Cholesterol-regulated Translocation of NPC1L1 to the Cell Surface Facilitates Free Cholesterol Uptake*

Liqing Yu†, Shantaram Bharadwaj‡, J. Mark Brown#, Yinyan Ma§, Wei Du¶, Matthew A. Davis¸, Peter Michaely∥, Pingsheng Liu*, Mark C. Willingham‡, and Lawrence L. Rudel‡

From the †Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157-1040 and the ‡Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Although NPC1L1 is required for intestinal cholesterol absorption, data demonstrating mechanisms by which this protein facilitates the process are few. In this study, a hepatoma cell line stably expressing human NPC1L1 was established, and cholesterol uptake was studied. A relationship between NPC1L1 intracellular trafficking and cholesterol uptake was apparent. At steady state, NPC1L1 proteins localized predominantly to the transferrin-positive endocytic recycling compartment, where free cholesterol also accumulated as revealed by filipin staining. Interestingly, acute cholesterol depletion induced with methyl-β-cyclodextrin stimulated relocation of NPC1L1 to the plasma membrane, preferentially to a newly formed “apical-like” subdomain. This translocation was associated with a remarkable increase in cellular cholesterol uptake, which in turn was dose-dependently inhibited by ezetimibe, a novel cholesterol absorption inhibitor that specifically binds to NPC1L1. These findings define a cholesterol-regulated endocytic recycling of NPC1L1 as a novel mechanism regulating cellular cholesterol uptake.

Whole body cholesterol homeostasis is maintained through three major pathways: de novo synthesis, intestinal absorption, and biliary excretion. Mice lacking npc1l1 (Niemann-Pick C1-like 1) have a substantial reduction in intestinal cholesterol absorption and are resistant to high cholesterol diet-induced cholesterol accumulation (1–3). The phenotypes of npc1l1-null mice recapitulate the effect of ezetimibe (1, 2), a novel cholesterol absorption inhibitor (4–6), indicating that NPC1L1 is in the ezetimibe inhibitory pathway. Although both the annexin-2/caveolin-1 complex and aminopeptidase N have been reported previously to be the direct target of ezetimibe (7, 8), caveolin-1 knockout mice have a normal percentage of cholesterol absorption (9), and the physiological evidence for aminopeptidase N as the ezetimibe target has yet to be shown. On the other hand, ezetimibe was recently shown to specifically bind to NPC1L1 (10). All these data strongly support that NPC1L1 is the target of ezetimibe and resides within the cholesterol uptake pathway. However, the reconstitution of NPC1L1-dependent cholesterol transport in cultured cell systems has been unsuccessful, and tissue-specific cofactors were speculated to be needed (1), limiting further exploration of the molecular basis for cholesterol absorption.

The NPC1L1 gene was initially identified to be a homolog of NPC1 (Niemann-Pick C1) and was predicted to be involved in intracellular cholesterol trafficking (11) based on the fact that mutations in the NPC1 gene result in a lipid storage disease, Niemann-Pick disease type C1 (12, 13). NPC1L1 is widely expressed in many human tissues, with the highest expression in the liver and small intestine (1, 3, 11). The expression pattern varies among species. Mouse and rat npc1l1 mRNAs are much more abundant in the small intestine than in the liver (1, 3). The reason for the different tissue expression patterns among species is unknown.

The NPC1L1 protein is predicted to have a typical signal peptide, 13 putative transmembrane domains, extensive potential N-linked glycosylation sites, and a conserved N-terminal “NPC1” domain (1, 11). Interestingly, NPC1L1, like its homolog NPC1 (11–13), also contains a sterol-sensing domain (SSD), a region conserved in at least six other polytopic membrane proteins, all of which are implicated in cholesterol metabolism (14), including 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of cholesterol synthesis (15, 16), and sterol regulatory element-binding protein cleavage-activating protein (SCAP), a protein that controls the transport and proteolytic activation of sterol regulatory element-binding proteins, which are membrane-bound transcription factors governing the synthesis of cholesterol and other lipids (15–17). Intracellular trafficking of some SSD-containing proteins is regulated by cellular cholesterol levels (14). The subcellular localization of NPC1L1 proteins has been a matter of debate. Although NPC1L1 proteins have been found in the apical surface of enterocytes (1), intracellular localization has also been observed in a human hepatoma cell line (HepG2) and a colon cancer cell line (Caco-2) (3). Whether the intracellular itinerary of NPC1L1 is modulated by cellular cholesterol availability and whether trafficking affects its functionality remain to be elucidated. In this study, NPC1L1 trafficking and its regulation by cholesterol were explored, and a relationship between NPC1L1 intracellular trafficking and cholesterol uptake was apparent.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal antibodies to the Golgi proteins GM130, golgin-97, and TGN38 were purchased from Transduction Laboratories, Molecular Probes, and Affinity Bioreagents, respectively. Rabbit anti-α-mannosidase II serum was kindly provided by Dr. Kelly W. Moremen (University of Georgia). Mouse monoclonal antibody to P-glycoproteins (C219) was from Calbiochem. Iron-loaded transferrin-tetramethylrhodamine conjugate was obtained from Molecular Probes. Peptide N-glycosidase F, endoglycosidase H, and all restriction endonucleases were obtained from New England Biolabs Inc.

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Ezetimibe was a generous gift from Schering-Plough and Merck. [14C]Cholesterol was purchased from Amersham Biosciences. All cell culture media were purchased from Fisher. Methyl-β-cyclodextrin (MβCD), mevinolin, filipin, and all other chemicals and reagents were purchased from Sigma unless stated otherwise.

**Cloning of Human NPC1L1 cDNA**—Four PCR primers (5'-GACTG-ACTGGATCCCTGGTTATGCTGAGGCTTGCTGGGT-3', 5'-GACTGACGCTAGTCTGGGAGCCACCTGGCTAGGGCT-3', 5'-GACTGACTGCTAGTCTGGGAGCCACCTGGCTAGGGCT-3', 5'-GACTGACTGCTAGTCTGGGAGCCACCTGGCTAGGGCT-3') were designed to amplify two fragments of the human NPC1L1 gene (GenBank™ accession number AY437865) using a digested pMAL-c2X vector (New England Biolabs Inc.), which contains gel-purified, sequentially digested, and ligated into the EcoRI/BamHI-digested pMAL-c2X vector. Fusion protein was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.3 mM for 3 h after optimal bacterial growth. Cells were then harvested, resuspended in amylase column buffer (10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, and 1 mM sodium azide), and stored overnight at −20 °C, followed by sonication and centrifugation. The supernatant was loaded into a column containing 20 ml of amylase resin. After overnight binding at 4 °C, the resin was washed with amylase column buffer, and the fusion protein was then eluted with amylase column buffer with addition of 10 mM maltose. The fusion protein was dialyzed extensively in 20 mM NaHPO4 and 28 mM NaCl (pH 7.5). An aliquot of the fusion protein was reduced and run on 10% polyacrylamide gel in the presence of SDS to verify the purity. The purified fusion protein was sent to Chemicon International, Inc. (Temecula, CA) for injection into rabbits to produce polyclonal antiserum.

**Establishment of Stable Cell Lines Expressing Either EGFP or NPC1L1-EGFP Fusion Protein**—McArdle RH7777 rat hepatoma cells from the Tissue Culture Core in our department were maintained in Medium A (4.5 g/liter glucose Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% fetal bovine serum) in a humidified 5% CO2 incubator at 37 °C. Transfection was performed using the Nucleofector system (Amaxa GmbH, Cologne, Germany) according to the manufacturer's instructions. Briefly, 1.5 × 106 cells were harvested and resuspended in 100 μl of Solution V (supplied by the manufacturer) along with with 5 μg of plasmid DNA (either pEGFP-N1 or pL1-EGFP). Following Program T28 for nuleofection, the cells were gently removed from the cuvette, immediately plated into 6-well plates containing prewarmed Medium A, and cultured in a CO2 incubator. Cells were allowed to recover for 24 h, after which G418 (Geneticin disulfate) selection was started at a concentration of 800 μg/ml. The concentration of G418 was reduced to 400 μg/ml after 2 weeks of selection, as almost all surviving cells were EGFP-positive. Cell colonies were isolated using cloning cylinders, and pure clones were isolated using the limited dilution plating of cells in 96-well plates. Isolated clones were grown, expanded, and maintained in Medium A plus 200 μg/ml G418.

**Cell Membrane Preparation and Immunoblot Analysis**—Cells were grown in 100-mm dishes to ~80% confluence, and the medium was aspirated. The cells were scraped in 5 ml of cold phosphate-buffered saline (PBS) and spun down at 1000 × g for 10 min at 4 °C. The cell pellet was resuspended in 1 ml of cold buffer consisting of 20 mM Tris-HCl (pH 7.5), 2 mM MgCl2, and 0.25 μM sucrose along with proteinase inhibitors at one tablet of Complete protease inhibitor mixture (Roche Diagnostics)/10 ml of buffer and incubated on ice for 10 min. The cell pellets were then ruptured by passing through a 22-gage needle 30 times and spun down at 1000 × g for 10 min at 4 °C. The supernatant was transferred to tabletop ultracentrifuge tubes and spun in a TL100 rotor at 55,000 rpm for 30 min at 4 °C. The cell membrane pellet was resuspended in ~100 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 80 mM NaCl, 2 mM CaCl2 and 1% Triton X-100) with protease inhibitors at one tablet of Complete protease inhibitor mixture/10 ml of lysis buffer.

The protein concentrations of the cell membranes were determined using the BCA kit (Pierce). Peptide N-glycosidase F and endoglycosidase H digestions were performed according to the manufacturer's protocol. Proteins (50 μg) were fractionated on 6.5% polyacrylamide gel in the presence of SDS and transferred to Hybond-C Extra nitrocellulose filters (Amersham Biosciences). The filters were then blocked in blocking buffer (PBS (pH 7.4) containing 0.05% Tween 20 (Sigma), 5% powdered milk, and 5% newborn calf serum) at room temperature for 2 h, followed by incubation in fresh blocking buffer containing L1Ab (1:2000 dilution) at room temperature for 1 h. After washing the non-bound primary
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antibody, the filter was incubated in fresh blocking buffer with 1:5000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) at room temperature for 30 min and then washed. The immunoblot was visualized with the SuperSignal substrate system (Pierce) at room temperature for 2 min and exposed to Kodak X-Omat XLS-1 films at room temperature for 10–120 s.

Measurement of Cell-surface Enrichment of NPC1L1 in Cholesterol-depleted HepG2 Cells—Cholesterol depletion and biotinylation of cell-surface proteins were performed as described previously (18–20). In brief, 1 g of MβCD was dissolved in 10 ml of DMEM and filtered to produce a 10% stock solution of MβCD. 5 ml of the stock solution was warmed to 50 °C and vortexed gently while 100 µl of 20 mg/ml cholesterol in ethanol was added to produce a 10% MβCD and 0.4 mg/ml cholesterol stock solution. Stock solutions were diluted with warm DMEM to produce 2% MβCD and 2% MβCD and 0.08 mg/ml cholesterol working solutions. HepG2 cells were treated with DMEM alone, 2% MβCD, or 2% MβCD and 0.08 mg/ml cholesterol for 0.08 min in a 37 °C incubator. Cells were then washed with ice-cold Buffer A (20 mM sodium phosphate (pH 7.2) and 150 mM NaCl) and treated with 1 mg/ml sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce) in Buffer B (20 mM sodium phosphate (pH 8.0) and 150 mM NaCl) for 30 min at 4 °C. Cells were washed with cold Buffer C (20 mM Tris-HCl (pH 8.0) and 150 mM NaCl) and incubated in Buffer C for 30 min at 4 °C. Cells were washed with cold Buffer A three times and collected by scraping in 500 µl of Buffer D (10 mM Tris-HCl (pH 8.0), 4 mM EGTA, and 1% Triton X-100). Volumes were adjusted to 1 ml with additional Buffer D, and 800 µl of each sample (500 µg of total cell proteins) was added to 100 µl of 50% (v/v) NeutrAvidin-agarose (Pierce) and rotated overnight. The agarose beads from each sample were then washed three times with Buffer E (15 mM Tris-HCl (pH 8.0), 500 mM NaCl, 4 mM EGTA, and 0.5% Triton X-100) and one time with Buffer F (15 mM Tris-HCl (pH 8.0), 4 mM EGTA, and 0.5% Triton X-100). Biotinylated proteins bound to the beads were eluted by addition of an equal volume (50 µl) of 5× SDS-PAGE sample buffer and heating to 100 °C for 10 min. One-fifth of the eluted samples was size-fractionated by 5–17% gradient SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore Corp.), and processed for immunoblot analysis using L1Ab and the preimmune serum from the same rabbit as described above.

Fluorescence Immunocytochemistry—Cells were grown in 35-mm Corning dish cultures, fixed at room temperature for 10 min in 3.7% formaldehyde in PBS for GM130 and golgin-97 staining or in methanol for TGN38 staining, and washed three times with PBS, followed by permeabilization with 0.2% (v/v) Triton X-100 in PBS at room temperature for 5 min. To stain α-mannosidase II, cells were fixed in 3% paraformaldehyde in PBS at 37 °C for 15 min and then at room temperature for 15 min, washed twice with PBS containing 50 mM NH4Cl for 15 min each at room temperature, and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 5 min on ice. All permeabilized cells were washed three times with PBS; blocked in PBS containing 1% (w/v) bovine serum albumin (BSA; 1% BSA/PBS) at room temperature for 10 min; and incubated in 1% BSA/PBS containing an antibody to GM130 (10 µg/ml), golgin-97 (1 µg/ml), or TGN38 (1:1000 dilution) at room temperature for 30 min. After three washes with PBS at room temperature for 5 min each, the cells were incubated in 1% BSA/PBS containing 15 µg/ml rhodamine-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc.) at room temperature for 30 min, followed by rinsing three times with PBS and once with deionized H2O. The circular coverslip was then mounted on the cell surface using a ProLong antifade Kit (Molecular Probes). The cells were examined under a ×63 objective using a Model 510 laser scanning confocal microscope (Carl Zeiss AG, Göttingen, Germany) with fluorescein isothiocyanate and rhodamine channels set up on a sequential mode.

To stain NPC1L1 and the canalicular P-glycoproteins in monkey liver, cryosections were cut at 5 µm, thaw-mounted on a glass slide, fixed in 3.7% formaldehyde in PBS at room temperature for 10 min, and washed three times with PBS at room temperature for 5 min each. The tissue section was then blocked in 1% BSA/PBS at room temperature for 10 min and incubated in 1% BSA/PBS containing LIAb (1:200 dilution) at room temperature for 30 min. After three PBS washes at room temperature for 5 min each, the tissue section was incubated in 1% BSA/PBS containing 15 µg/ml rhodamine-conjugated goat anti-rabbit IgG at room temperature for 30 min, washed with PBS, and incubated at room temperature for 30 min in 1% BSA/PBS containing antibody C219 (1:100 dilution), followed by incubation in 1% BSA/PBS containing 15 µg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG at room temperature for 30 min. The section was then post-fixed in 3.7% formaldehyde in PBS at room temperature for 10 min, stained with 100 ng/ml 4′,6-diamidino-2-phenylindole in methanol at room temperature for 5 min, and washed three times with PBS at room temperature for 5 min each. The coverslip was mounted on a slide using 90% glycerol in PBS and immediately examined using an Axioplan 2 fluorescence microscope (Carl Zeiss AG).

Filipin Staining—Cells were grown in a 35-mm Corning dish in Medium A. After three washes with PBS, cells were fixed in 3% paraformaldehyde (fresh) for 1 h at room temperature, rinsed three times with PBS, and then incubated in 1 ml of 1.5 mg/ml glycerol in PBS for 10 min at room temperature to quench the paraformaldehyde. The cells were stained with 1 ml of filipin working solution (0.05 mg/ml filipin, 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 2 g/liter glucose) at room temperature for 2 h, followed by three PBS washes. The cells were then visualized using an Axioplan 2 fluorescence microscope using an ultraviolet excitation filter set.

Measurement of Cellular Cholesterol—Cells were seeded in 150-mm dishes and allowed to grow to ~70% confluency in Medium A. The media in a subset of dishes were then changed to Medium B (4.5 g/liter glucose DMEM containing 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 5% calf lipoprotein-deficient serum) with 2% MβCD for 1 h to deplete the cells of cholesterol. All cells were washed twice with PBS, and total cellular lipids were extracted by the method of Bligh and Dyer (21). Total lipid extracts were dried under an N2 stream and resuspended in 4 ml of 1% Triton X-100 in chloroform. After evaporation of the chloroform, the detergent-solubilized lipid was resuspended in 1 ml of distilled H2O by vortexing, and duplicate aliquots of 50 µl were used to determine total and free cholesterol mass using total cholesterol reagent (Roche Applied Science) and free cholesterol C (Wako Chemicals), respectively, following the manufacturers’ protocol. Cholesterol ester was calculated as the difference between total and free cholesterol. For normalization, the protein concentration of parallel cultures was determined using the BCA colorimetric assay.

Cholesterol Uptake Assay—McArtht R77777 rat hepatoma cells stably expressing the human NPC1L1-EGFP fusion protein (referred to as L1-EGFP cells) or control EGFP cells were seeded in 35-mm dishes at a density of 2 × 10^4 cells/cm^2 and allowed to proliferate for ~36 h in Medium A. The media in a subset of dishes were then changed to Medium B containing 2% MβCD for 1 h to deplete the cells of cholesterol. Following cholesterol depletion, all plates were washed once with PBS and then supplemented with 1 ml of assay medium (serum-free DMEM). Immediately thereafter, 0.1 µCi of [4-14C]cholesterol was added per plate, and the cultures were incubated in a humidified 5% CO2 incubator at 37 °C for the indicated times. Prior to isotope addition,
the original [4,14C]cholesterol/toluene suspension was dried down under N2 and resuspended in 50 μl of 100% ethanol. The ethanolic [4,14C]cholesterol was diluted 30-fold into PBS containing 1.5% fatty acid-free BSA, which, when diluted into the assay medium, resulted in final concentrations of 0.0375% fatty acid-free BSA and 0.0833% ethanol. Following labeling, cells were washed three times with PBS and subsequently lysed in 0.1% SDS in PBS. The total cell lysate was subjected to liquid scintillation counting to determine cellular cholesterol uptake. To control for uncontracted cell-associated [4,14C]cholesterol, a set of cultures was briefly exposed to [4,14C]cholesterol (<30 s) and immediately washed and harvested; the resulting radioactivity was subtracted from all experimental results. For normalization, the protein concentration of parallel cultures was determined using the BCA kit. In ezetimibe inhibition studies, ezetimibe was solubilized in dimethyl sulfoxide at a stock concentration of 100 μM and further complexed to fatty acid-free BSA at a 1:1 (v/v) ratio using a 7.5% fatty acid-free BSA stock solution. This preparation was then diluted into assay medium to give the desired final concentration (0–30 μl), and fatty acid-free BSA was supplemented to give all cultures the same final concentration of 0.0045%. Ezetimibe was added 1 h prior to MβCD treatment and throughout the assay.

Statistical Analysis—Data are expressed as the means ± S.E. All data were analyzed using one-way analysis of variance, followed by Student’s paired t test for multiple comparisons. Differences were considered significant at p < 0.05 or 0.01. All analyses were performed using JMP Version 5.0.12 software (SAS Institute Inc, Cary, NC).

RESULTS

NPC1L1-EGFP Fusion Proteins Are Peptide N-Glycosidase F-sensitive and Endoglycosidase H-resistant—To define intracellular trafficking of NPC1L1 and to reconstitute NPC1L1-dependent cholesterol uptake in vitro, a stable cell line (L1-EGFP) expressing human NPC1L1 tagged with EGFP was established, as described under “Experimental Procedures,” from McArdle RH7777 rat hepatoma cells, which have a low endogenous npc1L1 mRNA level, if any (data not shown). In addition, this hepatoma cell line was chosen because previous reports have demonstrated that NPC1L1 is highly expressed in human liver (1, 3), yet its hepatic function is unknown. To visualize protein trafficking in living cells, one copy of EGFP was appended to the C terminus of NPC1L1. C-terminal tagging has been shown not to alter NPC1L1 subcellular localization (3). Furthermore, NPC1L1 has a typical signal peptide that is cleaved; and hence, this end of the protein is not suitable for EGFP tagging unless the precise cleavage site is mapped. Fusion of EGFP to the C terminus of NPC1 (homolog of NPC1L1) has been shown previously not to alter NPC1 trafficking and function (22, 23).

Expression of the human NPC1L1 protein in L1-EGFP cells was confirmed by immunoblotting using L1Ab (developed as described under “Experimental Procedures”). A specific protein band whose size was larger than the calculated molecular mass of 175 kDa for the fusion protein (145 kDa for NPC1L1 plus 30 kDa for EGFP) was revealed (Fig. 1, lane 2). It has been reported that the NPC1L1 protein is N-glycosylated (1, 24). Consistent with this, the NPC1L1-EGFP fusion protein was sensitive to peptide N-glycosidase F digestion, which removed the attached N-glycan chains and reduced the protein size to 175 kDa (Fig. 1, lane 4).

The NPC1L1-EGFP fusion protein was also found to be resistant to endoglycosidase H digestion (Fig. 1, lanes 6). The sensitivity to peptide N-glycosidase F and the resistance to endoglycosidase H indicated that the fusion protein had folded properly and trafficked beyond the endoplasmic reticulum. Surprisingly, after incubation in peptide N-glycosidase F- or endoglycosidase H digestion buffer, NPC1L1 proteins were less immunoreactive (Fig. 1, lanes 3 and 5). The reason for this observation is unknown, but it is not likely to be the result of protein degradation because a larger nonspecific protein band remained unchanged in all lanes and because the NPC1L1 immunoreactivity was restored after removing N-glycan chains with peptide N-glycosidase F under the same conditions (Fig. 1, lane 4). The NPC1L1-EGFP Fusion Protein Has Little Colocalization with Golgi Proteins—EGFP tagging allowed us to visualize the subcellular localization of NPC1L1. When L1-EGFP cells were grown in normal medium, NPC1L1-EGFP fusion proteins were present in both the intracellular compartments and the plasma membrane, with more apparent plasma membrane localization observed in confluent cells than in proliferating cells (Fig. 2A), indicating that the NPC1L1 protein traffics between the intracellular compartments and the cell surface. Nevertheless, the protein localized predominantly to the perinuclear region and diffusely in the cytoplasmic tubulovesicles (Fig. 2A), consistent with the reported subcellular localization of endogenous NPC1L1 in HepG2 and Caco-2 cells (3). The pericentriolar NPC1L1-EGFP protein had little colocalization with four Golgi proteins: cis-Golgi matrix protein GM130, Golgi cisternal protein α-mannosidase II, trans-Golgi protein golgin-97, and trans-Golgi network protein TGN38 (Fig. 2, B and C). Z-stack sections of confocal microscopic images revealed that cytoplasmic NPC1L1-EGFP proteins resided abundantly in a tubulovesicular network above TGN38-positive structures (Fig. 2C).

The NPC1L1-EGFP Fusion Protein Colocalizes with Transferrin and the Free Cholesterol Repository—To document the identity of NPC1L1-positive intracellular compartments, L1-EGFP cells were labeled with iron-loaded transferrin-rhodamine conjugate. Transferrin is a unique marker for the endocytic recycling compartment (ERC), which usually localizes in the perinuclear region of non-polarized cells (25, 26) and is a major repository of free cholesterol (27–29). Interestingly, the pericentriolar NPC1L1-positive compartment had extensive colocalization with transferrin (Fig. 3A), demonstrating that NPC1L1-EGFP fusion proteins are located in the endocytic recycling pathway. Intriguingly, the ERC in L1-EGFP cells was also found to be enriched with free cholesterol as revealed by its positive staining with filipin (Fig. 3B), an antibiotic that specifically binds free cholesterol (30). It is unlikely that expression of NPC1L1-EGFP fusion proteins is required for both the formation of the ERC and the retention of free cholesterol in the ERC because a similar structure with cholesterol deposition was also
Expression of NPC1L1 Fusion Proteins Increases Cellular Cholesterol Content—As a protein involved in the pathway of cholesterol uptake, NPC1L1, when overexpressed, is hypothesized to transport more cholesterol into cells. Consistent with this hypothesis, under normal culture conditions, total and free cholesterol mass was significantly increased in cells expressing NPC1L1-EGFP compared with control EGFP cells (Fig. 4) and ranged from 30 to 100% in replicated experiments. No significant difference in cholesterol ester content was observed between the two cell lines (Fig. 4). Surprisingly, the increased free cholesterol in the L1-EGFP cells failed to suppress the mRNA and protein levels of the low density lipoprotein receptor, which is a sensor of cholesterol biosynthesis (data not shown), indicating that the NPC1L1-derived cholesterol is largely excluded from the common cholesterol regulatory pool. When these cells were grown in Medium B containing 2% MβCD for 1 h, their total and free cholesterol content was reduced by >50%, with no change in cholesterol ester mass compared with the cells grown in Medium A (Fig. 4). However, the magnitude of the difference in total and free cholesterol levels remained the same between L1-EGFP and control EGFP cells after filipin staining (data not shown).

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Acute M subdomain formation because this structure could be observed in control EGFP cells (Fig. 4), indicating that the efficiency of MβCD-mediated cholesterol efflux is unaffected by NPC1L1 expression, which is consistent with the observation that the percentage of cholesterol efflux to MβCD over a 4-h time period was identical between the two cell lines (data not shown).

Acute Cholesterol Depletion Relocates NPC1L1-EGFP Fusion Proteins to the Cell Surface, Preferentially to an “Apical-like” Subdomain—To determine the relationship of NPC1L1 trafficking with cellular cholesterol availability, L1-EGFP cells were cholesterol-depleted for 1 h in Medium B containing 2% MβCD. This treatment resulted in an ~50% reduction in total and free cellular cholesterol content as shown in Fig. 4 and caused a rapid relocation of NPC1L1-EGFP fusion proteins from intracellular compartments (Fig. 5A, panel 1) to the cell surface, prominently in a subdomain of the plasma membrane (panel 2). Similar structures were also seen in cells treated for 24 h with the cholesterol synthesis inhibitor mevinolin (50 μM), but to a lesser extent (data not shown). An oval structure was often formed between two indented subdomains in the plasma membrane of two adjacent cells (Fig. 5, A, panel 2; and B). Expression of NPC1L1-EGFP fusion proteins was not required for the subdomain formation because this structure could be observed in cholesterol-depleted control cells despite the difficulty in identifying the structure in the absence of NPC1L1-EGFP proteins (data not shown). Acute MβCD treatment also raised the amount of transferrin on the cell surface (Fig. 5B). However, despite apparent cell-surface localization, transferrin was not concentrated in the NPC1L1-positive oval structure (Fig. 5B), implying that the subdomain location is protein-specific. The effect of cholesterol depletion on NPC1L1 translocation was reversible. Addition of free cholesterol (complexed with MβCD) to cholesterol-depleted L1-EGFP cells could drive cell-surface NPC1L1 back to the intracellular compartment within 1 h (data not shown).

Enrichment of NPC1L1 Proteins on the Cell Surface of Cholesterol-depleted HepG2 Cells—To make our observations in the transfected cells more physiologically relevant, HepG2 cells, which express endogenous NPC1L1, were used to study the plasma membrane translocation of NPC1L1 in response to cellular cholesterol availability. Biotinylation has been widely used for quantification of cell-surface proteins (18, 20). We biotinylated the cell-surface proteins of HepG2 cells that had been treated with MβCD or cholesterol/MβCD at 37 °C for 30 min as described under “Experimental Procedures.” Consistent with the finding in the transfected cells, cholesterol depletion resulted in an enrichment of endogenous NPC1L1 protein on the cell surface, and on the other hand, NPC1L1 proteins were undetectable on the cell surface of cholesterol-replete HepG2 cells (Fig. 5C). Immunostaining of the same biotinylated sample with the preimmune serum revealed no protein bands at the same position (Fig. 5C). The nonspecific biotinylated cell-surface protein with a much smaller molecular mass on the L1Ab blot was used as a loading control and remained unchanged under two conditions (Fig. 5C).

NPC1L1 Proteins Localize to the Canalicular Membrane of Hepatocytes in Monkey Liver—The NPC1L1-positive oval structure was occasionally formed even in L1-EGFP cells grown in normal medium (Fig. 5D). Interestingly, in cells forming the apical-like structure, NPC1L1 proteins disappeared from the pericentriolar region and reappeared in a transferrin-positive subapical compartment (Fig. 5), indicating the polarization of cells. The preferential accumulation of NPC1L1-EGFP fusion proteins in an apical-like structure of cultured hepatoma cells implies that the endogenous NPC1L1 protein might localize to the canalicular membrane of hepatocytes in an intact animal liver. To examine the hepatic localization of NPC1L1, frozen sections of African green monkey liver were used for fluorescence immunohistochemistry with L1Ab. A typical canalicular staining was revealed, with a complete colocalization with the staining of antibody C219, which specifically recognizes canalicular P-glycoproteins (Fig. 5E, upper panels). No similar pattern and colocalization were observed when the preimmune serum from the same rabbit was used (Fig. 5E, lower panels).

Translocation of NPC1L1 to the Cell Surface Facilitates Free Cholesterol Uptake—To define the function of NPC1L1, a cholesterol uptake assay was performed as described under “Experimental Procedures.” In this assay, a trace amount of isotopic free cholesterol was complexed with fatty acid-free BSA and used as a cholesterol donor to avoid the cytotoxicity of the physiological donor, the bile acid–containing micelle, which is found to be toxic to L1-EGFP cells (data not shown). Normal cultured L1-EGFP cells had only a slightly higher but non-significant free cholesterol uptake compared with control cells expressing only EGFP (Fig. 6A). The majority of NPC1L1 proteins may have been localized intracellularly under this condition (Fig. 2A). The cholesterol uptake assay was then performed in cells pretreated with 2% MβCD for 1 h. Intriguingly, MβCD treatment substantially increased cholesterol uptake in a time-dependent manner in L1-EGFP cells (~4.7-fold increase at 2 h compared with untreated L1-EGFP cells) (Fig. 6A). The data largely represent cholesterol uptake instead of a balance between uptake and efflux of cholesterol. In support of this, we found that the percentage of cholesterol efflux to MβCD, apoA-I, high density lipoprotein, and fetal bovine serum over a 4-h time period was similar between L1-EGFP and control EGFP cells (data not shown).

Ezetimibe Inhibits NPC1L1-mediated Cholesterol Uptake—To substantiate that the increased cholesterol uptake was NPC1L1-mediated, the NPC1L1-specific inhibitor ezetimibe was included in the assay as described under “Experimental Procedures.” Ezetimibe had no effect on the subcellular localization and MβCD-induced asymmetric movement of NPC1L1 (data not shown). As expected, it dose-dependently inhibited cholesterol uptake in MβCD-treated L1-EGFP cells, but not in MβCD-treated control EGFP cells (Fig. 6B); this was presumably via its binding to NPC1L1 on the cell surface (10). Without MβCD pretreatment, cholesterol uptake in L1-EGFP cells was very similar to that in control EGFP cells and was not affected by ezetimibe (Fig. 6B). These data suggest that the increased cholesterol uptake in MβCD-treated L1-EGFP cells was specifically mediated by NPC1L1.
DISCUSSION

The major findings in this study include the following. 1) NPC1L1 proteins traffic between the plasma membrane and intracellular compartments through the endocytic recycling pathway in cultured hepatoma cells. 2) The endocytic recycling of NPC1L1 proteins is apparently regulated by cellular cholesterol availability. 3) Acute cholesterol depletions relocate NPC1L1 to the cell surface, preferentially to a newly formed apical-like subdomain, resulting in an increased uptake of free cholesterol through NPC1L1, which can be dose-dependently inhibited by a novel cholesterol absorption inhibitor, ezetimibe.

The subcellular localization of NPC1L1 remains controversial. One group reported that NPC1L1 was located mainly in the plasma membrane (1, 24), and the other group found it predominantly in the perinuclear region (3). We observed that, in McArdle RH7777 cells, the NPC1L1-EGFP fusion protein exists simultaneously in both intracellular compartments and the cell membrane (Fig. 2A), and its subcellular distribution is dependent on cell growth state (Fig. 2A) and cholesterol availability (Fig. 5, A–C). These data indicate that the protein traffics between intracellular compartments and the cell surface. However, the precise intracellular itinerary of NPC1L1 has not been delineated. The NPC1L1-positive tubulovesicles seen in the cytoplasm of L1-EGFP cells (Fig. 2C) are identical to sorting endosomes described in two reports (25, 26). A previous study also showed that the NPC1L1 protein has extensive colocalization with a sorting endosome marker (Rab5A) in HepG2 cells (3). Although an obvious pericentriolar location was observed in this study and in a previous study in HepG2 cells (3), NPC1L1 exhibits very little colocalization with a variety of Golgi markers (3). We define this perinuclear organelle as the ERC because it has extensive colocalization with transferrin (Fig. 3A), a unique ERC marker. The same group that reported that NPC1L1 has extensive colocalization with the Rab5A sorting endosome marker failed to show colocalization of NPC1L1 with transferrin (Fig. 3A), a unique ERC marker. The ERC is a unique ERC located in the ERC for recycling back to the plasma membrane (25). Because transferrin turns over quickly (25, 26), a pulse labeling of cells with transferrin may or may not reveal the transient colocalization of two signals.

![Image](https://example.com/image.png)

**FIGURE 5.** Cholesterol depletion relocates NPC1L1 proteins to the cell surface, preferentially to an apical-like subdomain. A, L1-EGFP cells grown on coverslips were incubated in cholesterol-rich Medium A (panel 1) or in Medium B containing 2% MJCD for 1 h to deplete cells of cholesterol (panel 2). The cells were then fixed in 3.7% formaldehyde in PBS, mounted on glass slides using a Prolong antifade kit, and visualized using a fluorescence confocal microscope. B, live L1-EGFP cells were incubated in serum-free DMEM containing 20 μg/ml iron-loaded transferrin-rhodamine conjugate for 30 min, followed by three washes with PBS and 1 h of incubation in Medium B with 2% MJCD. Cells were then visualized using a confocal microscope. DIC, differential interference contrast. C, HepG2 cells were treated with DMEM containing 2% MJCD or DMEM containing 2% MJCD and 0.08 mg/ml cholestrol for 30 min at 37 °C. Surface proteins were then biotinylated at 4 °C and precipitated with NeutrAvidin-agarose. Equal amounts of precipitated proteins were run on 5–17% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted for human NPC1L1 with L1Ab. The much smaller nonspecific protein bands were included as loading controls. The preimmune serum was used to show the specificity of L1Ab. D, L1-EGFP cells were grown in a 35-mm Corning culture dish in Medium A for 3 days, fixed, and visualized using an Axioplan 2 fluorescence microscope. The NPC1L1-positive ‘canalculus-like’ structure was occasionally observed in L1-EGFP cells. E, snap-frozen liver samples from African green monkey were sectioned, fixed, and immunostained as described under “Experimental Procedures” using antibody C219 (raised against canalicular P-glycoproteins), L1Ab, and the preimmune serum from the same rabbit. Images were taken using an Axioplan 2 fluorescence microscope and processed with Photoshop software. Rhodamine signal (red arrows) indicates serum staining, and fluorescein isothiocyanate signal (green arrows) denotes antibody C219 staining. Yellow color represents colocalization of two signals.
Cholesterol Uptake and Intracellular Trafficking of NPC1L1

which cholesterol depletion promotes polarization of L1-EGFP cells is unclear. It seems that overexpression of NPC1L1 is not required because the same polarized structures can sometimes be seen in cholesterol-depleted control cells (data not shown).

Intriguingly, NPC1L1-EGFP fusion proteins were concentrated in the apical-like structure in polarized L1-EGFP cells induced by cholesterol depletion (Fig. 5, A and B). The mechanism underlying this preferential sorting remains to be defined. However, it may be attributable to the nature of this protein and its interaction with microdomains of the plasma membrane. The molecular mechanisms responsible for the apical sorting of proteins in polarized cells are also largely unknown. NPC1L1 may share the same machinery with other apical proteins for apical sorting. Whatever the mechanisms are, our data suggest that cholesterol plays an important role in regulating the process. The apical location of NPC1L1 in hepatoma cells predicts a canalicular distribution of NPC1L1 in vivo, which was confirmed in monkey liver (Fig. 5E). The physiological significance for a canalicular location of NPC1L1 in hepatocytes remains to be elucidated. Both enteroctyes and hepatocytes are polarized cells with their apical surface exposed to micelles consisting of free cholesterol, phospholipids, and bile acids. NPC1L1 mediates intestinal cholesterol absorption from micelles in the intestinal lumen, and it may also promote cholesterol re-uptake from micelles in the canalicular bile. This canalicular re-uptake mechanism may have evolutionarily evolved to protect the body against excessive loss of cholesterol, a critical component of cell membranes. It is tempting to speculate that the pharmacological efficacy of the cholesterol absorption inhibitor ezetimibe may be partially attributed to the blocking of this canalicular re-uptake mechanism.

Previous in vitro reconstitution of NPC1L1-dependent cholesterol uptake has been unsuccessful (1), and the reason for this remains clear. In this study, we were able to reconstitute NPC1L1-mediated cholesterol uptake in cholesterol-depleted NPC1L1-expressing cells. Because NPC1L1 localized predominantly to intracellular compartments, a cholesterol uptake assay was performed in cells depleted of cholesterol, which relocated intracellular NPC1L1 to the cell surface. It is assumed that the cell-surface location is required for NPC1L1 to transport cholesterol from the medium into cells. Alternatively, the NPC1L1 protein may function as a free cholesterol receptor in the plasma membrane, or NPC1L1-mediated cholesterol uptake may depend on cholesterol binding to the protein. The normal culture medium was enriched with cholesterol as a result of addition of 10% fetal bovine serum. Under this condition, NPC1L1 proteins may bind to unlabelled cholesterol, becoming cholesterol-saturated, which leaves no more binding site(s) for the binding of isotope labeled cholesterol. The dependence of NPC1L1-mediated cholesterol uptake on cholesterol binding to the NPC1L1 protein may also explain why cholesterol is accumulated and NPC1L1 is localized predominantly to the intracellular compartment in normal cultured L1-EGFP cells (Figs. 4 and 5). Under this condition, the binding of unlabeled cholesterol from the cholesterol-rich medium to the NPC1L1 protein may result in 1) facilitated uptake of unlabeled cholesterol through NPC1L1 proteins, which in turn causes an accumulation of cholesterol in these cells because of unchanged cholesterol efflux and biosynthesis (data not shown); 2) trafficking back of NPC1L1 proteins from the plasma membrane to the intracellular compartment due to cholesterol-regulated translocation (Fig. 5A), and 3) failure in further detection of the NPC1L1-facilitated uptake of labeled cholesterol (Fig. 6). Depletion of free cholesterol by MβCD releases NPC1L1 binding sites for free cholesterol, allowing binding of exogenous labeled cholesterol to the protein. Thus, NPC1L1-dependent labeled cholesterol uptake could be measured in the cholesterol-depleted cells. In support

FIGURE 6. Cholesterol depletion enhances NPC1L1-dependent cholesterol uptake, which can be inhibited by the cholesterol absorption inhibitor ezetimibe. A, free cholesterol uptake was performed as described under “Experimental Procedures” with L1-EGFP and control EGFP cells with or without pretreatment with 2% MβCD for 1 h. Data are expressed as dpm/mg of cell proteins and represent the means ± S.E. of three independent experiments (n = 7). *, p < 0.05 (MβCD-treated cells versus untreated cells at each time point); **, p < 0.01 (MβCD-treated cells versus untreated cells at each time point). B, L1-EGFP or EGFP cells were pretreated with the indicated amounts of ezetimibe in Medium A for 1 h, followed by 2% MβCD treatment for 1 h. The cholesterol uptake assay was performed in the presence of ezetimibe as described under “Experimental Procedures.” Data are expressed as dpm/mg of cell proteins and represent the means ± S.E. of one of three independent experiments (n = 3). Values not sharing a common letter differ significantly (p < 0.01).

ports the ERC location of NPC1L1. The ERC contains recycling molecules, but lacks molecules destined for lysosomes (25, 26, 31). Thus, it is concluded that NPC1L1 proteins traffic via the endocytic recycling pathway.

An apparent relationship between NPC1L1 intracellular trafficking and cellular cholesterol content has been revealed in this study. Acute cholesterol depletion results in translocation of the NPC1L1 protein to the cell surface. A similar response was observed for transferrin trafficking (Fig. 5B), and it has been shown previously to be a result of blocked endocytosis instead of facilitated recycling (32). Whether the increased cell-surface location of NPC1L1 is caused by reduced endocytosis or enhanced recycling remains to be elucidated. Additionally, the signaling responsible for this cholesterol-regulated translocation has yet to be determined.

L1-EGFP cells are derived from a hepatoma cell line and have the potential to polarize. Interestingly, cholesterol depletion causes NPC1L1 proteins to disappear from the pericentriolar region and to reappear in a transferrin-positive subapical compartment and an apical-like structure (Fig. 5), suggesting that acute cholesterol depletion promotes polarization of L1-EGFP cells. Previous studies have shown that polarized HepG2 cells accumulate lipids and transferrin in the subapical compartment instead of the ERC as seen in non-polarized cells (29, 33). Similar apical-like structures were also observed in isolated rat hepatocyte couplets, polarized HepG2 cells, and WIF-B cells and, in these cases, were defined as a “bile canaliculus” (34–38). The mechanism by
of this explanation, cholesterol was recently shown to covalently bind to the purified SSD of SCAP, which is another SSD-containing protein that controls its own synthesis through receptor-ligand interaction, and SCAP was defined as an endoplasmic reticulum receptor for free cholesterol (39). The SSD-containing protein NPC1 (homolog of NPC1L1) was also shown to bind to photoactivable cholesterol (40). It would be interesting to address whether and through which sites NPC1L1 binds to free cholesterol.

The delineation of the intracellular itinerary of NPC1L1 and its regulation by cholesterol availability provides a platform for further study of the molecular mechanisms governing intracellular cholesterol trafficking. The in vitro reconstitution of NPC1L1-dependent cholesterol uptake provides an opportunity to dissect signaling pathways controlling cholesterol absorption from the intestine and perhaps re-uptake from the canalicular bile as well, which in turn may lead to the discovery of new approaches for the management of whole body cholesterol homeostasis.

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