The Niemann-Pick C1 Protein Resides in a Vesicular Compartment Linked to Retrograde Transport of Multiple Lysosomal Cargo*

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Niemann-Pick C disease (NP-C) is a neurovisceral lysosomal storage disorder. A variety of studies have highlighted defective sterol trafficking from lysosomes in NP-C cells. However, the heterogeneous nature of additional accumulating metabolites suggests that the cellular lesion may involve a more generalized block in retrograde lysosomal trafficking.

Immunocytochemical studies in fibroblasts reveal that the NPC1 gene product resides in a novel set of lysosome-associated membrane protein-2 (LAMP2)(+)/mannose 6-phosphate receptor(−) vesicles that can be distinguished from cholesterol-enriched LAMP2(+) lysosomes. Drugs that block sterol transport out of lysosomes also redistribute NPC1 to cholesterol-laden lysosomes. Sterol relocation from lysosomes in cultured human fibroblasts can be blocked at 21 °C, consistent with vesicle-mediated transfer. These findings suggest that NPC1 vesicles may transiently interact with lysosomes to facilitate sterol relocation.

Independent of defective sterol trafficking, NP-C fibroblasts are also deficient in vesicle-mediated clearance of endocytosed [14C]sucrose. Compartmental modeling of the observed [14C]sucrose clearance data targets the trafficking defect caused by mutations in NPC1 to an endocytic compartment proximal to lysosomes. Low density lipoprotein uptake by normal cells retards retrograde transport of [14]Csucrose through this same kinetic compartment, further suggesting that it may contain the sterol-sensing NPC1 protein.

We conclude that a distinctive organelle containing NPC1 mediates retrograde lysosomal transport of endocytosed cargo that is not restricted to sterol.

Niemann-Pick C disease (NP-C)1 is an autosomal-recessive, fatal, neurodegenerative lysosomal storage disorder (1). Human NPC1 (2) and its ortholog in the mouse (3) have recently been identified by positional and complementation cloning. NPC1 is predicted to be a polytopic membrane-spanning protein with a domain of striking homology to the putative sterol-sensing regions of other proteins that are essential for managing cellular cholesterol homeostasis (2, 4). NPC1 also has a terminal di-leucine motif that targets proteins to late endocytic compartments (2).

The most recognized cellular lesion in NP-C cells is impaired lysosomal relocation of endocytosed low density lipoprotein (LDL)-derived cholesterol to other intracellular sites, such as the plasma membrane and endoplasmic reticulum (5–7). Cholesterol accumulates in lysosomes as well as the Golgi apparatus (8, 9). Induction of cholesterol-mediated homeostatic responses is proposed to be delayed because of deficient enrichment of regulatory sterol pools in the endoplasmic reticulum (10, 11).

NP-C is also characterized by lysosomal accumulation of other metabolites. Phospholipids, glycosphingolipids, sphingoid bases, cystine, and glycoproteins, as well as cholesterol, are stored in a tissue-specific pattern (1, 12). To date, it has been assumed that the transport defect in NP-C cells is specific to cholesterol and that other metabolites accumulate secondarily to cholesterol. However, in the brain, where functional impairment is progressive and ultimately fatal, massive accumulation of glycolipids occurs, with little, if any, excessive cholesterol storage (1, 12). Extensive glycolipid storage in fetal human NP-C liver further suggests that this storage is an early event in the development of the disorder (12). These glycolipid storage anomalies do not readily integrate into a unified pathogenic mechanism involving a primary defect in sterol trafficking. The possibility that the NP-C lesion might alternatively reflect a more generalized defect in trafficking out of lysosomes has not been previously explored.

Here we provide evidence that sterol transport from lysosomes is vesicle-mediated, as suggested by a previous report (13). We show that NPC1 resides in a novel set of vesicles that

GalNAcβ1–4(NeuAcrα2–3)Galβ1–4Glcα1–Cer: LAMP, lysosome-associated membrane protein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; MPR, mannose 6-phosphate receptor; NPC1, Niemann-Pick C1 protein; LRSC, lissamine rhodamine sulfonyl chloride.
NPC1 Vesicle Mediates Lysosomal Retroendocytosis

Experimental Procedures

Materials—Fetal bovine serum was obtained from HyClone Laboratories, Inc., Logan, UT. Lipoprotein-deficient bovine serum (LPDS) and human LDL were prepared by Intracel Corp. (Rockville, MD). Glass and plastic microculture wells (Lab-Tek) were purchased from Thomas Scientific. [14C]Sucrose (442 mCi/mM) was purchased from NEN Life Science Products. Filipin was purchased from Polysciences (Warrington, PA). Progesterone, purchased from Sigma, and U18666A (3β-2(diethylamino)ethoxy)androst-5-en-17-one), generously supplied by Dr. W. Andrus (The Upjohn Co.), were stored as 1 and 10 mg/ml stock solutions, respectively, in ethanol at −20 °C. Mouse anti-human lysosome-associated membrane protein-2 (LAMP2) and lysosomal integral membrane protein I antibodies, developed by Dr. J. T. August, were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Monoclonal anti-γ-adaptin (AP-1) and anti-α-adaptin (AP-2) antibodies were obtained from Sigma. Mouse anti-human cathepsin D antibodies were obtained from Chemicon (Temecula, CA). Antibodies to the 300-kDa catenin-independent mannose 6-phosphate receptor (MRP) were a gift of Dr. Suzanne Pfeiffer. Fluorescein isothiocyanate- and LRSC-labeled secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Tissue Culture—Normal and NP-C fibroblasts were derived from volunteers and confirmed patients of the Developmental and Metabolic Neurology Branch under the guidelines approved by the NINDS Institutional Review Board. Five different normal cell lines (ENZ123, ENZ125, ENZ143, GM5656, and GM1652) and three NP-C cell lines (GM3123, DMN92.31, and DMN87.57) were used. A null mutant NP-C cell line (DMM89.16), negative for NPC1 mRNA and protein expression,2 was used solely in immunocytochemical studies. Fibroblasts were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine, and 100 units of penicillin/streptomycin/ml in humidified 95% air and 5% CO2 at 37 °C. For biochemical analyses, fibroblasts seeded at a density of 80,000 cells/well in plastic 6-well dishes (Costar, Cambridge, MA) were incubated for 5–7 days in McCoy's medium with 5% LPDS supplemented as above. 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 microscope wells (Nunc, Inc., Naperville, IL) coated with human fibronectin.

Biochemical Measurement of Fluid-phase Endocytosis—Fluid-phase endocytosis was measured using [14C]sucrose as described (14–16). Briefly, cells were washed once with McCoy's/5% LPDS medium and then incubated for 3 h at 37 °C in prewarmed medium containing 5 μCi of [14C]sucrose (specific activity, 11,000 dpm/μmol). The labeling medium was removed, and the cells were washed six times with ice-cold serum-free McCoy's medium. For reflux experiments, cells were recultured in medium at 37 °C. At specified times, the medium was transferred to tubes and centrifuged at 4 °C for 5 min at 1500 rpm (to pellet any cells present). Cell monolayers were washed with cold serum-free McCoy's medium and then solubilized in 1% SDS for 30 min. The cell protein was determined by the method of Lowry et al. (17). [14C]Sucrose activity was assayed by counting aliquots of the medium and the solubilized cell monolayers in a scintillation counter (Tri-Carb, model 4000; Packard Instrument Co., Downer's Grove, IL). The percentage of [14C]sucrose in cells = (cell-associated [14C]sucrose)/cell-associated [14C]sucrose + medium [14C]sucrose) × 100.

Kinetic Data Analysis—[14C]Sucrose data were analyzed using WinSAAM, the Windows version of the SAAM (Simulation, Analysis and Modeling) program (18–20) and a four-compartment model. Compartments 1–3 represent intracellular pools of [14C]sucrose that turn over at distinct rates; compartment 4 represents the culture medium. This is a modified version of the model proposed by Blomhoff et al. (21), which contained compartments that turned over in 10, 18, and 330 min and were considered to represent early, late and terminal (lysosomal) endocytic compartments, respectively. Because only a few sampling times were available before 30 min, we retained the first compartment in the model but fixed its turnover to be 10 min. Loss of tracer from compartment 4 is directed to the medium, with no assumptions made about the pathway of delivery involved. Based on our immunocytochemical findings (see under “Results”), we directed loss from compartment 3 (lysosomes) back into compartment 2 (late endosomes) rather than into the medium.

Results

NPC1 Protein Resides in a Distinct Vesicular Compartment—We established the intracellular localization of NPC1 in cultured human fibroblasts using antibodies against three distinct 19-amino acid peptide sequences of the protein (Fig. 1). Although all three antisera immunostained similar structures, NPC1-C antisera was routinely used because it provided the brightest staining. Wild type and some mutated forms of NPC1

2 E. B. Neufeld, M. Wastney, S. Patel, S. Suresh, A. M. Cooney, N. K. Dwyer, C. F. Roff, K. Ohno, J. A. Morris, E. D. Carstea, J. P. Incardona, J. F. Strauss III, M. T. Vanier, M. C. Patterson, R. O. Brady, P. G. Pentchev, and E. J. Blanche-Mackie, unpublished data.

3 S. Patel, manuscript in preparation.
localized in granular structures (Fig. 2, A and B). NPC1 immunostaining is specific because it is blocked by co-incubation with NPC1-C peptide and is absent in “null mutant” NP-C fibroblasts that do not express NPC1 (Fig. 2C).

To further characterize the cellular structures containing NPC1, we compared its distribution to LAMP2, a marker for both late endosomes and lysosomes (22), in normal fibroblasts enriched with LDL cholesterol (Fig. 3). NPC1 extensively colocalizes with LAMP2 (Fig. 3A). However, NPC1 is found in some, but not all, LAMP2(+) vesicles. Thus, it appears that a subset of LAMP2(+) vesicles contains NPC1. NPC1(+) vesicles are not enriched by either endocytosed LDL-derived cholesterol (Fig. 3B), a lysosomal marker (8), or endocytosed DiIC16(3), a lipophilic fluorescent dye (23) that colocalizes with endocytosed LDL-derived cholesterol (data not shown). NPC1(+) vesicles in some NP-C fibroblast lines (such as GM3123) also do not become enriched with endocytosed cholesterol and can be clearly distinguished from cholesterol-laden LAMP2(+) lysosomes typical of such mutant cells (data not shown). NPC1(+) vesicles are also marked by antibodies to lysosomal integral membrane protein I, but not by antibodies to adaptins and cathepsin (data not shown). MPR, a specific marker for late endosomes (24), does not localize in NPC1(+) vesicles (Fig. 4). Thus, NPC1 appears to reside in a novel NPC1(+) /LAMP2(+) vesicle that is neither a MPR(+) late endosome nor a cholesterol-enriched lysosome.

**Drugs That Block Transport of Cholesterol out of Lysosomes Induce Lyosomal Sequestration of NPC1 Protein**—Because the documented mutations of NPC1 indicate that it plays a critical role in transporting endocytosed cholesterol from lysosomes, one might expect that this protein can interact with cholesterol-enriched lysosomes. We tested whether drugs such as U18666A (25) and progesterone (26), which block cholesterol transport out of lysosomes, might also alter the cellular distribution of NPC1. Cytocchemical analysis reveals that in addition to the sequestration of cholesterol in lysosomes (25, 26), U18666A (Fig. 5) and progesterone (not shown) also trap the majority of NPC1 protein within these same organelles. When progesterone is washed out of cells at 37 °C, to re-establish cholesterol transport (26), distinct NPC1(+) /LAMP2(+) and NPC1(-) /LAMP2(-) vesicles cleared of cholesterol are formed (data not shown). These observations suggest that a normally transient interaction of NPC1 with cholesterol-laden lysosomes is required to relocate cholesterol to other cellular sites.

**Cholesterol Transport From Lysosomes Is Temperature-dependent**—The localization of NPC1 to LAMP2(+) vesicles and its drug-induced relocation to cholesterol-filled lysosomes suggest that sterol transport from lysosomes is vesicle-mediated.

To further evaluate this potential mode of trafficking, progesterone was again used to reversibly accumulate LDL-derived cholesterol in lysosomes (7, 26). During the subsequent progesterone washout phase, the temperature was reduced to determine whether lysosomal cholesterol relocation could be blocked at temperatures that perturb vesicular trafficking (27). Cytocchemical analysis reveals that the pool of cholesterol sequestered in lysosomes (Fig. 6A) was readily mobilized during progesterone washout at 37 °C (Fig. 6B) but not at 21 °C (Fig. 6C). In similar experiments, the relocation of lysosomal [3H]cholesterol to the plasma membrane was also blocked below 21 °C (Fig. 7). Taken together, these results suggest that NPC1 participates in a vesicle-mediated transport of cholesterol from lysosomes.

**Clearance of Endocytosed [14C]Sucrose Is Impaired in NP-C Fibroblasts**—Because a vesicle-mediated mechanism of sterol transport from lysosomes allows potential cotransport of addi-
ional lysosomal cargo, we examined whether NP-C cells are also defective in clearing other endocytosed material. [14C]Sucrose, a fluid-phase marker for vesicular transport (14–16), was used to monitor endocytic trafficking. We tested whether mutated NPC1 alters vesicular transport in the absence of lysosomal cholesterol storage. Lysosomes of normal and NP-C fibroblasts were cleared of sterol by extended incubation in lipoprotein-deficient medium prior to loading with [14C]sucrose (8) and then were maintained in such medium during subsequent [14C]sucrose loading and clearance. As illustrated in Fig. 8, clearance of endocytosed [14C]sucrose was delayed in all NP-C cell lines examined. The difference in clearance was apparent at 30 min and was maintained thereafter.

Compartmental Modeling Predicts That NP-C Fibroblasts Are Defective in Clearance of [14C]Sucrose from a Late Endocytic Compartment—In order to identify the cellular compartments through which sucrose transport might be altered by a nonfunctional NPC1 protein, we constructed a compartmental model for the endocytic uptake and release of tracer [14C]sucrose in normal and NP-C fibroblasts (Fig. 9). In normal fibroblasts, the relative accumulation of endocytosed cargo (Table I) and the calculated turnover times of compartments 1, 2, and, 3 (10 min, 1 h, and ≥24 h, respectively) correspond to previously reported values for early endocytic, late endocytic, and lysosomal compartments (15, 16, 21, 28).

In NP-C fibroblasts, the calculated rates of [14C]sucrose efflux from intracellular compartments 1 and 3 were identical to those for normal cells, but the rate from compartment 2 was...
retarded (47% of normal, as shown in Fig. 9 for egress data displayed in Fig. 8). Release from compartment 2 into the medium was significantly lower in NP-C cells compared with normal cells (0.71 ± 0.23/h versus 0.44 ± 0.17/h (mean ± S.D.), respectively; p < 0.01 for eight paired experiments). Thus, the rate of transport from compartment 2 into the medium is nearly one-half the rate seen in normal cells (62.6 ± 14.4%; n = 8). Uptake into compartment 1 from the medium was 3.63E-5 ± 0.8E-5/h in normal versus 4.35E-5 ± 0.9E-5/h in NP-C fibroblasts (n = 8). Movement into compartment 3 from compartment 2 (0.54 ± 0.16/h) and release from compartment 3 (0.05 ± 0.01/h) were the same in both normal and NP-C cells. The modeling reveals that the impaired movement from late endosomes back into the medium accounts for the observed delayed clearance of [14C]sucrose from NP-C cells (as depicted in Fig. 8).

The compartmental modeling also predicts the NP-C phenotype, namely, excessive lysosomal storage of endocytosed cargo (see Table I). Most (88%) of the excess endocytosed cargo retained in the three NP-C fibroblast lines studied is predicted to accumulate in compartment 3 (lysosomes). The [14C]sucrose mass predicted to accumulate in lysosomes of NP-C cells is about 2-fold greater than normal cells, consistent with the previously reported 2-fold increase in the fluid-phase volume (29, 30) and endocytosed sterol content (6, 7, 10) of NP-C lysosomes.

**Cellular Cholesterol Enrichment Retards Clearance of Endocytosed [14C]Sucrose from Normal Fibroblasts**—We explored the possibility that endocytosed cholesterol is not only a cargo of the NPC1-mediated retrograde lysosomal transport pathway but may also modulate this pathway through the sterol-sensing domain of the NPC1 protein. Lipoprotein-depleted normal fibroblasts were incubated in the absence or presence of LDL during both the endocytic uptake and subsequent clearance of [14C]sucrose. As seen in Fig. 10, cellular sterol enrichment significantly retarded [14C]sucrose clearance in normal fibroblasts.

In order to identify the cellular compartments potentially targeted by this cellular sterol enrichment, we applied the same compartmental model (Fig. 9). Sterol enrichment significantly reduced [14C]sucrose release from compartment 2 back into the medium (0.71 ± 0.23/h versus 0.57 ± 0.21/h, p < 0.05; for −/+LDL versus (+)LDL, respectively) in three different normal fibroblast lines. Consistent with a recent study (31), cellular sterol enrichment did not alter the rate of trafficking of bulk flow markers between early endocytic compartments and the cell surface.

**DISCUSSION**

The well documented lesions in lysosomal sterol processing that occur in Niemann-Pick C disease have established a role for NPC1 in sterol transport out of lysosomes. The identification of putative sterol-sensing domains in the recently cloned NPC1 gene further links this protein to cellular sterol processing (2). Our present studies reveal that in cultured human fibroblasts, NPC1 identifies a novel vesicle that appears to interact with cholesterol-filled lysosomes. We provide evidence that NPC1 mediates a vesicular form of transport out of lysosomes and that the cargo transported along this pathway is not restricted to sterol.

We have shown that NPC1 protein (Fig. 2) resides in a novel set of vesicles that are LAMP2(+) (Fig. 3) and MPR(−) (Fig. 4) and that do not accumulate endocytosed lysosomal markers (Fig. 3). The ganglioside GM3 exclusively localizes to NPC1(+) vesicles and not to lysosomes containing endocytosed cholesterol, confirming their unique identity.4 We found that drugs that block sterol transport out of lysosomes (U18666A and progesterone) also trap NPC1 in cholesterol-laden lysosomes (Fig. 5). Sterol relocation from lysosomes can be blocked by low temperature (Figs. 6 and 7) and drugs (13) that affect vesicular trafficking. Taken together, these findings suggest that NPC1 vesicles transiently interact with lysosomes to transfer sterol to other cellular sites. Definitive characterization of the novel NPC1(+) vesicular compartment as well as other organelles that may participate in retrograde lysosomal transport is cur-

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4 E. J. Blanchette-Mackie, manuscript in preparation.
Several independent lines of research have provided evidence for retrograde lysosomal transport. Lysosomal membrane proteins appear to continuously cycle between lysosomes and the plasma membrane via endosomal compartments (32–39). Endocytosed fluid-phase cargo also transfers from lysosomes back to late endocytic compartments (40, 41). Physical interactions between MPR\(^{1}\)-late endosomes and lysosomes that allow content mixing have been reported (41–46). The NPC1\(^{1}\) cholesterol-laden lysosome induced by drugs in the current studies (Fig. 5) could represent a hybrid organelle analogous to that which forms from the fusion of a late endosome with a lysosome (44, 45). Drugs that retard cholesterol transport from lysosomes may allow fusion of NPC1 vesicles with lysosomes but block their subsequent fission. The observed reappearance of NPC1 vesicles as well as presumed lysosomes cleared of sterol after removal of progesterone from cell cultures (data not shown) is consistent with a reversible mechanism of drug interdiction involving blocked fission of a hybrid organelle.

NP-C fibroblasts were found to be defective in vesicle-mediated retrograde transport of \[^{14}\text{C}\]sucrose in the absence of lysosomal cholesterol storage (Fig. 8). Compartmental modeling was used to identify the kinetic compartment associated with this intracellular trafficking defect. The modeling targets the defect in retrograde transport to a late endocytic compartment (Fig. 9) and predicts that this defect is primarily responsible for the lysosomal storage (Table I) seen in NP-C cells. The extent of the predicted defect in the rate of \[^{14}\text{C}\]sucrose transport from the late endocytic compartment to the cell surface is comparable to the reported defect in endocytosed sterol transport from lysosomes to the cell surface in NP-C fibroblasts (approximately 50%) (6, 7). The kinetically defined late endo-

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**FIG. 6.** Cytochemical assessment of lysosomal clearance of LDL-derived cholesterol at 21 °C. Normal human fibroblasts were incubated in McCoy’s/5% LPDS medium at 37 °C for 4 days to deplete sterols from cellular membranes. A, cells were then incubated in fresh medium containing LDL (50 μg/ml) and progesterone (10 μg/ml) for 24 h to load lysosomes with cholesterol. A portion of the cultures was washed in phosphate-buffered saline and then incubated in McCoy’s/5% LPDS medium for 24 h at either 37 °C (B) or 21 °C (C). Confocal microscopy revealed abundant perinuclear lysosomal filipin cholesterol fluorescence in cells following endocytic LDL loading (A), which cleared 24 h after incubation in LPDS-containing medium at 37 °C (B) but not at 21 °C (C).
Cytic compartment likely represents the immunocytochemically defined NPC1 vesicles. The role that endocytosed sterol plays in NP-C disease may be quite different than previously envisioned. The defect in vesicle-mediated egress of both \(^{14}\text{C}\)sucrose and cholesterol suggests that the NPC1-mediated transport pathway is not restricted to sterol mobilization. Defective cellular clearance of a fluid-phase marker in the absence of lysosomal cholesterol storage in NP-C suggests that generalized, bulk retrograde transport of vesicular components is impaired. Several studies have provided evidence that endocytosed lipids (47) and proteins (48) recycle to the cell surface from late endocytic compartments. Increasingly, attention has focused on the sorting and trafficking of endocytosed plasma membrane glycolipids (49). The defect in retrograde vesicular transport that we presently report could account for the excessive GM2 accumulation seen in endosomal compartments of some NP-C cell lines (50). Recently we demonstrated that endocytosed GM2 is a specific marker for the NPC1 vesicle, \(^5\) consistent with a role for this organelle in the intracellular trafficking of both glycolipids and cholesterol. Disruption of glycolipid transport might contribute to the cellular pathology associated with NP-C. Glycolipids, such as GM2, accumulate extensively in the absence of excessive cholesterol storage in NP-C neurons (51, 52) and in the whole brain (1, 12). Interestingly, glycolipids and cholesterol are the major lipid components of membrane microdomains found in rafts; detergent-insoluble, glycolipid-enriched complexes; and caveolae (49). Preliminary cellular extraction studies show that NPC1 has the solubility properties of a raft-associated protein. \(^5\) Future studies will clarify the possible

\(^5\) J. Incardona, unpublished observations.

**Table I**

| Compartments       | Normal | NP-C   | Ratio of NP-C to normal |
|--------------------|--------|--------|-------------------------|
|                    | %      | %      |                         |
| 1 (early endocytic)| 100    | 497 ± 200 | 2.05 ± 0.65               |
| 2 (late endocytic) | 1057 ± 699 | 9790 ± 2505 | 1.79 ± 0.54               |
| 3 (lysosomal)      | 5710 ± 1734 | 5710 ± 1734 | 1.23 ± 0.30               |

**Fig. 8.** The effect of NPC1 mutations on the clearance of endocytosed \(^{14}\text{C}\)sucrose from cholesterol-depleted cells. Normal and NP-C human fibroblast cell lines were incubated in McCoy's/5% LPDS medium at 37 °C for 5–7 days to clear endocytosed cholesterol from lysosomes. Cells were incubated in McCoy's/5% LPDS medium containing 5 µCi/ml \(^{14}\text{C}\)sucrose for 3 h at 37 °C to load endocytic compartments with the tracer, washed at 4 °C, and then incubated in McCoy's/5% LPDS medium at 37 °C for the indicated times. \(^{14}\text{C}\)Sucrose associated with the cell monolayer and medium was measured as described. Each data point represents the mean value ± S.D. of triplicate culture wells. The graph represents the results of a single representative paired experiment. Data points are shown as squares for normal fibroblasts (GM5565) and as diamonds for NP-C fibroblasts (GM3123). The calculated fits to the data, using the model shown in Fig. 9, are represented by solid and dashed curves for normal and NP-C fibroblasts, respectively. The model is available at http://biomodel.georgetown.edu/model.

**Fig. 9.** Model for cellular uptake and release of \(^{14}\text{C}\)sucrose in fibroblasts. Circles represent compartments, numbers identify compartments, and arrows represent movement between compartments. Compartment 4 represents the extracellular medium, and compartments 1–3 are intracellular. Values above the arrows represent the fraction of compartment transferred to an adjacent compartment per hour, for a representative experiment. The initial conditions in compartment 4 at the start of the loading was 8 x 10^6 dpm, and uptake into compartment 1 was 4.7E-5/h and 3.5E-5/h, in normal and NP-C fibroblasts, respectively. The turnover time is represented by the reciprocal of the sum of the losses from each compartment, i.e. the turnover time of compartment 2 in normal fibroblasts = 1/(0.612/h + 0.459/h) = 56 min. Data and model calculated fits for cellular clearance in this representative study are shown in Fig. 8 (normal versus NP-C) and Fig. 10 (normal versus normal + LDL).

**Table 1**

Predicted percentage of accumulation of internalized \(^{14}\text{C}\)sucrose in endocytic compartments of cultured fibroblasts.

Values were calculated using the model in Fig. 9 and normalized to compartment 1 of normal fibroblasts. The values represent mean ± S.D. of eight paired experiments involving five normal and three NP-C cell lines.
**NPC1 Vesicle Mediates Lysosomal Retroendocytosis**

The presence of LDL (50 μg/ml) for 3 h at 37 °C to load endocytic compartments with the tracer. Cells were washed and then incubated in McCoy’s/5% LPDS medium in the absence or presence of LDL (50 μg/ml) for 24 h and then in McCoy’s/5% LPDS medium containing 5 μCi/ml [14C]sucrose in the absence or presence of LDL (50 μg/ml) for 3 h at 37 °C to load endocytic compartments with the tracer. Cells were washed and then incubated in McCoy’s/5% LPDS medium in the absence or presence of LDL (50 μg/ml) for 37 °C for the indicated times. [14C]Sucrose associated with the cell monolayer and microdomains might share.

Data points are shown as squares and diamonds for cells in the absence and presence of LDL, respectively. The calculated fits to the data, using the model shown in Fig. 9, are represented by solid and dashed curves for cells in the absence and presence of LDL, respectively.

**Fig. 10.** The effect of endocytosed LDL on the cellular clearance of endocytosed [14C]sucrose. Normal human fibroblast cell lines were incubated in McCoy’s/5% LPDS medium at 37 °C for 5–7 days to deplete membrane sterol. Cells were incubated in McCoy’s/5% LPDS medium in the absence or presence of LDL (50 μg/ml) for 24 h and then in McCoy’s/5% LPDS medium containing 5 μCi/ml [14C]sucrose in the absence or presence of LDL (50 μg/ml) for 3 h at 37 °C to load endocytic compartments with the tracer. Cells were washed and then incubated in McCoy’s/5% LPDS medium in the absence or presence of LDL (50 μg/ml) for 37 °C for the indicated times. [14C]Sucrose associated with the cell monolayer and medium was measured as described. Each data point represents the mean ± S.D. of triplicate culture wells. The graph represents the results of a single representative paired experiment using normal fibroblasts (GM5565). Data points are shown as squares and diamonds for cells in the absence and presence of LDL, respectively. The calculated fits to the data, using the model shown in Fig. 9, are represented by solid and dashed curves for cells in the absence and presence of LDL, respectively.

structural and functional relationships that NPC1 and lipid microdomains might share.

We also provide preliminary evidence that endocytosed sterol itself may modulate functions of NPC1. Cellular sterol enrichment retards the clearance of endocytosed [14C]sucrose from normal fibroblasts (Fig. 10) and, like NPC1 mutations, specifically targets a late endocytic compartment (Fig. 9). Thus, in addition to being cargo, endocytosed sterol appears to alter the rate of vesicular trafficking along this pathway, possibly interacting with the sterol-sensing domain of NPC1.

In conclusion, the present findings suggest that a novel NPC1 organelle mediates retrograde lysosomal trafficking of a potentially wide spectrum of lysosomal cargo. The tissue-specific pattern of metabolites that accumulate in NP-C disease likely reflects the diverse cargo that is transported along this pathway. In the liver, where endocytosed lipoprotein processing can be significant, sterol may represent a major portion of the endocytosed cargo utilizing this pathway. Alternatively, in neurons and other types of cells in which little sterol may be exogenously derived, NPC1 may predominantly regulate retroendocytic glycolipid trafficking.

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