.
20. Harder, T., Kellner, R., Parton, R. G., and Gruenberg, J. (1997) Mol. Biol. Cell 8, 533–545.
21. Oso, A., Kumanonoh, H., Yasaka, K., Sokawa, J., Hidaka, H., Sokawa, Y., and Maekawa, S. (2001) J. Neurosci. Res. 64, 235–241.
22. de Diego, I., Schwartz, F., Siegfried, H., Dauterstedt, P., Heeren, J., Beisiegel, U., Enrich, C., and Grewal, T. (2002) J. Biol. Chem. 277, 32187–32194.
23. Pons, M., Grewal, T., Russ, E., Schubert, H., and Enrich, C. (2001) Exp. Cell Res. 269, 13–22.
24. Choudhury, A., Dominguez, F., Puri, V., Sharmah, D. K., Narita, K., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2002) J. Clin. Invest. 109, 1541–1550.
25. Lebrand, C., Corti, M., Goodson, H., Cossen, F., Cavalli, V., Mayran, N., Faure, J., and Gruenberg, J. (2002) EMBO J. 21, 1299–1305.
26. Zervas, M., Somers, K. L., Thrall, M. A., and Walkley, S. U. (2001) Can. J. Biochem. Physiol. 80, 139–149.
27. Mayran, N., Parton, R. G., and Gruenberg, J. (2000) J. Cell Biol. 150, 1639–1649.
28. Lisanti, M. P., Tang, Z., Scherer, P. E., and Sargiacomo, M. (1995) Methods Enzymol. 259, 655–668.
29. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
30. van Echten, G., Iber, H., Stutz, H., Takatsuki, A., and Sandhoff, K. (1990) Eur. J. Cell Biol. 51, 135–139.
31. Miller Podraza, H., Mansson, J. E., and Svennerholm, L. (1992) Can. J. Biochem. Physiol. 70, 87–91.
32. Sillence, D. J., Ruggers, R. J., Neville, D. C. A., Harvey, D. J., and van Meer, G. (2000) J. Lipid Res. 41, 1220–1230.
33. Vance, D. E., and Sweeney, C. C. (1967) J. Lipid Res. 8, 621–630.
34. Hunnam, V., Harvey, D. J., Priestman, D. A., Bateman, R. H., Bordoli, R. S., and Arvedson, E. (2001) J. Am. Soc. Mass Spectrom. 12, 1220–1225.
35. Francey, R. J., and Amador, E. (1998) Clin. Chim. Acta 265, 255–263.
36. Merrill, A. H., Caligan, T. B., Wang, E., Peters, K., and Oj, J. (2000) Methods Enzymol. 312, 3–9.
37. Neville, D. C. A., Coquard, V., Priestman, D. A., te Vruchte, D., Sillence, D. J., Dwek, R. A. P., and Butter, T. D. (2004) Anal. Biochem., in press.
38. Gu, F., and Gruenberg, J. (2000) J. Biol. Chem. 275, 8154–8160.
