Role of Niemann-Pick Type C1 Protein in Intracellular Trafficking of Low Density Lipoprotein-derived Cholesterol

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Niemann-Pick type C (NPC) is a disease that affects intracellular cholesterol-trafficking pathways. By cloning the hamster ortholog of NPC1, we identified the molecular lesions in two independently isolated Chinese hamster ovary cell mutants, CT60 and CT43. Both mutants lead to premature translational terminations of the NPC1 protein. Transfecting hamster NPC1 cDNA complemented the defects of the mutants. Investigation of the CT mutants, their parental cells, and an NPC1-stable transfectant allowed us to present evidence that NPC1 is involved in a post-plasma membrane cholesterol-trafficking pathway. We found that the initial movement of low density lipoprotein (LDL)-derived cholesterol to the plasma membrane (PM) did not require NPC1. After reaching the PM and subsequent internalization, however, cholesterol trafficking back to the PM did involve NPC1. Both LDL-derived cholesterol and cholesterol originating from the PM accumulated in a dense, intracellular compartment in the CT mutants. Cholesterol movement from this compartment to the PM or endoplasmic reticulum was defective in the CT mutants. Our results functionally distinguish the dense, intracellular compartment from the early endocytic hydrolytic organelle and imply that NPC1 is involved in sorting cholesterol from the intracellular compartment back to the PM or to the endoplasmic reticulum.

Niemann-Pick type C (NPC) is an autosomal recessive, neurovisceral disease. The hallmark of the NPC syndrome is the intracellular accumulation of unesterified cholesterol and other lipids in various tissues and organs (1). In NPC fibroblasts, delayed homeostatic responses toward the regulatory effects of low density lipoprotein (LDL)-derived cholesterol have been demonstrated (2, 3). In these cells, the movement of LDL-derived cholesterol from the cell interior to the plasma membrane (PM) is defective (4, 5). Recent evidence at the microscopic level, however, illustrates that in NPC cells, cholesterol instead accumulates in the late endosomes (13, 14). Another study showed that in NPC-like cells, the movement of LDL-derived cholesterol from the lysosomes to the PM is not defective (15). This study also demonstrates a slow equilibrium between the PM and the abnormal, buoyant lysosomes present in the NPC-like cells when cells are overloaded with cholesterol. However, this study did not clearly identify at what step the cholesterol-trafficking defect may be in these NPC-like cells. The NPC1 protein may be also involved in translocating cholesterol from the PM to the ER for esterification (16, 17). How NPC1 mediates this step is not clear. Thus, based on current literature, it is not obvious what actual step(s) in the LDL cholesterol-trafficking pathway cause cholesterol accumulation in NPC cells.

In this work, we used two independently isolated cholesterol-trafficking mutants defective in NPC1, an NPC1 stable transfectant and their parental cells as tools to examine the role of NPC1 in intracellular cholesterol trafficking. We then provide evidence that NPC1 is involved in post-PM cholesterol trafficking, that is NPC1 cycles cholesterol from an intracellular compartment to the PM or to the ER after, but not prior to, newly hydrolyzed LDL-derived cholesterol appears in the PM. Our results allow us to explain the discrepancies that currently exist among various laboratories.

EXPERIMENTAL PROCEDURES

Cell Lines—The parental cell 25RA is a CHO cell line resistant to the cytotoxicity of 25-hydroxycholesterol (18) and contains a gain of function mutation in the SREBP cleavage-activating protein (SCAP) (11). Mutant CT43 was isolated along with CT60 (8) but was uncharacterized until this study. Cell hybridization studies revealed that CT60 and CT43 belong to the same complementation group (data not shown).

Cell Culture—Cells were grown in medium A (Ham’s F-12, 10% fetal bovine serum) as monolayers at 37 °C with 5% CO2 unless stated otherwise. When used at 37 °C, medium D refers to Ham’s F-12 with 5% delipidated fetal bovine serum (19), 35 μM oleic acid, 1.5 mM CaCl2; when used at 17–19 °C or at 4 °C, medium D refers to the same medium.
without sodium bicarbonate and with 15 mM HEPES, pH 7. 1,2-\(^3\)H]Cholesterol (50 Ci/mmol) was from American Radiolabeled Chemicals; 1,2,6,7-\(^3\)H]cholesterol linolate (30–60 Ci/mmol) was from American Sham Pharmacia Biotech; 2-hydroxypropyl-\(\beta\)-cyclodextrin (used for the cholesterol efflux studies) was from Sigma; methyl \(\beta\)-cyclodextrin (used for the \(\beta\) cyclodextrin labeling studies) was a gift from Corden-Pharm, Inc.; the acyl-coenzyme A cholesterol transferase (ACAT) inhibitor CI976 (20) was a gift of Parke-Davis. All media contained 10 \(\mu\)g/ml gentamicin.

**Cloning of Hamster NPC1 cDNA—** Total RNA was prepared from wild-type (WT) CHO cells by using Trizol reagent (Life Technologies, Inc.). The first strand cDNA was synthesized using SuperScript (Life Technologies, Inc.) reverse transcriptase (RT) and oligodT(25–30), (Life Technologies, Inc.). Based on the human and mouse NPC1 nucleotide sequence (6, 21), primers were designed to amplify the internal hamster NPC1 DNA by PCR. By using the primer set (forward primer, CTGTTGTGGTATG-GAGAGTTGGAAGAGG; reverse primer, GACACTGGTCCTCTTGGAAGAA-CCGCACTTGGG), a 2.9-kb product was subcloned into pOEM-T vector (Promega) and sequenced using at least three independent clones. The full-length hamster hNPC1 cDNA sequence has been submitted to GenBank™. For transformation studies, the full-length hNPC1 cDNA was released from the pOEM vector with EcoRI and SpeI. This cDNA fragment was then inserted into the expression vector pDNA3 (Invitrogen) linearized with NcoI and XhoI. The expression construct was named phmNPC1.

**RNase Cleavage Assay—** cDNA covering the coding region of hNPC1 was divided into five overlapping regions (size averaging 1.0 kb) and was amplified by PCR. The primer sets used were as follows (forward and reverse, respectively): set 1, GGTGGACGGCAAGCAGAGG; set 2, GTAAGAAGAATCCTGGGCAGAGTCT; set 3, CGCACTTGGG; set 4, CCTCTACAAGGCACTTACGAGGAGTTCG; set 5, TTTTGCACAGTCTTCATTGCAGAGTTGAC. The resulting RNA containing the mutation was subject to RNase cleavage in the presence of sense or antisense RNA. The resulting RNA from the same region was then transcribed by T7 or SP6 polymerase to generate \(5'\)- and \(3'\)-cDNA ends (\(5'\)-RACE and \(3'\)-RACE) was performed using Marathon cDNA Amplification system (CLONTECH). Poly(A)\(^+\) RNA was isolated from WT CHO cells using the kit from Invitrogen. Double-stranded cDNA was synthesized and ligated to adaptor cDNA to serve as a template. The gene-specific \(5'\) end primer (CTCTACAACGCCACTCACCAGTTTTGC) and \(3'\) end primer (GCCGAAGAAGAATCCTGGGCAGAGTTCC) were employed to generate the \(5'\)- and \(3'\)-RACE products, respectively. The amplified products were sequenced using three independent clones. The full-length hamster hNPC1 cDNA sequence has been submitted to GenBank™. For transfection studies, the full-length hNPC1 cDNA was released from the pOEM vector with EagI and SpeI. This cDNA fragment was then inserted into the expression vector pDNA3 (Invitrogen) linearized with NcoI and XhoI. The expression construct was named phmNPC1.

**Labeling the PMs with \(3H\) Cholesterol—** The PMs were labeled with \(3\)H]cholesterol by using one of three methods (15, 17, 23) as follows: (a) \(\text{[3H]}\)cholesterol and \(\text{[3H]}\)cholesterol oleate in cell extract and \(\text{[3H]}\)cholesterol in medium. Protein determinations were as described (22).

**Cell Fractionations and Percoll Gradient Analyses—** These were performed as described (8). The percent choleseryl efflux was calculated as the amount of \(\text{[3H]}\)cholesterol in medium divided by the sum of \(\text{[3H]}\)cholesterol and \(\text{[3H]}\)cholesterol oleate in cell extract.

**Labeling the PMs with \(4\)EFP-GFP—** The PMs were labeled with \(4\)EFP-GFP. For transfection studies, the full-length hNPC1 cDNA was released from the pcDNA3 vector with EcoRI and SpeI. This cDNA fragment was then inserted into the expression vector pDNA3 (Invitrogen) linearized with NcoI and XhoI. The expression construct was named phmNPC1. Arrows indicate a single GFP-expressing cell that stained negative with filipin. E, quantitation of result in D on a single glass coverslip, indicating the number GFP-expressing cells that stained negative with filipin.

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**Figure 1.** CT60 and CT43 are NPC1 mutants. A, NPC1 mutation in CT60. B, NPC1 mutation in CT43; small dot, stop codon. C, filipin staining of 25RA and CT43 grown in medium D plus or minus 100 \(\mu\)g/ml LDL for 24 h. D, representative filipin staining of CT43 transiently co-transfected with pEGFP-N3 and pcDNA3 or with pEGFP-N3 and phmNPC1. Arrows indicate a single GFP-expressing cell that stained negative with filipin.
Cells grown in 150-mm dishes were washed 3 times with cold PBS, scraped, and centrifuged. The cell pellets were resuspended in 500 μl of buffer A (250 mM sucrose, 20 mM HEPES, and 1 mM EDTA, pH 7.3) and homogenized on ice with tight-fitting, 1 ml Dounce homogenizers (Kontes); 50–100 strokes were needed to ensure extensive cell breakage. Postnuclear supernatants (800 μl) were layered onto 9 ml of 11% (v/v) Percoll in buffer A and centrifuged (20,000 × g, 40 min, 4 °C) using a Beckman model 50Ti rotor. Ten fractions were collected from the top. As determined by immunoblot analysis, more than 80% of the PM marker (Na+/K+-ATPase α-1) was concentrated in fractions 1 and 2, whereas more than 80% of the late endosomal/lysosomal marker (LAMP-1) and the lysosomal marker (LAMP-2) were concentrated in fractions 9 and 10. Anti-Na+/K+-ATPase α-1 was from Upstate Biotechnology, Inc.; anti-LAMP-1 and anti-LAMP-2 were from Santa Cruz Biotechnology.

**Semi-quantitative RT-PCR Analysis**—This was according to procedures described in the booklet by CLONTECH Laboratories on quantitative RT-PCR, using total RNA from 25RA, CT43, and CT43NPC1 cells. The PCR primer set, CCGGTGCCACCCCGTGTACTCGTCGGTGG(5)/AGGTTAAAGATGGTGTCATCAATG(3) flanks the region deleted from CT43 NPC1 mRNA such that a shorter product (524 bp) is produced from mutant mRNA, whereas a longer product (640 bp) is produced from WT mRNA. To serve as an internal standard, a region (371 bp) of TCAGGGCTTCTT(f)/GGTCAACATTCAGGGCTCCATCAAA(r). from WT mRNA. To serve as an internal standard, a region (371 bp) of TCAGGGCTTCTT(f)/GGTCAACATTCAGGGCTCCATCAAA(r).

**RESULTS**

**Sequence Analysis of the Hamster NPC1 Mutants**—To begin to characterize the CT60 and CT43 mutants at the molecular level, we determined the hmNPC1 cDNA sequence from WT CHO cells by performing RT-PCR and 5' and 3' RACE. hmNPC1 cDNA encodes a protein of 1277 amino acids, sharing high homology with the human and mouse NPC1 proteins (6, 21) with 87/94% and 92/96% identity/similarity, respectively. The hmNPC1 mutations in CT60 and CT43 cells were located by RNase cleavage assays followed by sequencing (see “Experimental Procedures”). The CT60 mutant contains a Cys to Thr mutation at nucleotide 355, causing premature translational termination after only 118 amino acids (Fig. 1A). Previous studies indicate that the NPC1 protein cannot be detected in CT60 cells by immunoblot analysis (24, 25). The CT43 mutant contains a mutation that causes an 116-bp deletion from nucleotides 2796 to 2911 in exon 19 (26), creating a frameshift that leads to premature translational termination after 933 amino acids (Fig. 1B). Based on recent functional analysis on human NPC1 (9, 24), both CT mutants produce a non-functional hmNPC1 protein. The mutation analyses along with the RT-PCR result (Fig. 6A) indicate that both CT60 and CT43 mutants contain only one NPC1 allele at the RNA level. Additional results show that the parental cell line (25RA) from which the CT mutants were derived contain a normal NPC1 allele (data not shown).

**Transient Expression of hmNPC1 Causes Disappearance of Filipin Staining in the CT43 Mutant**—One distinguishing feature of the NPC phenotype is the inability to deliver properly LDL-derived cholesterol to various organelles within the cell, thereby leading to accumulation of free cholesterol in an acidic cellular compartment that can be stained by filipin. Filipin is a fluorescent compound that specifically binds free cholesterol. When CT60 and CT43 cells were incubated with LDL for 24 h and then stained with filipin, they exhibited a strong fluorescent intracellular staining pattern relative to their parental 25RA cells or when incubated without LDL (Fig. 1C). To quantitate NPC1 cDNA expression, we used a method previously described (24); co-transfecting CT43 cells with the constructs phmNPC1, which expresses WT NPC1, and pEGFP-N3, which expresses the green fluorescent protein (GFP), resulted in the disappearance of filipin staining in a significant portion of GFP-expressing cells. Co-transfecting the empty vector pcDNA3 and pEGFP-N3 failed to produce the same effect (Fig. 1, D and E). These results demonstrate that the cloned hmNPC1 cDNA is biologically active.

**Pre-PM LDL-derived Cholesterol Trafficking Is NPC1-independent**—The parental 25RA cells and CT mutants contain a gain of function mutation in the protein SCAP, rendering these

**Fig. 2. The initial arrival of LDL-derived cholesterol to the PM is not impaired in the CT mutants.** 25RA and CT60 (A) or 25RA and CT43 (B) were plated at 2 × 10^4 cells/well in 6-well dishes in medium A for 24 h, washed, and incubated with medium D for 48 h. Cells were pulsed with 60 μg/ml [3H]CL-LDL in medium D for 1 h at 37 °C (protocol A), washed 3 times with PBS, and incubated with medium D at 37 °C with or without 2% CD for indicated times. 25RA and CT60 (C) or 25RA and CT43 (D) were grown as described in A, pre-chilled at 4 °C for 30 min, pulsed at 17 °C with 60 μg/ml [3H]CL-LDL for 4.5 h (protocol B), washed, and incubated in medium D with 2% CD at 37 °C for 60 min; at 17 °C for 120 min (C); and at 37 °C for indicated times (D). Values are the averages of duplicate dishes for A; results are representative of four independent experiments; for B–D, values are the averages of triplicate dishes. Error bars indicate sizes of 1 S.E.
cells resistant to sterol-dependent transcriptional regulation. This phenotype should not affect the general applicability of our results because we use these cells to study intracellular cholesterol trafficking, not sterol-dependent transcriptional control. To study the role of a single gene mutation (i.e., NPC1), we chose to compare directly the cholesterol trafficking activities of 25RA cells, rather than WT CHO cells, to the CT mutants.

When cells are incubated with LDL at 19 °C, lipid transport from the sorting endosome to the lysosome is blocked (27). Upon warming to 37 °C, the majority of the LDL is rapidly hydrolyzed in the lysosome within 10–20 min; the free cholesterol is then available for efflux at the PM, using high density lipoprotein as an acceptor, 40–50 min later (28, 29). Cyclodextrins are compounds that can be used to monitor the flux of cholesterol through the PM of living cells. When cells are treated with cyclodextrin, PM cholesterol cannot be reinternalized into the cell interior (5). Taking advantage of these properties of cyclodextrin and considering the short time needed for cholesterol transport from the lysosome to the PM, we designed an assay in intact cells to monitor the arrival of newly hydrolyzed LDL-derived cholesterol at the PM.

We used two different protocols to radiolabel cells prior to measuring cholesterol efflux with cyclodextrin at 37 °C as follows: protocol A, pulse cells with [3H]CL-LDL for 1 h at 37 °C; protocol B, pulse cells with [3H]CL-LDL for 4–5 h at 17–19 °C, allowing [3H]CL-LDL to accumulate in a pre-lysosomal compartment. To avoid continuously loading the cells with cholesterol, which may cause secondary consequences, we used cells grown in cholesterol-free media for 48 h as the starting culture and used cholesterol-free media throughout the experiments. After labeling, the cells were washed several times and immediately treated with 2% cyclodextrin at 37 °C to monitor cholesterol movement to the PM.

When protocol A was used to label the cells, we found that the initial cholesterol efflux rates to the PM for 25RA and CT60 cells were essentially identical (Fig. 2A). Control experiments showed that the presence of cyclodextrin was necessary to cause significant cholesterol efflux (Fig. 2A). We next performed similar experiments, using 25RA and CT43 cells, but inspected earlier time points after the short pulse period. Again, we found that no difference in cholesterol efflux rates could be observed between 25RA and CT43 cells (Fig. 2B). Additional control experiments showed that the cholesterol efflux rates of 25RA, CT60, and CT43 cells examined under this condition were not altered if endogenous cholesterol synthesis in these cells was blocked by adding mevinolin, a cholesterol synthesis inhibitor, to the growth medium (data not shown).

When protocol B was used to label the cells, we confirmed that cholesterol efflux in the CT mutants was not diminished as compared with 25RA cells (Fig. 2, C and D). In fact, the cholesterol efflux rate observed in CT43 cells was consistently slightly higher than that in 25RA cells (Fig. 2D). Control experiments indicated that cyclodextrin added to cells maintained at 17 °C for 120 min failed to cause significant cholesterol efflux (Fig. 2C, open columns), suggesting that under this condition, the bulk of the hydrolyzed [3H]CL-LDL remains in a pre-lysosomal compartment inaccessible to cyclodextrin. 2

**Post-PM LDL-derived Cholesterol Trafficking Is NPC1-de-**

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2 In protocol B, after incubation for 4 to 5 h at 17–19 °C, 20–25% of [3H]CL-LDL was hydrolyzed in 25RA and CT cells. At 17–19 °C, with or without, a small percentage of [3H]CL-LDL (1–2% of total or 5–7% of hydrolyzed) could be reesterified to form [3H]cholesteryl oleate in 25RA and CT cells. This result is consistent with the observation (35, 30) that a small portion of cholesterol may be transported directly to ACAT for reesterification without traversing the PM. This result could also be explained by the finding that a small but significant percentage of ACAT may reside in a non-ER cytoplasmic organelle (31, 32).
The above findings indicate that early endocytic cholesterol trafficking to the PM is not affected by the NPC1 lesion. This result suggests that the impaired cholesterol movement to the PM in NPC1 cells observed by other investigators may actually occur after LDL-derived cholesterol reaches the PM. To test this prediction, we performed pulse-chase experiments. Instead of immediately incubating cells with cyclodextrin after pulse-labeling them with LDL, we incubated, or chased, the cells at 37 °C for several hours to allow cholesterol arriving at the PM to be subsequently internalized. We then monitored the movement of cholesterol back to the PM by measuring cholesterol efflux with cyclodextrin. The results are presented in Fig. 3.

When cells were labeled by protocol A and chased for 6 h, a partial defect in cholesterol efflux to the PM was now observed in CT60 and CT43 cells (Fig. 3, A, C and D). To rule out the possibility that the defect observed in the CT mutants is a consequence of LDL overloading during the pulse-chase period, we increased the chase period to 24 h and obtained essentially the same result (Fig. 3B). To avoid possible interference/complication by the cellular cholesterol reesterification process when measuring cholesterol efflux, a specific ACAT inhibitor was included throughout the experiments. The presence of ACAT inhibitor in the growth medium did not affect cholesterol efflux (Fig. 3D) or lysosomal cholesteryl ester hydrolysis (Fig. 3E) in either 25RA or CT43 cells. When ACAT inhibitor is not
present, cholesterol arriving at the ER can be reesterified to form cholesteryl oleate by ACAT1, an enzyme located mainly in the ER (33). We measured the amount of [3H]cholesteryl oleate produced, and we found that CT43 cells are severely defective in utilizing LDL-derived cholesterol for reesterification (Fig. 3F). The inability to reesterify the cholesterol in the ER was not due to defects in ACAT activity (data not shown). A similar result has been previously observed in CT60 cells (8).

To provide physical evidence that defective cholesterol-trafficking pathways lead to accumulation of cholesterol in an intracellular compartment, we treated cells with protocol B (pulse with [3H]CL-LDL for 4–5 h at 17–19 °C), chased the cells at 37 °C for the indicated times, and then prepared cell homogenates for Percoll gradient analyses. For both 25RA and CT43 cells, at a 0-h chase, the majority of the free [3H]cholesterol remained in the heavy fractions rich in the late endosomes/lysosomes (Fig. 4A). After a 1.5-h chase, a significant amount of [3H]cholesterol was located in the light fractions rich in PM in both 25RA and CT43 cells; the free cholesterol ratio in light fractions versus heavy fractions was very similar in both cell types (Fig. 4B, inset). After an 8-h chase, for CT43 cells, more free cholesterol accumulated in the heavy fractions (Fig. 4C); the free cholesterol ratio in light fractions versus heavy fractions was roughly one-third in CT43 cells as compared with 25RA cells (Fig. 4C, inset). These biochemical experiments confirm and are consistent with the kinetic experiments described in Figs. 2 and 3. Together, these studies illustrate that NPC1 is involved in the trafficking of LDL-derived cholesterol after, but not prior to, newly hydrolyzed cholesterol reaches the PM.

PM to ER Cholesterol Trafficking Is NPC1-dependent—The results described in Fig. 3F imply that NPC cells exhibit a severe defect in reesterifying LDL-derived cholesterol that is internalized from the PM. If the NPC defect is a post-PM cholesterol-trafficking defect, it is reasonable to think that all cholesterol originating from the PM is defective in moving to the ER. To examine this issue further, we employed three methods to label the PM with [3H]cholesterol and then monitored esterification at 37 °C. The results revealed that CT43 cells exhibit a serious defect in esterifying PM cholesterol relative to 25RA cells (Fig. 5, A–C). Control experiments showed that esterification in 25RA cells was completely inhibited when cells were treated with an ACAT inhibitor during the experiment (data not shown).

The result described above raises the possibility that in CT43 cells, cholesterol originating from the PM may subsequently accumulate in an intracellular compartment similar to that of LDL-derived cholesterol (see Fig. 4). To examine this, we pulse-labeled the PM of cells with [3H]cholesterol, chased the cells for
18 h at 37 °C, and then analyzed cell homogenates on Percoll gradient. The result shows that this is indeed the case. In 25RA cells, most of the [3H]cholesterol was located in the light fractions, whereas in CT43 cells, a significant fraction of [3H]cholesterol was located in the dense fractions rich in the late endosomes/lysosomes (Fig. 5D). The free cholesterol ratio of light fractions versus heavy fractions was roughly one-third in CT43 cells as compared with 25RA cells (Fig. 5D, inset). Earlier, by cholesterol mass analysis, the accumulation of free cholesterol in dense fractions on Percoll gradient has been observed in CT60 cells grown in cholesterol-free medium (17). These results provide the rationale to explain why cells with the NPC phenotype are defective in esterifying PM cholesterol as follows: cholesterol that is internalized from the PM enters a dense, intracellular compartment, and NPC1 is required to translocate cholesterol from the internal compartment to the ER.

Stable Expression of hmNPC1 at Low Levels Partially Restores the Intracellular Cholesterol-trafficking Defects in the CT43 Mutant—Although attempts have been reported, the creation of NPC1 stable transfectants has yet to be described in the current literature. After transfecting CT43 cells with phmNPC1, we were able to isolate several independent candidate stable transfectants; one of them, designated as the CT43NPC1 cell line, was fully characterized. Semi-quantitative RT-PCR analysis was undertaken to measure the relative expression levels of the NPC1 gene (WT) in 25RA and CT43NPC1 cells (Fig. 6A). The primer set was selected to flank the deleted region within the NPC1 mRNA in the CT43 mutant (as indicated by the two flanking arrows in Fig. 6A). Based on the sequence analysis (Fig. 1B), a shorter PCR product (524 bp) should be produced from the mutant mRNA, whereas a longer PCR product (640 bp) should be produced from the WT NPC1 mRNA. The ubiquitously expressed α-tubulin gene was used as an internal control for quantitation. This analysis confirms that the transfected (WT) NPC1 cDNA was expressed in CT43NPC1 cells. As expected, CT43NPC1 cells also express the mutant NPC1 mRNA found in stable clones expressing the empty vector pcDNA3 (CT43neo1).

When we examined the intracellular cholesterol trafficking activities of CT43NPC1 cells, we found that all the defects manifested in the CT43 mutant were partially restored in CT43NPC1 cells. Comparing pre-PM cholesterol efflux of CT43NPC1, CT43neo1, and 25RA cells confirmed the finding that pre-PM cholesterol transport does not require NPC1 (Fig. 6B). We have found that post-PM cholesterol transport is partially defective in the CT43 mutant (see Figs. 3–5). When this pathway was analyzed, we show that CT43NPC1 cells transport...
We propose a working model describing the intracellular trafficking of LDL-derived cholesterol in mammalian cells, emphasizing the involvement of NPC1 in sorting internalized cholesterol from an intracellular cholesterol sorting compartment to the PM (step 4a) or to the ER for esterification (step 4b). See text for details.

DISCUSSION

In this article, we report the cloning of the hmNPC1 gene; molecular analysis of the CT60 and CT43 mutants reveal that each possess mutations in the NPC1 coding region, leading to nonfunctional NPC1 proteins. We next dissect the cholesterol-trafficking pathways in these two CT mutants, and we provide new insights regarding the role of NPC1 in intracellular cholesterol trafficking. By corroborating our data with those from other investigators, we propose a model for the role of NPC1 in intracellular cholesterol trafficking (Fig. 7). In supporting this model, we provide evidence that the initial movement of LDL cholesterol derived from the early hydrolytic/degradative organelle to the PM (step 2) does not require NPC1. We then demonstrate that, upon reaching the PM, LDL-derived cholesterol is internalized into an intracellular compartment, designated as the cholesterol sorting compartment (step 3). In CT60 and CT43 cells, cholesterol derived from LDL or from the PM accumulates in this compartment. We show that NPC1 is involved in the movement of cholesterol from this intracellular compartment back to the PM (step 4a) and to the ER for esterification (step 4b).

Our results implicate that, in the CT mutants, the delayed post-PM cholesterol movement originates from an intracellular compartment that is functionally distinct from the organelle wherein the majority of the cholesteryl esters of LDL are hydrolyzed. At present, there is uncertainty as to what the exact identity of the subcellular organelle responsible for the early endocytic hydrolysis/degradation of LDL may be (for example, see Ref. 34 for discussion). For simplicity, we operationalize define this compartment as the lysosomes. A previous study (15) demonstrated normal transport from the lysosome to the PM in NPC-like fibroblast cells; however, this study did not identify at what step the cholesterol-trafficking defect may be in these cells. The partial defects in cholesterol efflux to the PM in the CT mutant cells described here are in agreement with previous studies reported from other laboratories, in which NPC fibroblast cells were continuously labeled with \(^{3}H\)-CL-LDL (4, 5, 13). The important difference between our current study and the previous studies is that we demonstrate that the delay in cholesterol movement from the cell interior to the PM can only be seen at later time points, not during the early endocytic hydrolysis and release step.

We use cyclodextrin to monitor the movement of cholesterol from the cell interior to the PM. The advantage of cyclodextrin over other extracellular cholesterol acceptors, such as HDL or small unilamellar vesicles, is that the removal of cholesterol by cyclodextrin is extremely rapid, thus allowing us to examine the early time points after the LDL pulse period. When we analyze the initial movement of newly hydrolyzed LDL-derived cholesterol to the PM using cyclodextrin (Fig. 7; step 2), cholesterol is not internalized into the cell interior. This is evidenced in the lack of cholesterol reesterification (or trafficking to the ER) in 25RA cells when immediately incubated with cyclodextrin (data not shown). When, however, LDL pulse-chase experiments are performed to monitor the arrival of internalized cholesterol back to the PM (Fig. 7; step 4a), we know cholesterol has been internalized during the chase period because 25RA cells are able to reesterify the cholesterol (Fig. 3F). In addition, our results from subcellular fractionation studies, analyzing the distribution of intracellular cholesterol pools during the pulse-chase period, are fully consistent with the kinetic studies using cyclodextrin. These results cannot be adequately explained as a possible secondary consequence of lipid loading the cells during the chase period for the following reasons: we perform LDL pulse-chase experiments in cells grown in cholesterol-free medium, instead of continuously labeling with LDL in cells grown in cholesterol-containing medium. Also, we show that after cells were chased for 24 h in cholesterol-free medium, the partial defect in cholesterol efflux still remains in the CT mutants (Fig. 3B).

Lysosomes and late endosomes co-migrate as dense fractions on Percoll gradient; however, endosomes, unlike lysosomes, are involved in various sorting processes (35). Based on recent microscopic evidence (13, 14), it is possible that the internal compartment, depicted as the cholesterol sorting compartment in our model, may consist of the late endosomes. From the late endosomes, LDL-derived cholesterol either cycles back to the PM or moves to the ER (converted to cytoplasmic lipid droplets for storage). In NPC1 cells, the cholesterol-sorting process (steps 4a and 4b) is defective, causing cholesterol to accumulate within the late endosomes. The sorting process may take place by vesicular trafficking (15, 36). Steps 4a and 4b may also involve the Golgi apparatus (37). In NPC cells, the apparent rate for step 4a is slower but not completely defective. It is possible that in the absence of a functional NPC1 protein, the late endosomes may fuse with the lysosomes, permitting cholesterol to release from the lysosomes to the PM via step 2. It is also possible that other cholesterol-trafficking route(s) between the late endosomes and the PM may exist.

To test the model, it will be important to dissect the cellular and molecular nature of each step depicted in Fig. 7. In particular, it will be important to isolate and examine the intracel-
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Lalar cholesterol sorting compartment (or vesicle). Ohno and colleagues (38) recently demonstrated that the specific glycosphingolipid GM₂ ganglioside accumulates in NPC1 human fibroblasts when cells are grown in cholesterol-free medium. It will be important to understand how a defect in cycling internalized cholesterol back to the PM can lead to an abnormal accumulation of GM₂ in the PM. NPC is a fatal, neurodegenerative disorder that presently has no therapeutic cure. To understand fully the etiology of the NPC disease, it will be necessary to elucidate the various intracellular lipid trafficking processes in cells isolated from the central nervous tissues that are affected by NPC.

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REFERENCES

1. Pentchev, P. G., Vanier, M. T., Suzuki, K., and Patterson, M. C. (1995) in The Metabolic and Molecular Basis of Inherited Disease (Scriber, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2625–2639, McGraw-Hill Inc., New York.

2. Pentchev, P. G., Comly, M. E., Kruth, H. S., Tokoro, J., Butler, J., Sokol, M., Filling-Katz, M., Quirk, J. M., Marshall, D. C., Patel, S., Vanier, M. T., and Brady, R. O. (1987) FASEB J. 1, 40–45.

3. Liscum, L., and Faust, J. R. (1987) J. Biol. Chem. 262, 17002–17008.

4. Liscum, L., Ruggiero, M. R., and Faust, J. R. (1989) J. Cell Biol. 106, 1625–1636.

5. Neufeld, E. B., Coley, A. M., Pitta, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) J. Biol. Chem. 271, 21604–21613.

6. Carstea, E. D., Morris, J. A., Coleman, K. G., Lufts, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Kritzman, D. B., Nagle, J., Polymerypolus, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kanese, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T. Y., Liscum, L., Straus, J. F., III, Ohno, K., Zeigler, M., Carmi, R., Sokol, J., Markie, D., O'Neill, R. R., Van Diggelen, O. P., Elleder, M., Robbins, E. B., Brady, R. O., Brady, R. O., Patel, S., and Brady, R. O. (1996) Biochim. Biophys. Acta 1254, 283–294.

7. Carstea, E. D., Morris, J. A., Carstea, E. D., Gu, J. Z., Cummings, C., Brown, A., Elison, J., Ohno, K., Rosenfeld, M. A., Tagle, D. A., Pentchev, P. G., and Pavan, W. J. (1997) Science 277, 232–235.

8. Cadigan, K. M., Spillane, D. M., and Chang, T. Y. (1990) J. Biol. Chem. 265, 1657–1662.

9. Watari, H., Blanchette-Mackie, E. J., Neufeld, E. B., Brady, R. O., Pentchev, P. G., and Straus, J. F., III (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 805–810.

10. Watari, H., Vanier, M. T., Patterson, M. C., Brady, R. O., Pentchev, P. G., and Straus, J. F., III (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 85–90.

11. Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Nishizawa, M., Musiek, F. S., and Elleder, M. (1997) J. Biol. Chem. 272, 11218–11224.

12. Brown, M. S., and Goldstein, J. L. (1986) Science 232, 34–47.

13. Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. D., Inardonna, J. P., Strauss, J. F., III, Vanier, M. T., Patterson, M. C., Brady, R. O., Pentchev, P. G., and Blanchette-Mackie, E. J. (1999) J. Biol. Chem. 274, 9627–9635.