Sterol-modulated Glycolipid Sorting Occurs in Niemann-Pick C1 Late Endosomes*

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The Niemann-Pick C1 (NPC1) protein and endocytosed low density lipoprotein (LDL)-derived cholesterol were shown to enrich separate subsets of vesicles containing lysosomal associated membrane protein 2. Localization of Rab7 in the NPC1-containing vesicles and enrichment of lysosomal hydrolases in the cholesterol-containing vesicles confirmed that these organelles were late endosomes and lysosomes, respectively. Lyso-bisphosphatidic acid, a lipid marker of the late endosomal pathway, was found in the cholesterol-enriched lysosomes. Recruitment of NPC1 to Rab7 compartments was stimulated by cellular uptake of cholesterol. The NPC1 compartment was shown to be enriched in glycolipids, and internalization of GalNAcβ1–4[NeuAc2–3]Galβ1–4Glcβ1–1-ceramide (Gβ2) into endocytic vesicles depends on the presence of NPC1 protein. The glycolipid profiles of the NPC1 compartment could be modulated by LDL uptake and accumulation of lysosomal cholesterol. Expression in cells of biologically active NPC1 protein fused to green fluorescent protein revealed rapidly moving and flexible tubular extensions emanating from the NPC1-containing vesicles. We conclude that the NPC1 compartment is a dynamic, sterol-modulated sorting organelle involved in the trafficking of plasma membrane-derived glycolipids as well as plasma membrane and endocytosed LDL cholesterol.

The generation of unesterified cholesterol from endocytically derived cholesterol esters in lysosomes does not itself initiate metabolic responses in cells. This lysosomal pool of cholesterol remains metabolically inert until it has been delivered to other cellular organelles. The metabolic significance and mechanisms of lysosomal cholesterol trafficking have begun to be revealed through the study of Niemann-Pick C (NP-C) disease (1). The prominent cellular feature of this metabolic disorder is the extensive sequestration and accumulation of LDL cholesterol in lysosomes resulting from a defect in the translocation of this sterol pool to other cellular membranes (2–4). The recognition of this genetically induced sterol-trafficking defect introduced the concept of specific protein-mediated egress of cholesterol from lysosomes. This notion was further supported by the subsequent recognition of two separate defective gene loci for NP-C disease (5). The gene most commonly mutated in this disorder (NPC1) was recently cloned, and its sequence predicts a unique multiple membrane-spanning protein of 1278 amino acids (6, 7). Analysis of the primary sequence suggests that the NPC1 protein has several different domains likely to have functional significance. In addition to a signal peptide for endoplasmic reticulum insertion (amino acid residues 1–22), the N terminal of NPC1 contains a region (amino acid residues 55–165) that has been termed the “NPC1 domain” because of its high degree of conservation in a wide array of existing NPC1 orthologs. Downstream of the NPC1 domain are five predicted transmembrane domains (at amino acid residues 615–797) in a region with strong homology to the putative sterol sensing domains of several other proteins, including the sterol response element binding protein cleavage-activating protein, the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and the Hedgehog signaling protein, Patched. The C terminus of NPC1 contains a dileucine motif suggested to be required for the targeting of proteins to lysosomes. The functional significance of these domains was recently confirmed in studies using site-directed mutagenic mapping strategies (8, 9).

Earlier studies demonstrated that the NPC1 protein is located in late endosomes and that the clearance of endocytosed [14C]sucrose as well as cholesterol was defective in NP-C cells (10). These findings suggested that general retroendocytic trafficking and mobilization of multiple lysosomal cargo are defective at a late endosomal trafficking step. This concept provided an explanation for the accumulation of multiple lipids in NP-C cells and tissues (1). The present study characterizes the NPC1 compartment with respect to glycolipid content using antibodies to glycolipids. The glycolipid profile of the NPC1 compartment was found to be modulated by cholesterol. This report also documents an

LIMP, lysosomal integral membrane protein; LBPA, lysobisphosphatidic acid; M6PR, mannose-6-phosphate receptor; apoD, apolipoprotein D; CHO cells, Chinese hamster ovary cells; FITC, fluorescein isothiocyanate; LRSC, lissamine rhodamine B sulfonyl chloride; DiI-LDL, octadecyl (C18) indocarbocyanine; GFP, green fluorescence protein; Cy5, indodicarbocyanine.

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The abbreviations used are: NP-C, Niemann-Pick C disease; NPC1, Niemann-Pick C1 protein; Gβ2, GalNAcβ1–4[NeuAc2–3]Galβ1–4Glcβ1–1-ceramide; Gβ3, NeuAc2–8NeuAc2–3Galβ1–4Glcβ1–1-ceramide; CTH, Galβ1–4Galβ1–4Glcβ1–1-ceramide; LPDS, lipoprotein-deficient bovine serum; GFP, green fluorescent protein; LAMP, lysosomal associated membrane protein;
unusual mode of tubular membrane trafficking for the NPC1 compartment that appears to be linked to the NPC1 protein. Based on these results we discuss the concept of a multiple functional repertoire for the NPC1 compartment that includes the sorting of glycolipids and cholesterol.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc., Logan, UT. Lipoprotein-deficient bovine serum (LPDS) and human low-density lipoprotein (LDL) were prepared by Intracel Corp., Rockville, MD. Glass and plastic chamber slides (Lab-Tek) were purchased from Thomas Scientific. Rabbit polyclonal anti-serum to human NPC1 generated with a conjugated synthetic peptide of residues 1256–1274 was employed as described previously (10). Mouse anti-human LAMP2 and LIMP1 (CD63) antibodies, developed by Dr. Tadashi Tai, Tokyo Metropolitan Institute of Medical Science. Mouse anti-NeuAc$^2$-2–8NeuAc$^b$-2–3Gal$^b$-b-ceramide (G D3) was obtained from Dr. Shutish Patel, Veterans Affairs Medical Center, University of Iowa. Rabbit anti-adaptin (AP-1) and anti-adaptin (AP-2) antibodies were generously provided by Dr. J. T. August, who obtained the adapted form from Sigma Chemical Co. (St. Louis, MO). antibodies to the 300-kDa cation-independent mannose-6-phosphate receptor were gifts from Dr. Suzanne Pfeffer (11), Stanford University, Stanford, CA. Mouse anti-human cathepsin D and mouse anti-Rab7 antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). antibodies to alpha- and beta-Hexosaminidase (a- and b-Hexosaminidase) (IgG) were generously provided by Dr. Richard Proia (13), Genetics and Biochemistry Branch, NIDDK, National Institutes of Health. Rabbit anti-apolipoprotein D (D) was obtained from Dr. R. Bansal, University of Connecticut Health Center, Farmington, CT. The O1 antibody (15) has been shown to recognize lactosylceramide (16), galactosylceramide, and other galactolipids (15). Mouse anti-human LAMP2 and LAMP3 (lysosomal–associated membrane proteins 2 and 3), and LIMP1 (lysosomal integral membrane protein-1) were obtained from Polysciences (Warrington, PA).

Comparison of the NPC1 containing vesicles with the cholesterol enriched vesicles: immunocytochemical and endocytic marker characterization

| NPC1-containing vesicles | Cholesterol-enriched vesicles | Immunocytochemical and endocytic markers of vesicle content |
|--------------------------|-------------------------------|----------------------------------------------------------|
| Positive                 | Positive                      | LAMP2, LAMP3 (lysosomal-associated membrane proteins 2 and 3), and LIMP1 (lysosomal integral membrane protein-1) |
| Positive                 | Negative                      | Rab7 (GTPase)                                           |
| Negative                 | Negative                      | M6PR (mannose-6-phosphate receptor)                     |
| Negative                 | Negative                      | Adaptins 1 and 2                                        |
| Negative                 | Positive                      | ApoD (apolipoprotein D)                                 |
| Positive                 | Positive                      | LBPA (lysobisphosphatidic acid)                         |
| Positive                 | Positive                      | Cathepsin D                                            |
| Positive                 | Positive                      | Dextran                                                 |
| Negative                 | Positive                      | DII-LDL                                                 |
| Positive                 | Negative                      | GM2                                                     |
| Positive                 | Negative                      | Gal-Cer/Lac-Cer                                         |
| Negative                 | Positive                      | G3m                                                    |
| Negative                 | Negative                      | CTH                                                     |
| Positive                 | Negative                      | GD3                                                     |
| Positive                 | Negative                      | Gal-Cer/Lac-Cer                                         |
| Negative                 | Positive                      | Dextran                                                 |
| Negative                 | Positive                      | DII-LDL                                                 |
| Positive                 | Negative                      | GM2                                                     |
| Positive                 | Negative                      | Gal-Cer/Lac-Cer                                         |
| Negative                 | Positive                      | G3m                                                     |
| Negative                 | Negative                      | CTH                                                     |

and 100 units of penicillin/streptomycin/ml in humidified 95% air and 5% CO$_2$ at 37 °C. For immunocytochemical analyses, fibroblasts were seeded at a density of 20,000 cells/well in McCoy's/5% LPDS medium in 9.5-cm glass chamber slides (Nunc, Inc., Naperville, IL) coated with human fibronectin. Immunochemical Analyses—Cells in glass chamber slides were washed in phosphate-buffered saline and fixed in 3% paraformaldehyde for 30 min. Cells were immunocytochemically labeled using an indirect procedure in which all incubations (quench, primary and secondary antibodies, and washes) were performed in blocking solution containing filipin (0.05%) and IgG (2.5 mg/ml) of the secondary antibody species. Primary antibody solutions were used at the following dilutions; NPC1 (1:1000), LAMP2 (1:50), LBPA (1:50), apoD (1:100), M6PR (1:50), Rab7 (1:100), G3m (1:50), O1 anti-Gal-Lac-Cer (1:100), CTH (1:50), and G3m (1:50). Secondary FITC Cy5 and LSRC-labeled antibodies were used at 1:100 dilution. Fluorescence was viewed with a Zeiss 410 laser confocal scanning microscope using an Omnicrone model krypton-argon laser (American Laser System, Salt Lake City, UT) with excitation wavelengths of 488, 633 and 568 nm, for FITC Cy5 and LSRC, respectively. Filipin fluorescence was viewed with an Enterprise model argon laser (Coherent, Santa Clara, CA) with an excitation wavelength of 360 nm.

Galactosylceramide—Normal (ENZ143) and NPC fibroblasts expressing mutated (GM3123) or no detectable (DMNB98.16) NPC1 protein were seeded at a density of 500,000 cells in plastic 100-mm dishes (Costar, Cambridge, MA) and incubated in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine medium, 1% nonessential amino acids, 2 mm glutamine, and 100 units of penicillin/streptomyocin/ml in humidified 95% air and 5% CO$_2$ at 37 °C.

Cells were cultured for 72 h in medium containing 10 µg/ml of N-acetyl-$\beta$-D-glucosaminyl-$\alpha$-D-mannosamine. Gangliosides were isolated according to the method of Fishman (19) and separated on high-performance thin-layer chromatography plates developed in chloroform/methanol/0.25% KCl (v/v/v). The radioactivity of gangliosides identified using authentic standards was determined by liquid scintillation counting.

Plasmid Construction—The mammalian expression vector, pNNE, was constructed by inserting the Nael/BglII fragment (4057 base pairs) from plasmid 1-1 (6) encoding NPC1 into pEGFP-C1 (CLONTECH, Palo Alto, CA) at the BssEI site (filled with dNTP by T4 DNA polymerase) and BglII sites in-frame. The plasmid containing GFP-NPC1 cDNA was sequenced to confirm insertion at the appropriate sites. Another mammalian expression vector, pN-6, was constructed in multiple steps.

Briefly, the fragment encoding NPC1 was obtained by restriction digestion of plasmid 1-1 with EcoRI and was inserted into pSV-SPORT1 (Life Technologies, Gaithersburg, MD) at the EcoRI site. Directionality of this construct, pSN, was confirmed by restriction digestion. A linker encoding 6 histidine residues and a BamHI site was inserted at the 3′-end of NPC1 to replace the stop codon of NPC1. Finally, GFP from pEGFP-N1 (CLONTECH, Palo Alto, CA) was inserted in-frame into the BamHI site in the linker in pSN to create the plasmid pN-6 encoding an NPC1-GFP fusion protein.

Expression of NPC1 in CHO (CT-60) Cells or Null NPC1 Human NPC-C Fibroblasts—Functional expression of the plasmids pNNE and pN-6 was confirmed by complementation of the NPC-C phenotype in both CT-60 NPC-C mutant CHO cells (20) or null NPC1 human NPC-C fibroblasts. CT-60 cells and 93.41 primary skin fibroblasts from an NPC-C patient were cultured to ~70% confluency. Cells were transfected

M. Zhang and S. Patel, unpublished data.

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with pNNE, pNES-6, or the control plasmid pEGFP-C1, using LipofectAMINE 2000 (Life Technologies) according to manufacturer’s instructions. 48–72 h after transfection and incubation in 10% FBS, cells were fixed with 3% paraformaldehyde for 30 min and stained with filipin to assess cholesterol clearance (6, 8). The percentage of cells that expressed the GFP (from pNNE) or NPC1-GFP chimera (from pNES-6) and complemented the NP-C phenotype was determined (9). Expression of NPC1 was confirmed by indirect immunofluorescence using polyclonal antibodies directed against a C-terminal peptide of NPC1 (10).

RESULTS

Cytochemical Identification of the NPC1 Compartment—Earlier immunocytochemical studies showed that the NPC1 protein is located in a unique set of LAMP2-containing vesicles that do not contain mannose 6-phosphate receptor (M6PR) or LDL-derived cholesterol (10). We have now further defined the intracellular distribution of the NPC1 protein in cultured fibroblasts with additional markers of the late endosomal pathway. The data are summarized in Table I and are collected from fibroblasts incubated with LDL for 24 h. As previously reported, NPC1 and cholesterol are located in separate vesicles (Fig. 1, B and G), but both are positive for LAMPs 2 and 3 and LIMP (Table I). The NPC1 protein-containing vesicle contains the late endosomal marker Rab7 (Fig. 1, C and D, and Table I). The NPC1 compartment is not enriched in cathepsin D (Fig. 1, E and F), and NPC1 sparsely localizes as well with α and β-hexosaminidase (Table I). However, both cathepsin D and α- and β-hexosaminidase strongly colocalize with cholesterol-enriched LAMP-positive vesicles (Table I). The distribution of markers among the separate NPC1 and cholesterol-enriched vesicles confirms that the NPC1 protein is normally localized in a late endosomal compartment apart from cholesterol-enriched lysosomes. These two separate subsets of LAMP-positive vesicles of the endocytic pathway, NPC1-positive late endosomes and LDL-cholesterol enriched lysosomes, are present in all normal fibroblasts and most NP-C fibroblast cell lines studied. We have, however, found two NPC cell lines in which both NPC1 and LDL-derived cholesterol locate together in the same LAMP-positive vesicle. Although a single gene is affected by the NPC1 mutation, the protein has multiple domains each of which may be affected and produce a different phenotype.

In fibroblasts, LDL-cholesterol-enriched lysosomes contain also apolipoprotein D (Table I) and lysobisphosphatidic acid, a presumptive marker for the late endosomal compartments (12) (Fig. 2, A and B, and Table I), whereas neither is present in the NPC1 compartment. Adaptins 1 and 2 are not present in either the NPC1 vesicle or the cholesterol-enriched vesicle (Table I). Cellular uptake of the fluid phase marker, fluorescent dextran, by fibroblasts (Table I), and Chinese hamster ovary cells (CHO) enriched both the NPC1 vesicle (Fig. 1, B and G) and the cholesterol-containing (Fig. 2, C and D) compartment. Dil-LDL, an endocytosed marker for lysosomes (22), colocalized with cholesterol-enriched vesicles in both fibroblasts (Table I) and CHO cells (Fig. 2, E and F).

Sterol Modulation in the Cellular Distribution of NPC1—In cholesterol-depleted normal fibroblasts, NPC1 has a diffuse cellular distribution with little discernible vesicular staining (Fig. 3A). During a subsequent period of cellular cholesterol enrichment with LDL, prominent detection of NPC1 protein in endocytic vesicles is now noted (Fig. 3B). This marked alteration in the cytochemical detection of NPC1 appears to reflect changes in the organization and distribution of the protein, because Western blot comparisons of cell extracts from cholesterol-depleted and -enriched cultures did not show any differences in absolute levels (data not shown). Because the NPC1 protein has transmembrane domains, it is likely the diffuse cellular immunofluorescence under conditions of cholesterol depletion represents NPC1’s diffuse distribution in an exten-

FIG. 1. Cytochemical characterization of the NPC1-containing late endosomal compartment. Normal human fibroblasts (A–F) were incubated in McCoy's 5% LPDS medium at 37 °C for 4 days and then incubated in fresh medium containing LDL (50 μg/ml) for 24 h to enrich cellular membranes with sterol. Fibroblasts were immunostained for NPC1 (A, C, E, red), stained with filipin for cholesterol (B, blue), and immunostained for Rab7 (D, green) and cathepsin D (F, green). Confocal microscopy revealed that the NPC1 vesicles (A, arrows) are not enriched with endocytosed cholesterol (B, open arrowheads), and conversely, cholesterol-laden vesicles (B, arrows) do not contain NPC1 protein (A, open arrowheads). NPC1 vesicles (C, red) contain Rab7 (D, green) a marker for late endosomes. Arrows highlight vesicles that show clear colocalization between NPC1 and Rab7 in C and D. NPC1 vesicles (E, arrows) do not contain cathepsin D (F, open arrowheads), and conversely, cathepsin D-containing vesicles (F, arrows) do not contain NPC1 protein (E, open arrowheads). NP-C mutant CT-60 Chinese hamster ovary cells (G, H) transfected with human NPC1-GFP cDNA express fluorescent NPC1-GFP (G, green). These cells were incubated with fluorescent dextran (H, red), and confocal microscopy revealed that the fluorescent NPC1-GFP (G, arrows) is in the same compartment containing endocytosed dextran (H, arrows). Bars for A–H = 5 μm.
Glycolipid Sorting in the NPC1 Compartment

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Fig. 2. Cytocchemical characterization of the cholesterol-containing lysosomal compartment. Normal human fibroblasts (A, B) were incubated in McCoy’s/5% LPDS medium at 37 °C for 4 days and then incubated in fresh medium containing LDL (50 μg/ml) for 24 h to enrich cellular membranes with sterol. Fibroblasts were immunostained for lysocephosphatidic acid (LBPA) (A, green) and cytochemically stained with filipin (B, blue) to reveal the cellular distribution of endocytosed cholesterol. Arrows highlight vesicles that show clear colocalization between LBPA and cholesterol. CT-60 Chinese hamster ovary cells (C–F) were incubated with fluorescent dextran (C, red) and fluorescent DiI-LDL (E, red) and stained for cholesterol-loaded lysosomes (D, F; blue). Arrows highlight vesicles in which endocytosed fluorescent dextran or DiI-LDL colocalize with cholesterol-loaded lysosomes. Bars for A–F = 5 μm.

Fig. 3. Uptake of LDL induces NPC1 protein accumulation in vesicles. Normal human fibroblasts grown in lipid depleted serum (A) or serum supplemented with LDL for 24 h (B). Cells were immunostained for NPC1 protein. Incubation with LDL increases the amount of NPC1 protein (B, red) sequestered in late endosomes. Bar = 25 μm.
The NPC1 protein has proven to be a valuable biological tool in the study of intracellular lipid trafficking. The current report represents an extended immunocytochemical characterization of the positioning of NPC1 within the compartments of the late endosomal pathway as it relates to its role in sterol and glycolipid transport (see Table I for summary).

**Cellular Cholesterol Uptake Enriches the NPC1 Content of Late Endosomes**—Defective retroendocytic clearance of endocytosed [14C]sucrose from NP-C cells predicted a trafficking lesion in an endocytic compartment with the size and turnover rate of late endosomes (10). The present studies confirm the late endosomal nature of the NPC1-enriched compartment. In sterol-deprived cells, the NPC1 protein appears in a cellular dispersed state (Fig. 3A). When cells are enriched with lipoprotein-derived cholesterol, dispersed cellular NPC1 protein relocates to an enriched vacuolar state (Fig. 3B). These NPC1 vesicles contain Rab7 (Fig. 1F) a marker protein for late endosomes said to function in vesicular trafficking to lysosomes (24). Rab7 was not present in cholesterol-laden vesicles (Table I). Rab proteins, belonging to a superfAMILY of low molecular weight GTPase, are known to be crucial for vesicular transport and are found on the membranes of the pairs of organelles that interact during the transport cycle (25). Rabs are recruited onto the donor vesicle membrane where they are thought to direct targeting, docking, and fusion of those vesicles to the recipient organelle. Rab9, which has been suggested to regulate endosomal traffic to the trans-Golgi network (26), has also been reported to be associated with the NPC1-containing vesicle (27).

The location of both Rab7 and Rab9 in NPC1-enriched vesicles suggests this compartment is a late endosome having the potential to shuttle cholesterol and perhaps other endosomal products to lysosomes and Golgi complex. In this regard, it has been shown that NPC1 mutations disrupt the flow of LDL-derived cholesterol through both lysosomes and the trans-Golgi network (28–29). The mechanism of the NPC1-mediated transport between these organelles remains to be elucidated.

**Mannose-6-phosphate receptor (M6PR)** has been described in many cell types as a marker protein for prelysosomal and late-endocytic vesicles (30). M6PR's deliver newly synthesized acid hydrolases to the endocytic pathway and direct their return to the Golgi (11). However, in human fibroblasts we did not find M6PR in the NPC1-containing vesicles that we consider to be late endosomes (10). In other cells, including Hep-2 cells, M6PR was also not visualized in late endosomes at steady states and most of the receptor was found within the trans-Golgi network and in vacuolar structures in the peripheral cytoplasm (31). We found comparable locations for M6PR in normal fibroblasts (Fig. 4 in Ref. 10), suggesting that the M6PR does not become enriched in the late endocytic pathway. Lack of accumulation of the M6PR in late endosomes could reflect a rapid dissociation and egress of the receptor from the late endosomes. Thus, a stable M6PR-rich compartment, equivalent to the late endosome stage of the endocytic pathway, may not exist in human fibroblasts under the employed culture conditions thus reflecting the finding that the NPC1 vesicles are not M6PR-positive.

**LDL Uptake Enriches the Cholesterol Content of Lysosomes**—We examined the cytochemical distribution of the lysosomal enzymes, cathepsin D (24) and α- and β-hexosaminidases (13), with specific reference to the distinct LAMP/NPC1-positive and LAMP/cholesterol-containing compartments. Cathepsin D is considered to reside primarily in lysosomes, although precursor enzyme travels from the Golgi to late en-

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**Fig. 4.** Glycolipids are present in NPC1-containing vesicles in fibroblasts. Normal human fibroblasts grown in bovine serum were immunostained for NPC1 protein (A, C, E, G; red panels) and glycolipids (B, D, F, H; green panels). Glycolipids and NPC1 colocalize. Arrows highlight vesicles that show clear colocalization between NPC1 and Ga32 (A and B), NPC1 and Gal-Cer/Lac-Cer (C and D), NPC1 and Ga32 (E and F), and NPC1 and CTH (G and H). Bars = 5 μm.

tied of cholesterol (data not shown).

The cellular profile of Ga32 distribution in these transfected CT-60 cells differs entirely (Fig. 8). In cells that were not transfected (Fig. 8A), Ga32 was found in lysosomes filled with cholesterol (Fig. 8B). In cells in which NPC1-GFP protein still largely resides in the limiting membranes of lysosomes (Fig. 8C), Ga32 has now been cleared from the vesicles (Fig. 8D–F). In transfected CT-60 CHO cells cleared of cholesterol, where NPC1-GFP protein was distributed into the interior of endocytic vesicles (Fig. 8G), Ga32 has now been cleared from the vesicles (Fig. 8H). The extensions associated with NPC1-GFP vesicles, noted in Fig. 8G have been monitored in viable cells by time-lapse confocal microscopy at enhanced detection and have been found to represent flexible and kinetically mobile tubules that extend from the GFP-positive vesicles (data not shown).

**DISCUSSION**
dosomes en route to lysosomes (24). Cathepsin D was found infrequently in NPC1-containing vesicles (Fig. 1) but was consistently associated with LAMP/cholesterol-containing vesicles (Table I). In addition, α- and β-hexosaminidases were also infrequently associated with NPC1-positive vesicles and were consistently found in LAMP/cholesterol-containing vesicles (Table I). These distribution patterns of marker enzymes support previous reports (10, 27) that the endosomal compartment enriched with cholesterol during active LDL uptake is primarily lysosomes.

These cholesterol-enriched lysosomes contain lysobisphosphatidic acid (LBPA) (Fig. 2A). This particular marker is not located in NPC1 vesicles. LBPA was reported in baby hamster kidney (BHK) cells to be a lipid marker of late endosomal vesicles displaying the morphology of multivesicular bodies, which contain Rab7 (32). LBPA was later shown to reside in the cholesterol-laden vesicles in a particular NP-C mutant fibroblast cell line and in normal cells treated with a drug that blocks lysosomal cholesterol transport (12). Because these vesicles also contained M6P, they were considered to represent late endosomes (12). In contrast, the LBPA/cholesterol-containing vesicles we identified were not of the multivesicular form as

**Fig. 5.** Cellular uptake of LDL-cholesterol modulates the glycolipid profile of the NPC1 compartment. Cholesterol-depleted normal human fibroblasts grown in LDL (50 μg/ml) for 24 h were immunostained for NPC1 (A; red), gangliosides G₃M₂ (B; green), and G₃D₃ (D; green) and stained with filipin (C, D; blue) for cholesterol localization. Arrows highlight clear colocalization of NPC1 (A; red) with G₃M₂ (B; green). The merged image (C) shows that G₃M₂ (green) does not colocalize with cholesterol (blue). The color bar shows green, aqua, and blue to aqua is a merge of the green and blue, indicating colocalization. The merged image (D) shows that G₃D₃ colocalizes with cholesterol as indicated by the aqua color. Bars = 5 μm.

**Fig. 6.** NPC1 cDNA transfection of NPC1 null-mutant fibroblasts restores G₃M₂ to endocytic vesicles. NPC null mutant human fibroblasts, either nontransfected or transfected with GFP-NPC1 cDNA, were immunostained for NPC1 (A, C; red) and G₃M₂ (B, D; green). Nontransfected, NPC1 null mutant fibroblasts contained no specific staining for NPC1 (A; lack of red), and staining for G₃M₂ (B; green) appeared limited to the cell surface. Transfected cells showed clear colocalization between immunostained NPC1 vesicles (C; red) and internalized G₃M₂ (D; green), highlighted by arrows. Expression of NPC1 in cells was determined by the presence of GFP fluorescence in the cytosol and nucleus. Bars = 5 μm.
Table II
Ganglioside synthesis in normal and NP-C fibroblasts

|                | GM₁ | GM₂ | GM₃ |
|----------------|-----|-----|-----|
| **WT NPC1**    | 279 ± 145 | 620 ± 332 | 12,477 ± 5,830 |
| **Mutant NPC1**| 236 ± 18 | 427 ± 95 | 10,063 ± 1,237 |
| **Null NPC1**  | 462 ± 151 | 919 ± 398 | 6,209 ± 1,026 |

Table II shows the ganglioside synthesis in normal and NP-C fibroblasts. Normal and NP-C human skin fibroblasts were incubated with 10 μCi of N-acety[-d-3H]mannosamine for 72 h to label cellular pools of gangliosides and then lipids were extracted and analyzed as described under "Experimental Procedures."

The cholesterol-enriched lysosomes were immunocytochemically shown to also contain apolipoprotein D (Table I). Apolipoprotein D is a member of the lipocalin family of transport proteins whose roles are thought to include the binding and transport of small hydrophobic ligands such as progesterone and cholesterol (35). Although not previously shown to be a marker of cholesterol-laden lysosomes, apolipoprotein D levels and its metabolism are severely affected in NP-C disease (14).

The NPC1 Compartment Is a Sterol-sensitive Sorting Vesicle in the Endocytic Trafficking of Glycolipids—When fibroblasts are cultured in fetal bovine serum, NPC1-enriched vesicles were found to contain numerous glycolipids. Four different types of glycolipids (C₁₈:1, Gal-Cer/Lac-Cer, G₃₃, and G₄₃) were shown immunocytochemically to colocalize in NPC1/LAMP/Rab7-positive vesicles when normal fibroblasts are maintained in complete fetal bovine serum (Fig. 4). When cells are induced to endocytically process a large bolus of LDL-derived cholesterol, G₃₃ (Fig. 5) and Gal-Cer/Lac-Cer (data not shown) remain in the NPC1-positive vesicles but G₄₃ (Fig. 5) and CTH (data not shown) are redistributed to cholesterol-enriched lysosomes.

In those NP-C lines where NPC1 and cholesterol remain segregated in their respective LAMP-positive vesicles, LDL-induced vesicular glycolipid sorting was comparable to that noted in normal cells (data not shown). However, in human mutant NPC1 fibroblast cells expressing no NPC1 protein, endocytic accumulation of G₃₃ (Fig. 6) or Gal-Cer/Lac-Cer (data not shown) is absent. These null mutant NPC1 cells were not defective in their ability to synthesize such glycolipids (Table I), but the distribution of these lipids was predominately limited to the plasma membrane (Fig. 6). In these cells LDL uptake induced G₄₃ and CTH to internalize into cholesterol-laden lysosomes (data not shown). Transfection of the null mutant human fibroblasts with wild type NPC1 cDNA not only corrects the sterol transport lesion, but the defect in glycolipid sorting as well evidenced by the reappearance of G₃₃ in NPC1-containing vesicles (Fig. 6).

Similarly, in CT-60 NP-C CHO cells, G₃₃ does not accumulate endocytically and G₄₃ comes to be stored in cholesterol-loaded lysosomes (Figs. 7 and 8). Transfection of these cells with NPC1 cDNA also clears lysosomal sterol and promotes internalization of G₃₃ into NPC1-positive late endosomes and clears G₄₃ from lysosomes. Taken together, these studies reveal that normal endocytic trafficking and metabolism of glycolipids depends upon a functional NPC1 late endosomal compartment.

Tubulation as a Mechanism of NPC1 Vesicular Membrane Exchange—The budding, fission, and fusion of limiting membranes has defined the major mechanism by which vesicular trafficking occurs within the endocytic pathway (21). Unexpectedly, an additional mode of membrane communication was observed when CT-60 NP-C cells were biologically corrected by transfection with NPC1-GFP cDNA. Relatively short, flexible, and mobile NPC1-GFP (+) tubules were noted to emanate from and retract to NPC1-enriched vesicles (Fig. 8G). The potential biological importance of this mechanism in membrane/lipid transfer is underscored by the finding that drug-induced blocks in lysosomal cholesterol egress as well as targeted mutational alterations of NPC1 function suppress the morphology and kinetic activity of these tubules (data not shown). The physiological relevance of these tubular extensions in the transfer of cholesterol and glycolipids by the NPC1 protein is currently under active investigation.

Conclusion—Based on the results of the current studies we propose that the NPC1 compartment serves as a sorting station in the endocytic trafficking of both cholesterol and glycolipids. We suggest that enriching the cholesterol content of lysosomes recruits the NPC1 protein into endocytic vesicles containing glycolipids that are in transit from the plasma membrane. Further characterization of the topological relations of the glycolipid and cholesterol pools at the plasma membrane will need to be carried out in future studies. In the presence of elevated cholesterol levels, certain glycolipids (galactolipids and G₃₃, but not CTH and G₄₃) are restricted from entering the lysosomal compartment for degradation and are efficiently recycled in NPC1-sorting vesicles to the plasma membrane accompanied by cholesterol leaving the lysosomes. This NPC1 compartment-mediated recapture of glycolipids could serve to stabilize the ratio of glycolipids/cholesterol concentrations in the plasma membrane during LDL processing. Niemann-Pick C disease could, therefore, be envisioned as primarily a vesicular trafficking defect producing a disruption of glycolipid as well as cholesterol trafficking. In this regard, it is interesting to note that glycolipids that accumulate in NP-C cells and tissues, such as galactolipids and G₃₃ (1) are those sorted through the NPC1
FIG. 7. NPC1 cDNA transfection of CT-60 mutant NP-C CHO cells restores GM₃ in endocytic vesicles. CT-60 CHO cells were transfected with NPC1-GFP cDNA as described under “Experimental Procedures.” In transfected cell that have not yet cleared of cholesterol (A, and inset), NPC1-GFP appears as rings (A and inset; green), at the surface of cholesterol laden lysosomes (inset; blue). These cholesterol-laden cells do not contain intracellular GM₃ (B; no red immunostaining). In transfected cells that have cleared cholesterol from lysosomes, NPC1-GFP is in the lumen (C; green) of vesicles that now also contain GM₃ (D; red). Arrows highlight vesicles that show clear colocalization between NPC1 and GM₃ (C and D). Bars = 5 μm.

FIG. 8. NPC1 cDNA transfection of CT-60 mutant NP-C CHO cells causes clearing of cholesterol and GM₃ from lysosomes. CT-60 CHO cells were transfected with NPC1-GFP cDNA. Nontransfected cells (A; lack of green fluorescence) contain intracellular GM₃ (B; red). In transfected CT-60 CHO cells that have not yet cleared of cholesterol, NPC1-GFP appears as rings (C; green) at the surface of lysosomes and intracellular GM₃ (D; red) is present in the lysosomes. A highly magnified, merged image (E) shows NPC1-GFP present at the periphery of cholesterol-laden lysosomes (NPC1 appears as green rings around a blue filipin-stained core). A second, highly magnified merged image (F) shows the relationship between NPC1-GFP (F; green) and GM₃ (F; red). NPC1-GFP present at the periphery of lysosomes appears as green rings around a core containing red immunostained GM₃. Transfected CT-60 CHO cells (G and H), which have been cleared of lysosomal cholesterol, contain vesicular and tubular NPC1-GFP compartments (G; green) and no intracellular GM₃. Arrowheads in G and H mark the boundary of the transfected cell. Bars = 2.5 μm.
Glycolipid Sorting in the NPC1 Compartment

compartment, whereas nonaccumulating glycolipids such as CTH and GD3 are shown to traffic on to the lysosomes for probable degradation. Cholesterol-mediated sorting of the NPC1 compartment may suggest that G_{54} and galactolipids have higher affinity for membranes containing NPC1, whereas CTH and G_{53} have greater affinity for membranes enriched in cholesterol.

However, the specific manner in which the NPC1 protein affects glycolipid trafficking remains to be established. The possibilities of either a direct NPC1 interaction with these lipids, or alternatively, an indirect intervention related to the ability of this protein to modulate cholesterol trafficking through the late endosomal compartments are currently attractive.

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