Fate of Endogenously Synthesized Cholesterol in Niemann-Pick Type C1 Cells

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Mammalian cells obtain cholesterol via two pathways: endogenous synthesis in the endoplasmic reticulum and exogenous sources mainly through the low density lipoprotein (LDL) receptor pathway. We performed pulse-chase experiments to monitor the fate of endogenously synthesized cholesterol and showed that, after reaching the plasma membrane from the endoplasmic reticulum, the newly synthesized cholesterol eventually accumulates in an internal compartment in Niemann-Pick type C1 (NPC1) cells. Thus, the ultimate fate of endogenously synthesized cholesterol in NPC1 cells is the same as LDL-derived cholesterol. However, the time required for endogenous cholesterol to accumulate internally is much slower than LDL-derived cholesterol. Different pathways thus govern the post-plasma membrane trafficking of endogenous cholesterol and LDL-derived cholesterol to the internal compartment. Results using the inhibitor N-butyldeoxynojirimycin, which depletes cellular complex glycosphingolipids, demonstrates that the cholesterol trafficking defect in NPC1 cells is not caused by ganglioside accumulation. The ultimate cause of death in NPC disease is progressive neurological deterioration in the central nervous system, where the major source of cholesterol is derived from endogenous synthesis. Our current study provides a plausible link between defects in intracellular trafficking of endogenous cholesterol and the etiology of Niemann-Pick type C disease.

Genetic disorders have served as important model systems to identify the factors and mechanisms involved in intracellular lipid metabolism and trafficking. One example has been elegantly demonstrated by the elucidation of the low density lipoprotein (LDL) receptor pathway for regulation of intracellular cholesterol metabolism, using human fibroblast (Hf) cells from patients homozygous in familial hypercholesterolemia (1). Another disease that has provided important insights into cholesterol metabolism is the Niemann-Pick type C (NPC) disease (2). NPC disease is an autosomal recessive, neurovisceral disorder that presents with no therapeutic cure. It affects children who carry homozygous forms of the mutant NPC1 gene (3) and causes death before adulthood. Hf cells from NPC patients have been found to accumulate LDL-derived cholesterol as unesterified cholesterol in an intracellular compartment (4–6). Other lipids, particularly glycosphingolipids, have also been found to accumulate in NPC cells (7). We have previously isolated Chinese hamster ovary (CHO) cell mutants defective at the NPC1 locus (8, 9). By performing pulse-chase experiments in these cells, we found that LDL-derived cholesterol initially moves from the hydrolytic compartment/lysosome to the plasma membrane (PM) independent of NPC1. After reaching the PM, cholesterol is internalized into an intracellular compartment. We propose that NPC1 is involved in sorting post-PM cholesterol from the intracellular compartment back to the PM or to the ER for re-esterification (10).

In humans and mice, mutations in NPC1 cause neurodegeneration in the central nervous system. Cells in the brain acquire cholesterol mostly by endogenous synthesis (11, 12). Thus, to fully understand the etiology of the NPC disease, it is important to elucidate the intracellular trafficking of endogenous cholesterol from its site of synthesis in the ER and its movement to the PM. The majority of the newly synthesized cholesterol is rapidly transported from the ER to cholesterol-rich microdomains of the PM, termed caveolae (or lipid rafts) by an energy-dependent process (13–18), and the Golgi may play a partial role in the overall trafficking process (18). The initial movement of the newly synthesized cholesterol to the PM is independent of NPC1 (6, 19). However, the post-PM trafficking of endogenously synthesized cholesterol remains to be investigated.

In this report, we examine the role of NPC1 in the intracellular trafficking of endogenously synthesized cholesterol in both CHO and Hf cells defective in NPC1, testing the hypothesis that NPC1 is involved in the post-PM trafficking of both endogenously synthesized cholesterol and LDL-derived cholesterol. Our results reveal that the ultimate fate of both cholesterol sources with regards to NPC1 is similar: trafficking of both endogenously synthesized cholesterol and LDL-derived cholesterol between the PM and the ER involves NPC1. We also show that, after arriving at the PM, the initial fate of the two sources of cholesterol before entering the NPC1-associated compartment is different. Finally, we demonstrate that the trafficking defect of endogenously synthesized cholesterol in NPC1 cells is not caused by cellular ganglioside accumulation.

EXPERIMENTAL PROCEDURES

Materials—[4-3H]Acetate (20 Ci/mmol), [1-14C]acetate (56.7 mCi/mmol), [4-3H]cholesterol (50–60 mCi/mmol) were purchased from American Radiolabeled Chemicals; [1,2,6,7-3H]cholesterol, linolate...
CT43 cells were pulsed-labeled with [3H]acetate for 2–3 h at 14 °C. Cells were then (A) chased with 2% cyclodextrin (CD) in medium D at 37 °C and percentage of cholesterol efflux was determined or (B) chased with medium D at 37 °C for the indicated times and percentage of esterification was determined. C, 25RA, CT43, and three independently isolated NPC1 stable transfec tant cells were pulse-labeled with [3H]acetate for 1 h at 37 °C, chased with medium D for 5 h at 37 °C, and the percentage of esterification was determined. Values are the combined results of two independent experiments, each performed in triplicate dishes. Error bars indicate sizes of 1 S.E. In a separate experiment, similar results as shown in C were found when cells were pulsed with [3H]acetate for 2–3 h at 14 °C and chased with medium D for 6 h at 37 °C (data not shown). The total amount of [3H]-labeled sterols synthesized did not significantly differ between cell types (data not shown).

**Cholesterol Trafficking Experiments in Intact Cells**—CHO and Hf cells were grown and cultured in six-well dishes as described under “Cell Culture.” On day 4, CHO cells were prechilled in medium D for 30 min at 4 °C and then pulse-labeled for 2–3 h at 14 °C with 10 μCi of [3H]acetate/well for single-labeling experiments or with 1 μCi of [3H]CL-LDL and 0.5 μCi of [14C]acetate/well for double-labeling experiments, unless otherwise indicated. On day 4, Hf cells were subjected to pulse-chase conditions as described in the figure legends, using 25 μCi of [3H]acetate or 0.5 μCi of [14C]cholesterol/well. After the pulses, the cells were washed once with phosphate buffered saline (PBS) plus 5 mg/ml bovine serum albumin (BSA) and twice with PBS without BSA. For cholesterol efflux experiments in CHO cells: to measure pre-PM cholesterol trafficking, cells were immediately incubated with 2% cyclodextrin in medium D at 37 °C for the indicated times; to measure post-PM cholesterol trafficking, cells were chased in medium D at 37 °C for the indicated times and then incubated with 2% cyclodextrin in medium D for 30 min at 37 °C (10). To measure cholesterol esterification, after the pulse-labeling, cells were chased in medium D without cyclodextrin at 37 °C for the indicated times. The radiolabeled lipids were extracted and separated via TLC; the respective 14C or 3H label counts were measured in a liquid scintillation counter using a dual-labeling program. In [14C]- or [3H]acetate labeling experiments, both cholesterol and fatty acids were labeled. To analyze the label distribution of cholesterol esters in cholesterol and fatty acids, the labeled cholesterol esters scraped off the TLC underwent saponification and re-extraction; the separated cholesterol and fatty acid components were then quantified by TLC according to procedures previously employed (24, 25). The result showed that over 95% of the recovered label was cholesterol. Other methods, including the percentage of cholesterol efflux, percentage of (re)esterification, and percentage of hydrolysis were determined as described previously (19).

**Percoll Gradient Analyses**—Cells were grown and cultured in 2 x 150-mm dishes as described under “Cell Culture,” except that cells were incubated with medium D on day 3. On day 4, cells were prechilled in medium D for 30 min at 4 °C and then pulse-labeled for 3 h at 14 °C with 10 μCi of [3H]CL-LDL and 5 μCi of [14C]acetate/dish. The cells were washed once with PBS plus 5 mg/ml BSA, washed twice with PBS without BSA, and chased in medium D 2 or 24 h at 37 °C. The
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method to isolate the PM and internal membrane (IM) fractions, adapted from a previously described procedure (16), is described as follows. After the pulse-chase, all of the subsequent steps were conducted at 4 °C. Cells were washed two times with buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM Tris, pH 7.8), scraped, and collected by centrifugation (1400 × g, 5 min). The cell pellet was homogenized in 1 ml of buffer A, using 10 strokes of a 2-ml “Dura-Grind” stainless steel homogenizer (Wheaton). The homogenate was centrifuged (1000 × g, 10 min), the post-nuclear supernatant was collected, and the pellet was homogenized and centrifuged again. The post-nuclear supernatants were combined and loaded onto a Percoll gradient (50% in buffer A, 23.5 ml) and centrifuged (84,000 × g, 30 min), and 25 fractions (1 ml) were collected from the top. The PM usually localized to fractions 9 and 10, as evidenced by a visible white membrane band and the enrichment of Na+/K+ ATPase enzyme activity (26) and anti-caveolin-1 expression as determined by immunoblot analysis with rabbit polyclonal caveolin-1 IgG (Santa Cruz Biotechnologies). Protein determinations were as described (27).

Glycolipid Analyses—On day 1, cells were cultured in medium A. On day 2, the medium was changed to medium D supplemented with NB-DNJ as described in the figure legend. On day 5, cells were harvested by cell scraping, pelleted by centrifugation, and washed three times with PBS. For glycolipid analysis, a method previously described was employed (28). The lipids were extracted from the pellet three times by adding 1 ml of chloroform:methanol (2:1) with vortexing, first over-night at 4 °C and then for 3 h at room temperature, pooled, and dried under nitrogen. The lipid fraction was analyzed by TLC, using a solvent system of chloroform:methanol:water (65:25:4, v/v). The TLC plate was air-dried, and the glycolipids were identified by spraying with α-naphthol (1% w/v), in methanol) followed by 50% (v/v) sulfuric acid and heat-treated (80 °C for 10 min).

RESULTS

NPC1 Is Involved in Post-PM Trafficking of Endogenous Cholesterol to the ER for Esterification—We pulse-labeled mutant CT43 cells and their parental 25RA cells with [3H]acetate at 14 °C then chased the cells at 37 °C. Acetate is the principal metabolite from which cholesterol is biosynthesized. Early work has shown that, when cells are pulsed with radiolabeled acetate at 14 °C, most of the newly synthesized cholesterol remains in the ER as free, unesterified cholesterol; upon warming the cells at 37 °C, the labeled cholesterol moves to the PM within minutes (29). To monitor the arrival of cholesterol at the PM, we chased cells with cycloexdrin to measure cycloexdrin-mediated cholesterol efflux (10, 18, 19, 30). We found significant amounts of endogenous cholesterol rapidly appeared at the PM in both cell types within 10–20 min (Fig. 1A). In agreement with earlier studies (6, 19), this result indicates that the movement of endogenous cholesterol from the ER to the PM does not involve NPC1.

To monitor the post-PM fate of endogenous cholesterol, we used the same protocol for the pulse period, but chased the cells without cycloexdrin, then determined the amount of labeled cholesterol esterified by acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1), a resident ER protein (31). The results revealed that CT43 cells are severely defective in esterifying endogenous cholesterol throughout the chase period (up to 21 h) (Fig. 1B). The inability of CT43 cells to esterify endogenous cholesterol was not due to defects in the ACAT-1 enzyme, for ACAT-1 activity was normal in CT43 cells. By using a reconstituted ACAT enzyme assay (32), we found that the ACAT specific activity in 25RA and CT43 cells was 119.4 ± 12.3 and 99.2 ± 14.1 pmol/min/mg, respectively.

To ascertain that the observed defect in endogenous cholesterol esterification is due to mutation within the NPC1 gene in CT43 cells, we compared the percentage of endogenous cholesterol esterifications in 25RA, CT43, and three NPC1 stable transfectant cell lines independently isolated from CT43 cells. We found that the percentage of endogenous cholesterol esterification values in NPC1 stable transfectant cells were significantly higher (by up to 2.7-fold) than the value in CT43 cells, although not as high as the value in 25RA cells (Fig. 1C). The partial restoration in ability to esterify endogenous cholesterol may be explained by the low expression level of the transfected hamster NPC1 seen in the stable transfecant clones (10). These cumulative findings imply that NPC1 is functionally involved in the post-PM trafficking of endogenous cholesterol from the PM to the ER.

The Intracellular Cholesterol Trafficking Defect Is Manifested in Certain Hf NPC Cells—Both mutant CT43 cells and their parental 25RA cells contain a gain of function mutation in the protein SCAP (30, 21), rendering these cells resistant to sterol-dependent transcripational regulation. It may be argued that the phenotype demonstrated in CT43 cells may not be seen in NPC cells without the SCAP mutation. To address this concern, we examined two independent Hf NPC cells, NPC 93.22 and NPC 94.71. Both NPC 93.22 and NPC 94.71 cells stained positively with filipin, indicative of an accumulation of free intracellular cholesterol (Fig. 2A). We then examined the ability of these cells to esterify cholesterol delivered via differ-
ent routes and found that both NPC 93.22 and NPC 94.71 cells were defective in re-esterifying LDL-derived cholesterol (Fig. 2B), with NPC 94.71 cells exhibiting a more serious defect. When esterification of endogenously synthesized cholesterol (Fig. 2C) or cholesterol delivered from the growth medium (Fig. 2D) was examined, we discovered that only NPC 94.71 cells but not NPC 93.22 cells exhibited a defect relative to control cells. These results indicate that the various cholesterol esterification defects observed in CT43 cells can be seen in some but not all of Hf NPC cells.

Post-PM Trafficking of Endogenous Cholesterol Back to the PM Is Defective Only at Later Time Points in CT43 Cells—Once arriving at the PM, endogenous cholesterol may be internalized and recycle between an internal compartment and the PM in a similar manner as demonstrated for LDL-derived cholesterol (10). To test this possibility, we used the same protocol for the pulse period as described earlier but chased the cells in medium without cycloextrin (CD) in medium D for 30 min at 37 °C to measure percentage of efflux of [14C]- or [3H]-cholesterol. Values are representative of two independent experiments, each performed in triplicate. Error bars indicate sizes of 1 S.E. Note that, for endogenous cholesterol, the result of the single-labeling experiment was the same as that of the double-labeling experiment, indicating that the presence of LDL did not affect outcome of the results (see “Discussion” for details of its implication).

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**Fig. 3.** Post-PM efflux of endogenous and LDL-derived cholesterol. 25RA and CT43 cells were (A) pulsed-labeled with [3H]acetate or (B) pulse-labeled with [14C]acetate and [3H]CL-LDL for 2–3 h at 14 °C. Cells were then chased with medium D for the indicated times at 37 °C before treatment with 2% cycloextrin (CD) in medium D for 30 min at 37 °C to measure percentage of efflux of [14C]- or [3H]cholesterol. Values are representative of two independent experiments, each performed in triplicate. Error bars indicate sizes of 1 S.E. Note that, for endogenous cholesterol, the result of the single-labeling experiment was the same as that of the double-labeling experiment, indicating that the presence of LDL did not affect outcome of the results (see “Discussion” for details of its implication).

As the chase period increased, the defect in LDL-derived cholesterol in these cells gradually decreased. This result may be due to the existence of a default pathway that occurs in NPC1 cells, when the cholesterol sorting compartment fuses with the lysosome, permitting cholesterol to be released from the lysosome to the PM (10).
cells. We pulse-labeled cells with [14C]acetate and [3H]CL-LDL at 14 °C then chased (A, B) with or (C, D) without 2% cyclodextrin (CD) in medium D at 37 °C for the indicated times. A, the percentage of efflux of [14C]- or [3H]cholesterol; B, the percentage of hydrolysis of [3H]CL-LDL. In C the values represent the percentage of esterification of [14C]- or [3H]cholesterol derived from the indicated sources. The values in D are replots of results shown in C and represent the relative increase in esterification of cholesterol derived from the indicated sources in 25RA cells, using the average values between 0- to 50-min time points as 1.0. The results shown in A and B and in C and D are from the same set of experiments and are the averages of triplicate dishes. The same result was obtained in two independent experiments. Error bars indicate sizes of 1 S.E.

Endogenous Cholesterol Accumulates in an Intracellular Compartment at Later Time Points in CT43 Cells—To biochemically compare the initial and ultimate fates of endogenous cholesterol and LDL-derived cholesterol, we used the double-labeling procedure described earlier to perform pulse-chase experiments in 25RA and CT43 cells then analyzed the cell homogenates using a 30% Percoll gradient (16). Control experiments showed that PM fractions isolated from 25RA cells or CT43 cells are enriched with PM markers caveolin-1 (Fig. 5A) and Na+/K+ ATPase (Fig. 5B). The results revealed that, after a 2-h chase, the cholesterol distribution profile (plotted as the PM:IM cholesterol ratio) was similar for both 25RA and CT43 cells for endogenous cholesterol. However, for LDL-derived cholesterol, the PM:IM cholesterol ratio was much lower in CT43 cells than in 25RA cells (Fig. 5C).

Additional experiments showed that, with longer incubation time (>90 min), the LDL-derived cholesterol eventually became readily accessible to cyclodextrin (data not shown).

We noted that, at the zero time point, the percentage of esterification was higher for endogenous cholesterol versus LDL-derived cholesterol in both cell types (Fig. 4C), suggesting that a small portion of newly synthesized cholesterol in the ER is available as a substrate for ACAT-1 without first traversing the PM.
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Inhibition of Glycosphingolipid Synthesis Does Not Restore the Defect in Endogenous Cholesterol Esterification in CT43 Cells—NPC cells accumulate not only cholesterol but also other lipid species such as glycosphingolipids (7). In cells isolated from patients with various sphingolipid storage diseases, the accumulation and localization of cholesterol and sphingolipids have been shown to be intimately linked (33, 34). These results raise the possibility that the cholesterol trafficking defect seen in NPC cells is caused by the accumulation of glycosphingolipids. NB-DNJ, a specific inhibitor for the enzyme glucosylceramide synthase, has been shown to deplete the cellular glycosphingolipid pool in various lysosomal storage disorders in studies using an in vitro Gaucher’s disease model (35) or a mouse model of Tay-Sachs disease (36). We found that 25RA cells treated with NB-DNJ for 3 days exhibited significant depletion in cellular levels of gangliosides GM2 and GM1, reflecting reductions in complex glycosphingolipid syntheses in these cells (Fig. 6A). Similar results were found for CT43 cells (data not shown). NB-DNJ treatment did not affect sterol biosynthesis (Fig. 6B). More importantly, the defect in endogenous cholesterol esterification in CT43 cells was not restored, indicating that the defect was not a secondary consequence of complex glycosphingolipid accumulation in these cells.

DISCUSSION

In this report, we find that, similar to the fate of LDL-derived cholesterol reported earlier (10), the post-PM recycling of endogenous cholesterol back to the PM and to the ER for esterification is severely defective in CT43 cells. These cholesterol-trafficking defects cause eventual accumulation of endogenous cholesterol in an intracellular compartment. The defects cannot be explained as a consequence of a large buildup of a pre-existing cholesterol pool in CT43 cells, because we routinely employed cells cultured in cholesterol-free medium for 2 days before the experiments began. In addition, the defects cannot be justified in terms of radioisotope dilution of the cellular cholesterol pool, because the results of the double-labeling experiment (in which LDL was added in medium) agreed with the results of the single-labeling experiment (in which LDL was not added in medium), as evidenced by comparing results in Fig. 3 (A and B), Fig. 5 (C and D), and additional results not shown. The defects can be partially restored in CT43 cells that stably express low levels of normal hamster NPC1, supporting the conclusion that all the defects described are caused by a single gene mutation in NPC1. We also show that the drug NB-DNJ does not restore the impaired endogenous cholesterol esterification in CT43 cells, indicating that the defect is not a secondary consequence of complex glycosphingolipid accumulation in these cells. Our results involving the use of NB-DNJ are consistent with the recent in vitro finding that the genetic alleviation of neuronal ganglioside storage does not improve the clinical course of the NPC mouse (37).

Using one line of Hf NPC cells, other investigators (38) recently reported similar defects regarding the fate of LDL-derived cholesterol as we reported (10); however, their study did not support our finding that an impairment in the translocation of PM cholesterol to the ER is present in NPC cells (10). To clarify this discrepancy, we present evidence that the various trafficking defects seen in CT43 cells, including the PM to the ER step, can be demonstrated in one of the two Hf NPC cell lines that we analyzed (Fig. 2). These results, along with results from other investigators (4, 38, 39), suggest that, among the various Hf NPC cells, a large heterogeneity exists in their

Fig. 5. Percoll gradient analysis. A and B, 25RA cells were grown and cultured as described under “Experimental Procedures.” The cell extracts were homogenized and the post-nuclear supernatants were subjected to 30% Percoll gradient analysis as described. Percoll fractions (1 ml per fraction, 25 fractions in total) were analyzed by immunoblot analysis with rabbit polyclonal caveolin-1 IgG (A) and for Na+K+ ATPase activity (B). Only data from Percoll fractions 1 through 18 or 1 through 19 are shown; neither caveolin-1 signal nor Na+K+ ATPase activity was found in the remaining fractions. C and D, 25RA and CT43 cells grown in medium D were pulse-labeled with [14C]acetate and [3H]CL-LDL for 3 h at 14 °C and chased with medium D for 2 h (C) or 24 h (D) at 37 °C before subjecting cell homogenates to 30% Percoll gradient analysis. Plasma membrane (PM) and internal membrane (IM) fractions were analyzed for the amount of [14C] or [3H]cholesterol by TLC as described under “Experimental Procedures.” Values represent the PM:IM cholesterol ratio. Data are representative of two independent experiments. When these cell homogenates were analyzed by a 11% Percoll gradient (6, 10), similar results as shown in C and D, 25RA and CT43 cells grown in medium D were pulse-labeled with [14C]acetate and [3H]CL-LDL for 3 h at 14 °C and chased with medium D for 2 h (C) or 24 h (D) at 37 °C before subjecting cell homogenates to 30% Percoll gradient analysis. Plasma membrane (PM) and internal membrane (IM) fractions were analyzed for the amount of [14C] or [3H]cholesterol by TLC as described under “Experimental Procedures.” Values represent the PM:IM cholesterol ratio. Data are representative of two independent experiments. When these cell homogenates were analyzed by a 11% Percoll gradient (6, 10), similar results as shown in

CT43 cells than in 25RA cells (Fig. 5D).

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ability to esterify endogenous cholesterol and/or cholesterol delivered from the growth medium. Unlike the mutant CT43 cell line, which contains a well-defined frameshift mutation that causes complete inactivation of the NPC1 protein (10), the nature of mutations present in most of the Hf NPC cells used remains largely unknown. The failure to demonstrate the cholesterol-trafficking defect in some Hf NPC cells may be caused by the presence of leaky mutations that cannot completely inactivate the mutant NPC1 proteins.

We utilize a double-labeling protocol to directly compare the fates of endogenously synthesized cholesterol and LDL-derived cholesterol in 25RA and CT43 cells. We find that, in parental 25RA cells, endogenous cholesterol arrives at the PM faster than LDL-derived cholesterol, but its post-PM trafficking to the ER for esterification is slower than LDL-derived cholesterol. We also illustrate that the time required for endogenous cholesterol to accumulate in the internalized compartment in mutant CT43 cells is much longer (>8 h) than that for the LDL-derived cholesterol. These results imply that, unlike LDL-derived cholesterol, the majority of endogenous cholesterol arriving at the PM is not available for NPC1-mediated cholesterol trafficking until later times (>8 h). After arriving at the PM, endogenous cholesterol may remain in the PM or enter an internal pool that is in rapid equilibration with the PM cholesterol pool that is accessible to cyclodextrin-mediated efflux. The exact nature of the immediate post-PM endogenous cholesterol pool is unknown at present.

In NPC1 mice, the bulk of endogenous cholesterol efflux mediated through the SR-B1 pathway has been demonstrated to be normal, whereas the efflux of LDL-derived cholesterol through the same pathway is defective (40). However, this study does not critically address the post-PM efflux of endogenous cholesterol and cannot eliminate the possibility that, once internalized, endogenous cholesterol trafficking is defective in NPC1 cells as demonstrated in our current study.

We present a model (Fig. 7) to describe the role of NPC1 in the trafficking of endogenous cholesterol. Endogenous cholesterol synthesized in the ER moves to the PM, first appears in caveolae, and then is rapidly distributed within the PM (16, 18). Initially, the PM-associated endogenous cholesterol pool is distinct from the PM-associated LDL-derived cholesterol pool; only the latter is in rapid equilibration with the NPC1-associated internal cholesterol pool. Over time, most of the post-PM endogenous cholesterol is ultimately redistributed (>8 h) to the NPC1-associated intracellular cholesterol pool to be recycled back to the PM or to the ER for esterification. Previously, by studying a similar CHO cell mutant CT60 (containing a truncated NPC1 protein near its N-terminal (10)), we had found that when NPC cells were grown in cholesterol-free medium, a small but significant intracellular cholesterol pool was detectable by Percoll gradient analysis (26). Other investigators showed that a slow equilibration exists in Hf NPC cells between the intracellular cholesterol pool and the PM cholesterol pool (41). The model presented in Fig. 7 provides a kinetic mechanism to account for these early observations. To test the validity of this model, it will be necessary to identify the various cholesterol pools, as well as the various trafficking steps indicated in this diagram.

The ultimate cause of death in NPC disease is progressive neurological deterioration in the central nervous system. The
A much slower than the post-PM LDL-derived cholesterol. See text for cholesterol in mammalian cells. The diagram emphasizes the point that NPC1 in the intracellular trafficking of endogenous and LDL-derived

major source of cholesterol in neurons is derived from endogenously synthesized rather than from exogenous uptake of cholesterol (11, 12). Our current study suggests that the defect in endogenous cholesterol trafficking may be the major cause for NPC1 disease. Recently, embryonic striatal neurons isolated from NPC1 mice have been shown to exhibit deficits in LDL-derived cholesterol trafficking and metabolism (42). Furthermore, the ability of these embryonic NPC1 neurons to differentiate in vitro has been found to be defective, when the neurons are grown under cholesterol-free conditions (42). In the future, it will be important to examine whether neurons present in various regions of the NPC1 mouse brain exhibit the same defects in the post-PM trafficking of endogenously synthesized cholesterol as demonstrated in this work.

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