The Niemann-Pick C1 gene is downregulated by feedback inhibition of the SREBP pathway in human fibroblasts

William S. Garver,[1,*] David Jelinek,[*] Gordon A. Francis,[†] and Bruce D. Murphy[§]

Department of Pediatrics,* University of Arizona, Tucson, AZ 85724-5037; Department of Medicine,[†] James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research, University of British Columbia, Vancouver, British Columbia, Canada V6Z 1Y6; and Centre de Recherche en Reproduction Animale,[§] Faculté de Médecine Vétérinaire, Université de Montréal, St. Hyacinthe, Québec, Canada J2S 7C6

Abstract The Niemann-Pick C1 (NPC1) protein regulates the transport of cholesterol from late endosomes/lysosomes to other compartments responsible for maintaining intracellular cholesterol homeostasis. The present study examined the expression of the NPC1 gene and the distribution of the NPC1 protein that resulted from the transport of LDL-derived cholesterol through normal human fibroblasts. A key finding was that the transport of cholesterol from late endosomes/lysosomes to the sterol-regulatory pool at the endoplasmic reticulum, as determined by feedback inhibition of the sterol-regulatory element binding protein (SREBP) pathway, was associated with the downregulation of the NPC1 gene. Consistent with these results, fibroblasts incubated with LDL had decreased amounts of SREBP protein that interacted with sterol-regulatory element (SRE) sequences positioned within the NPC1 gene promoter region. Finally, partial colocalization of the NPC1 protein with late endosomes/lysosomes and distinct regions of the endoplasmic reticulum suggested that the NPC1 protein may facilitate the transport of cholesterol directly between these two compartments. Together, these results indicate that the transport of LDL-derived cholesterol from late endosomes/lysosomes to the sterol-regulatory pool, known to be regulated by the NPC1 protein, is responsible for promoting feedback inhibition of the SREBP pathway and downregulation of the NPC1 gene.—Garver, W. S., D. Jelinek, G. A. Francis, and B. D. Murphy. **The Niemann-Pick C1 gene is downregulated by feedback inhibition of the SREBP pathway in human fibroblasts. *J. Lipid Res.* 2008.** 49: **1090–1102.**

Supplementary key words cholesterol homeostasis • coated-pit pathway • late endosomes/lysosomes • low density lipoprotein-derived cholesterol • sterol-regulatory element binding protein

Niemann-Pick C1 (NPC1) disease is an autosomal-recessive cholesterol-storage disorder characterized by liver dysfunction, hepatosplenomegaly, and progressive neurodegeneration (1, 2). At the cellular level, NPC1 disease is characterized by an accumulation of cholesterol within late endosomes/lysosomes derived from both endogenously synthesized cholesterol and the endocytosis of lipoprotein-derived cholesterol through the coated-pit pathway (3–6). Consistent with these results, a number of studies performed using different cell types have determined that the NPC1 protein is primarily associated with a unique subset of late endosomes that transiently interact with cholesterol-enriched late endosomes/lysosomes (7–11). Moreover, it is generally accepted that the NPC1 protein regulates the transport of cholesterol from late endosomes/lysosomes to other cellular compartments, specifically the Golgi apparatus, plasma membrane, and endoplasmic reticulum (12–15). Additional studies have indicated that proper function of the NPC1 protein is required for oxysterol generation and the upregulation of ABCA1 to facilitate the transport of cholesterol from late endosomes/lysosomes to the plasma membrane for efflux mediated by apolipoprotein A-I (16, 17).

Consistent with a generalized defect in the transport of lipoprotein-derived cholesterol through the coated-pit pathway, early studies using human NPC1 fibroblasts revealed a defect in the regulatory responses necessary for maintaining intracellular cholesterol homeostasis (18–20). The molecular basis for maintaining intracellular cholesterol homeostasis has largely been defined and is commonly referred to as the sterol-regulatory element binding protein (SREBP) pathway (21). In brief, a series of stud-

Manuscript received 28 November 2007 and in revised form 18 January 2008.

Published, JLR Papers in Press, February 13, 2008.
DOI 10.1194/jlr.M700555-JLR200

Abbreviations: ChIP, chromatin immunoprecipitation; CREB, cAMP response element binding protein; FAFA, fatty acid-free albumin; FCS, fetal calf serum; INSIG, insulin-induced gene; LAMP-1, lysosome-associated membrane protein-1; LPDS, lipoprotein-deficient serum; NPC1, Niemann-Pick C1; PDI, protein disulfide isomerase; P/S, penicillin/streptomycin; SCAP, SREBP-cleavage-activating protein; SRE, sterol-regulatory element; SREBP, sterol-regulatory element binding protein.

[1] To whom correspondence should be addressed.
e-mail: wgarver@peds.arizona.edu

ies have determined that cholesterol and oxysterols specifically bind to the SREBP-cleavage-activating protein (SCAP) and insulin-induced gene (INSIG) protein, respectively, to inhibit the translocation and processing of precursor SREBP (p-SREBP) proteins to produce mature SREBP (m-SREBP) proteins that serve as nuclear transcription factors (22–26). The m-SREBP proteins have been shown to interact with highly variable *cis* elements (5′-nTCACnCCACn-3′) called sterol-regulatory element (SRE) sequences that are positioned within the promoter region of target genes (27, 28). With respect to how the NPC1 protein functions to alter the regulation of the SREBP pathway, studies performed using NPC1-deficient fibroblasts and mouse livers have demonstrated that, despite an increased amount of total cellular cholesterol, the decreased transport of cholesterol from late endosomes/lysosomes to the sterol-regulatory pool increases the transcription of SREBP genes and the processing of p-SREBP proteins, thereby promoting the activation of the SREBP pathway and the expression of target genes containing SRE sequences (18, 19, 29, 30).

To date, a number of studies performed in vitro and in vivo have reported that expression of the NPC1 gene is constitutive and not regulated by the amount of cellular cholesterol or the SREBP pathway (31–35). However, other studies performed in vitro using both human and porcine cells have confirmed the presence of consensus sequences contained within the 5′-flanking region of the NPC1 gene capable of binding various transcription factors, among them specificity protein-1, cAMP response element binding protein (CREB)/activating transcription factor-1, activating protein-1, modified zinc finger protein-1, and transcription factor-II (31, 36). Further studies performed using porcine steroidogenic cells have determined that the NPC1 gene is regulated by the cAMP-protein kinase A pathway, along with the phosphorylation of CREB, the recruitment of the coactivator CREB binding protein, and the phosphorylation/acetylation of the histone H-3 protein (37). Importantly, because these particular transcription factors are known to activate SRE sequences in conjunction with the SREBP pathway, it was suspected that expression of the NPC1 gene may also be regulated by the amount of cellular cholesterol.

To ascertain whether cholesterol may have a role in regulating the expression of the NPC1 gene, the present study was performed by incubating normal human fibroblasts in the absence or presence of LDL to increase the transport of LDL-derived cholesterol to the sterol-regulatory pool and feedback inhibition of the SREBP pathway. In brief, it was determined that feedback inhibition of the SREBP pathway, characterized by decreased amounts of SREBP mRNA and the processing of p-SREBP proteins, was directly correlated with decreased amounts of NPC1 mRNA and NPC1 protein. Because the relative amounts of NPC1 mRNA and NPC1 protein were directly correlated, expression of the NPC1 gene was primarily regulated at the transcriptional level. Moreover, it was determined that fibroblasts incubated in the presence of LDL had decreased amounts of m-SREBP protein that interacted with SRE sequences positioned within the NPC1 gene promoter region, which directly correlated with the decreased transcription of the NPC1 gene. These results were confirmed by cotransfection studies verifying that the m-SREBP protein promoted the transcription of an NPC1 gene promoter-luciferase reporter construct. Together, these results indicate that the NPC1 gene is downregulated by LDL-derived cholesterol feedback inhibition of the SREBP pathway.

MATERIALS AND METHODS

Materials

DMEM, Opti-MEM I, PBS, trypsin-EDTA, and penicillin/streptomycin (P/S) for cell culture were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal calf serum (FCS) and lipoprotein-deficient serum (LPDS) were purchased from Cocalico Laboratories (Reamstown, PA). Complete protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Indianapolis, IN). Purified cholesterol and cholesteryl oleate standards and fatty acid-free albumin (FAFA; fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). To extract RNA and generate cDNA, the Qiaquik PCR purification column, RNeasy Mini Kit, and RNase-Free DNase Kit were purchased from Qiagen (Valencia, CA). To transfect human granulosa cells, Effectene Transfection Reagent was purchased from Qiagen. The Luciferase Reporter Vector (pGL3) and Renilla Luciferase Vector (pRL.SV40) were purchased from Promega Corp. (Madison, WI). The TaqMan Gene Expression Assay, TaqMan PCR Master Mix, and the cDNA primers for NPC1 (Hs00264835_M1), SREBP-1 (Hs00231674_M1), and SREBP-2 (Hs00190237_M1) were purchased from Applied Biosystems (Foster City, CA). The human NPC1 antibody, generated against amino acids 1,254–1,273 (NKAKSCATEERYGTERER) of the human NPC1 protein, was produced, affinity-purified, and purchased from Invitrogen Corp. The antibody for protein disulfide isomerase (PDI; clone RL77) was purchased from Affinity BioReagents (Golden, CO). The antibodies for SREBP-1 (K-10) and lysosome-associated membrane protein-1 (LAMP-1; clone H4A3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for SREBP-2 (clone IgG-1C6) was purchased from BD BioSciences (San Diego, CA). The antibody for β-actin (clone AC-74) was purchased from Sigma Chemical Co. Peroxidase-, Cy2-, and Cy3-conjugated goat secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Aqua Poly/Mount for mounting glass coverslips onto slides was purchased from Polysciences, Inc. (Warrington, PA). The West Pico SuperSignal Substrate for Western Blotting and Bicinchoninic Acid Protein Assay Kits were purchased from Pierce Chemical Co. (Rockford, IL).

Preparation of low density lipoprotein

Human LDL (d = 1.019–1.063) was obtained from the pooled plasma of healthy volunteers and isolated using standard discontinuous density gradient ultracentrifugation (38).

Cell culture and harvest

Normal human fibroblasts (CRL-2097) were purchased from the American Type Culture Collection (Manassas, VA) and maintained in a humidified incubator at 37°C equilibrated with 5% CO_2 and 95% air. Fibroblasts were seeded onto plates or coverslips using DMEM containing 10% FCS and

1% P/S (DMEM, 10% FCS, 1% P/S). Once the fibroblasts reached ~50% confluence, the medium was removed and the cells were rinsed three times with PBS followed by changing the medium to DMEM containing 5% LPDS and 1% P/S (DMEM, 5% LPDS, 1% P/S), which has been shown to deplete cellular sterol pools, increase the expression of the LDL receptor, and thereby promote the endocytosis of LDL into the coated-pit pathway (39, 40). After the fibroblasts reached ~80% confluence (48 h), the medium was removed and changed to medium without LDL (DMEM, 5% LPDS, 1% P/S) or medium with LDL (DMEM, 5% LPDS, 1% P/S, with 50 μg/ml LDL) to promote the endocytosis of LDL (24 h) and the transport of LDL-derived cholesterol through the cell. Finally, to determine the effects of incubating fibroblasts in the absence of serum, fibroblasts were rinsed with PBS containing 2 mg/ml FAFA (PBS, 2 mg/ml FAFA) followed by changing the medium to DMEM containing 2 mg/ml FAFA and 1% P/S (DMEM, 2 mg/ml FAFA, 1% P/S) to promote the equilibration of cellular sterol pools (24 h).

For studies described in this report, the fibroblasts grown in medium without LDL (denoted LPDS) were considered the basal or control culture condition, whereas fibroblasts grown in medium with LDL (denoted LDL) were considered the test culture condition. For one study, the fibroblasts were also rinsed and incubated in the absence of serum to promote the equilibration of cellular sterol pools after growth in medium without LDL (denoted LPDS-E) and with LDL (denoted LDL-E). For each of these culture conditions, the fibroblasts were harvested for experimentation by one of the following methods: *i*) rinsing three times with ice-cold PBS followed by extraction of total lipids using hexane-isopropanol (cholesterol and cholesteryl ester analysis); *ii*) rinsing three times with ice-cold PBS followed by extraction of total RNA using the RNeasy Mini Kit (quantitative RT-PCR analysis); *iii*) rinsing three times with ice-cold PBS followed by collection of cells with PBS containing a protease inhibitor cocktail (immunoblot analysis); *iv*) cross-linking of protein associated with DNA using formaldehyde, then rinsing three times with ice-cold PBS and collection of cells with PBS containing a protease inhibitor cocktail [chromatin immunoprecipitation (ChIP) analysis]; or *v*) removal of medium and fixation of cells using PBS containing paraformaldehyde (fluorescence microscopy analysis).

Cholesterol and cholesteryl ester analysis

To determine the concentration of cholesterol and cholesteryl ester, the fibroblasts were rinsed three times with ice-cold PBS and total lipids were extracted with hexane-isopropanol (3:2, v/v). The organic phase was dried with nitrogen gas, and the resulting lipids were suspended into chloroform and separated using a conventional silica TLC plate developed with hexane-diethyl ether-glacial acetic acid (80:20:1, v/v/v). Cholesterol and cholesteryl ester were identified by comigration with purified standards after staining with iodine vapor. After destaining, the cholesterol and cholesteryl ester were scraped from the plate, hydrolyzed using ethanol containing 0.1 N KOH (1 h at 80°C), and then extracted with hexane-water (3:1, v/v). A fraction of the hexane phase was collected and dried with nitrogen gas. Finally, the amount of cholesterol and cholesteryl ester was determined using the cholesterol oxidase method (41). The mass of cholesterol and cholesteryl ester was normalized to the corresponding protein that was precipitated with organic solvent and measured using the bicinchoninic acid protein assay method to then determine the cellular concentration of cholesterol and cholesteryl ester, respectively.

Quantitative RT-PCR analysis

The relative amounts of NPC1, SREBP-1, and SREBP-2 mRNA were determined using quantitative RT-PCR analysis. Total RNA was extracted from fibroblasts using the RNeasy Mini Kit followed by treatment of the RNA with RNase-free DNase to remove contaminating DNA. The concentration of RNA was determined by absorbance at 260 nm using a spectrophotometer. Reverse transcription was performed using 1.0 μg of total RNA, 2.5 μM random hexamers, 4.0 mM dNTPs, 15 mM $MgCl_2$, 50 units of reverse transcriptase, and 100 units of RNA inhibitor in a 40 μl solution to produce cDNA. Finally, quantitative RT-PCR was performed using the cDNA, a TaqMan Gene Expression Assay containing a 20× mixture of unlabeled PCR primers and TaqMan MGB probe (FAM dye-labeled), and the TaqMan Universal PCR Master Mix. The detection and quantification of specific sequences converted to cDNA was performed using the ABI-PRISM Sequence Detection System (Applied Biosystems). The relative amounts of target cDNA and endogenous control (18S rRNA) were quantified and then normalized to the control.

Immunoblot analysis

The relative amounts of NPC1, SREBP-1, and SREBP-2 protein were determined using immunoblot analysis. Protein samples were separated using 6% or 12% SDS-PAGE under reduced conditions and then transferred to a nitrocellulose membrane (42). In brief, blocking buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk) was used to block nonspecific sites on the nitrocellulose membrane (2 h). The membranes were then incubated in blocking buffer containing the appropriate dilution of primary antibody (16 h at 4°C). For the SREBP-1 and SREBP-2 proteins, both the 125 kDa p-SREBP protein and the 68 kDa m-SREBP protein were detected using respective antibodies. The membranes were rinsed with blocking buffer (3 × 10 min) to remove residual primary antibody and then incubated in blocking buffer containing the appropriate dilution of peroxidase-conjugated goat secondary antibody (90 min). The membranes were rinsed with blocking buffer (3 × 10 min) to remove residual secondary antibody, and enhanced chemiluminescence was performed to obtain autoradiograms. The relative amounts of target protein and endogenous control protein (β-actin) were quantified within the linear range of film using a Bio-Rad model GS-700 Imaging Densitometer and then normalized to the control values.

NPC1 gene promoter activity analysis

A fragment of the human NPC1 gene promoter region spanning 1.9 kb upstream from the transcriptional start site was cloned into the Luciferase Reporter Vector (pGL3). A human granulosa cell line (SVG40), generously provided by Dr. Peter Leung from the University of British Columbia, was cultured in Opti-MEM I containing 5% FCS and 1% P/S. These cells were transfected with the pGL3 vector containing the NPC1 gene promoter region using Effectene Transfection Reagent (100 nM/well), followed by cotransfection with either 10 ng of the empty Renilla Luciferase Vector (pRL.SV40), which served as a control, or with 10 ng of this vector capable of expressing mature (and transcriptionally active) SREBP-1c protein to induce NPC1 gene promoter activity, as described in a companion study (N. Y. Gévry et al). To normalize the results for transfection efficiency, the cotransfection of pNPC1-LUC/pRL.SV40 was performed at a ratio of 10:1, respectively.

ChIP analysis

To cross-link the associated proteins with DNA, fibroblasts were treated with PBS containing 1.0% formaldehyde (10 min),

rinsed with ice-cold PBS containing a protease inhibitor cocktail (3 × 1 min), then scraped from the plate and collected by centrifugation (2,500 rpm for 4 min at 4°C). Cells were resuspended in 200 μl of lysis buffer (50 mM Tris, pH 8.1, 1.0% SDS, 10 mM EDTA, and a protease inhibitor cocktail) followed by incubation on ice (10 min), sonication, and removal of insoluble material by centrifugation (13,000 rpm for 10 min at 4°C). The resulting supernatent was diluted 10-fold in dilution buffer (16.7 mM Tris, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and a protease inhibitor cocktail). A 20 μl aliquot of the diluted supernatant was used for the purification of total DNA. To reduce nonspecific background, the samples were precleared by incubating in a 50% gel slurry that contained 40 μl of mouse serum and salmon sperm DNA/protein A-agarose (60 min at 4°C). After centrifugation to remove the beads, samples (2.0 ml) were divided equally and incubated with SREBP antibody (16 h at 4°C). The chromatin immunocomplex was collected using 50 μl of protein A-agarose (2 h at 4°C) and then rinsed once with each of the following buffers in sequence: low-salt rinse buffer (20 mM Tris, pH 8.1, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, and 2.0 mM EDTA); high-salt rinse buffer (20 mM Tris, pH 8.1, 500 mM NaCl, 0.1% SDS, 1.0% Triton X-100, and 2.0 mM EDTA); LiCl rinse buffer (10 mM Tris, pH 8.1, 0.25 M LiCl, 1.0% Nonidet P-40, 1.0% sodium deoxycholate, and 1.0 mM EDTA); and TE rinse buffer (10 mM Tris, pH 8.1, and 1.0 mM EDTA). The products were eluted using 250 μl of elution buffer (0.1 M $NaHCO_3$ and 1.0% SDS), and the cross-linked protein-DNA complex was reversed by incubation (6 h at 65°C) followed by treatment with proteinase K and purification of the DNA using a Qiaquik PCR purification column. A 255 bp fragment within the proximal NPC1 promoter region was amplified by PCR using both total and immunoprecipitated DNA. As a control, a sequence within the NPC1 gene open reading frame (353 bp) was amplified using both total and immunoprecipitated DNA. The specific primer sequences used for the PCR amplification of a region within the NPC1 gene promoter region containing the three SRE sequences, in addition to a region within the NPC1 gene open reading frame, are listed in **Table 1**. The resulting PCR products were resolved using agarose gels followed by visualization and quantification using the Alpha-Imager gel documentation system.

Fluorescence labeling

The fibroblasts grown on coverslips were removed from the incubator and the medium was immediately replaced with fixative buffer (PBS containing 3% paraformaldehyde) and incubated (30 min). After fixation, the fibroblasts were rinsed with PBS (3 × 5 min) and placed in quenching buffer (PBS containing 50 mM NH_4Cl) and incubated (15 min). Fibroblasts were rinsed with PBS (3 × 5 min) to remove residual NH_4Cl and placed in either blocking/cholesterol-staining buffer (PBS containing 10% goat serum and 0.05% filipin) or blocking/permeabilization buffer (PBS containing 10% goat serum and 0.05% saponin) and incubated (90 min). The second set of fibroblasts were then placed in blocking/permeabilization buffer containing the appropriate dilution of primary antibodies to label target proteins (90 min). Fibroblasts were rinsed with PBS (3 × 5 min) to remove residual primary antibody and then placed in blocking/permeabilization buffer containing the appropriate dilution of Cy2- and Cy3-conjugated goat secondary antibodies (90 min). Finally, both sets of fibroblasts were rinsed with PBS (3 × 5 min) to remove residual filipin or secondary antibodies, and the coverslips were mounted onto slides using Aqua Poly/Mount.

TABLE 1. Primer sequences used to perform PCR for the NPC1 gene promoter region and the NPC1 gene open reading frame

NPC1 gene promoter region	
P1	5′-GCACTACCCTTCGCTGAAACCTCG-3′
P2	5′-AGGAAGAAGGCGTCGTCGGCTC-3′
NPC1 gene open reading frame	
C1	5′-CAGATTACCTGTTTCGTGAGTC-3′
C2	5′-GGAAGAAGTGTAGTCGTGCC-3′

NPC1, Niemann-Pick C1.

Fluorescence microscopy

Conventional fluorescence microscopy was performed to visualize the distribution of cellular cholesterol. A fluorescence microscope equipped with a Nikon 20×, numerical aperture 0.75, objective and a Nikon 60×, numerical aperture 1.4, oil-immersion objective was used with an excitation wavelength of 360 nm, in conjunction with an emission filter of 460 nm. Confocal fluorescence microscopy was performed to visualize the relative distribution of NPC1 protein with LAMP-1 and PDI. A Bio-Rad MCR-1024 ES laser scanning confocal microscope equipped with a Nikon 100×, numerical aperture 1.4, oil-immersion objective was used with excitation wavelengths of 488 and 568 nm, in conjunction with fluorescein/Cy2 and Cy3 emission filters (522 DF 35 and HQ 598 40) and simultaneous recording. Images obtained using confocal microscopy were derived from a single optical section estimated to be 1.0 μm thick. For merged images, the separate Cy2 and Cy3 images were adjusted to similar intensities and then merged using Adobe Photoshop 6.0.

Statistical analysis

For all experiments, quantitative data are represented as means ± SD using five plates of cells (cholesterol and cholesteryl ester analysis) or three plates of cells (quantitative RT-PCR analysis, immunoblot analysis, NPC1 gene promoter analysis, and ChIP analysis) for the respective culture conditions. The reported results are representative of three independent experiments. Significant differences ($P \leq 0.05$) between groups of data were determined using the two-tailed Student's *t*-test assuming equal variance.

RESULTS

Concentration and distribution of cellular cholesterol

The concentration of cellular cholesterol and cholesteryl ester was determined in fibroblasts grown in medium without or with LDL (**Fig. 1**). As expected, compared with fibroblasts grown in medium without LDL, considered the basal or control condition, fibroblasts grown in medium with LDL had a significant increase in the concentration of cholesterol and cholesteryl ester (3- and 25-fold, respectively). With respect to the total amount of sterol that was internalized into the cell, approximately equal amounts of LDL-derived cholesterol contributed to the increased concentration of cholesterol and cholesteryl ester (Fig. 1A, B). The distribution of cellular cholesterol was also determined in fibroblasts grown in medium without or with LDL. The results indicated two prominent cellular distributions for cholesterol, with one distribution represented by various perinuclear cytoplasmic vesicles and the other represented by the plasma membrane (Fig. 1C, D). As expected, compared with fibroblasts grown

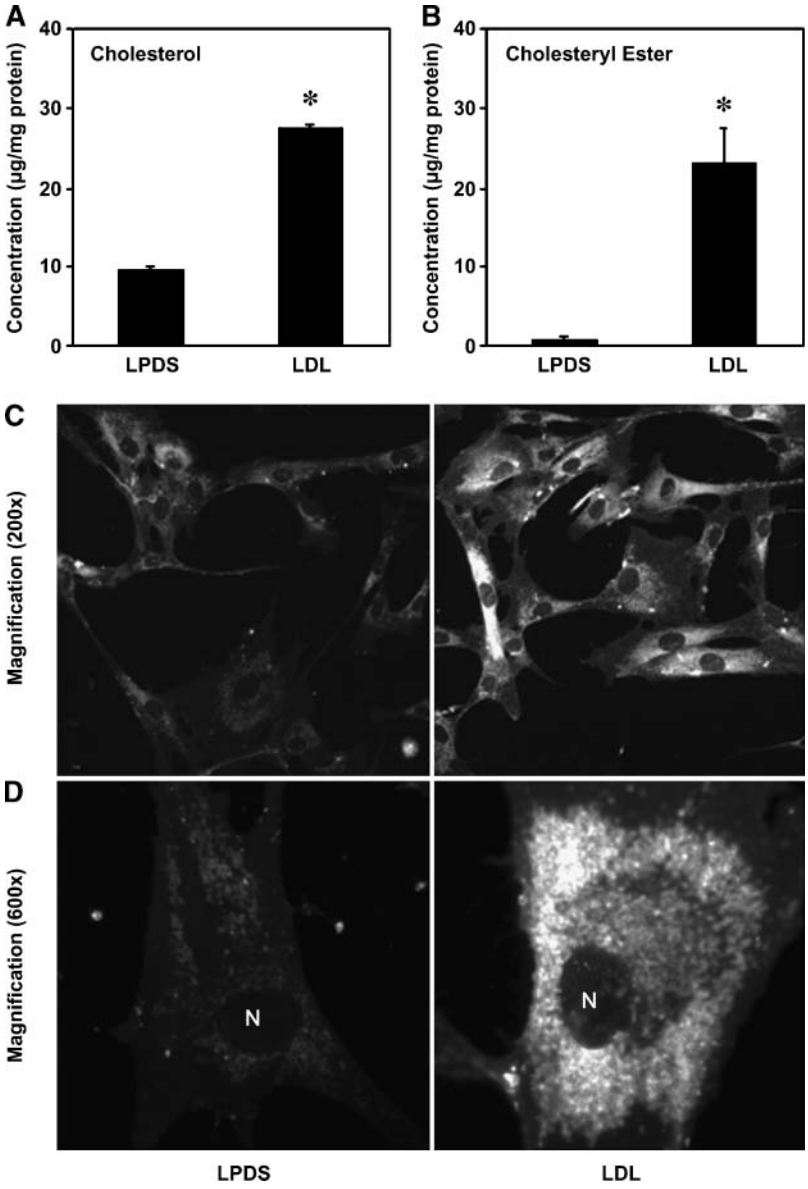

Fig. 1. Concentration and distribution of cellular cholesterol. The fibroblasts were cultured and processed as described in Materials and Methods. The fibroblasts are denoted as grown in medium without LDL [lipoprotein-deficient serum (LPDS)] or medium with LDL (LDL). The amount of cellular cholesterol and cholesteryl ester from each plate was normalized to the corresponding cell protein that was precipitated during extraction to provide the concentration of cellular cholesterol (A) and cholesteryl ester (B). The values represent means ± SD obtained from five plates of fibroblasts. * $P \leqslant 0.05$ compared with fibroblasts grown in medium without LDL. The distribution of cellular cholesterol was determined by staining fibroblasts with filipin and then performing fluorescence microscopy. Fluorescence images were obtained using a conventional fluorescence microscope equipped with a Nikon 20×, numerical aperture 0.75, objective to visualize a field of cells (C) and a Nikon 60×, numerical aperture 1.4, oil-immersion objective to visualize a single cell (D). Images are representative of other fibroblasts grown on the same coverslip. All images were acquired and processed using identical conditions. N, nucleus.

in medium without LDL, the fibroblasts grown in medium with LDL were characterized by enhanced staining of the cytoplasmic vesicles and plasma membrane, consistent with these cells having an increased concentration of cellular cholesterol. Therefore, the perinuclear cytoplasmic vesicles, suspected to represent primarily endosomes and lysosomes, served to store a large portion of the LDL-derived cholesterol.

Relative amounts of SREBP mRNA and SREBP protein

The relative amounts of SREBP-1 and SREBP-2 mRNA, and SREBP-1 and SREBP-2 protein, were determined in fibroblasts grown in medium without or with LDL (**Fig. 2**). The results indicated that compared with the amounts of SREBP-1 and SREBP-2 mRNA in fibroblasts grown in medium without LDL, the amounts of SREBP-1 and SREBP-2 mRNA were decreased significantly (to 56% and 22%, respectively) in fibroblasts grown in medium with LDL (Fig. 2A). With respect to the amount of p-SREBP-1 protein, there was no significant difference between fibroblasts grown in either culture condition (Fig. 2B). However, compared with fibroblasts grown in medium without LDL, the amount of p-SREBP-2 protein was decreased significantly (to 66%) in fibroblasts grown in medium with LDL (Fig. 2B). Finally, compared with fibroblasts grown in medium without LDL, the amounts of m-SREBP-1 and m-SREBP-2 protein were both decreased significantly (to 38% and 27%, respectively) in fibroblasts grown in medium with LDL (Fig. 2C). Therefore, fibroblasts grown in medium with LDL were characterized by decreased transcription of the SREBP genes, in addition to decreased processing of the p-SREBP proteins to produce m-SREBP proteins, indicating feedback inhibition of the SREBP pathway.

Relative amounts of NPC1 mRNA and NPC1 protein

The relative amounts of NPC1 mRNA and NPC1 protein were determined when fibroblasts were grown using four

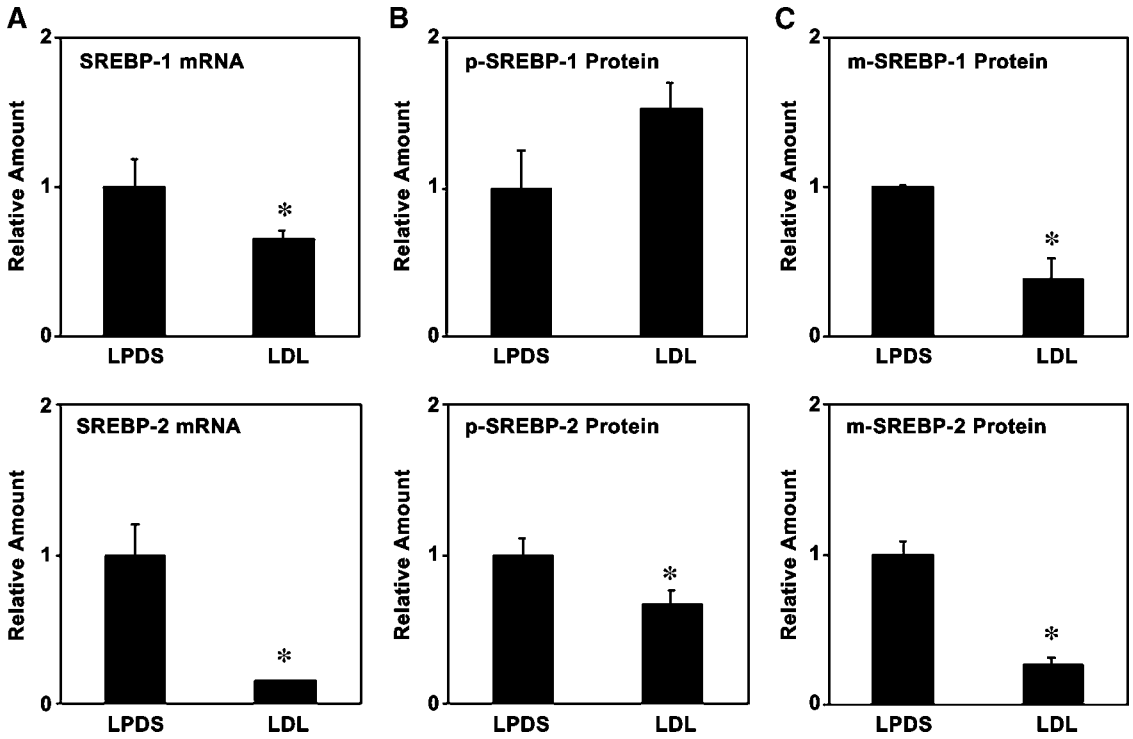

Fig. 2. Relative amounts of sterol-regulatory element binding protein (SREBP) mRNA and SREBP protein. The fibroblasts were cultured and processed as described in Materials and Methods. The fibroblasts are denoted as grown in medium without LDL (LPDS) or medium with LDL (LDL). The relative amounts of SREBP-1 mRNA and SREBP-2 mRNA (A) were determined using quantitative RT-PCR after normalization to the corresponding amounts of 18S rRNA (internal control). The average amounts of SREBP-1 mRNA and SREBP-2 mRNA for fibroblasts grown in medium without LDL were assigned an arbitrary value of 1.0, with the average amounts of SREBP-1 mRNA and SREBP-2 mRNA for fibroblasts grown in medium with LDL expressed as fold change. The relative amounts of p-SREBP-1 and p-SREBP-2 protein (B), in addition to m-SREBP-1 and m-SREBP-2 protein (C), were determined using immunoblot analysis after normalization to the corresponding amounts of β-actin (internal control). The average amounts of p-SREBP-1, p-SREBP-2, m-SREBP-1, and m-SREBP-2 protein for fibroblasts grown in medium without LDL were assigned an arbitrary value of 1.0, with the average amounts of p-SREBP-1, p-SREBP-2, m-SREBP-1, and m-SREBP-2 protein for fibroblasts grown in medium with LDL expressed as fold change. The values represent means ± SD. * $P \leq 0.05$ compared with fibroblasts grown in medium without LDL.

different culture conditions. The culture conditions consisted of fibroblasts grown in medium without LDL (LPDS) or with LDL (LDL) and fibroblasts incubated in the absence of serum to promote the equilibration of cellular sterol pools after growth in medium without LDL (LPDS-E) or with LDL (LDL-E) (**Fig. 3**). The amount of NPC1 mRNA in fibroblasts grown in medium with LDL was decreased significantly (to 39%) compared with the amount of NPC1 mRNA in fibroblasts grown in medium without LDL (Fig. 3A). Moreover, although the amount of NPC1 mRNA was not significantly different when fibroblasts were grown in LPDS and equilibrated in the absence of serum, the amount of NPC1 mRNA was decreased further (to 25%) when fibroblasts were grown in LDL and equilibrated in the absence of serum. Similarly, the amount of NPC1 protein in fibroblasts grown in medium with LDL was also decreased significantly, but to a lesser extent (to 70%), compared with the amount of NPC1 protein in fibroblasts grown in medium without LDL (Fig. 3B). Consistent with the relative amounts of NPC1 mRNA, the amount of NPC1 protein was not significantly different when fibroblasts were grown in LPDS and equilibrated in the absence of serum, whereas the amount of NPC1 protein was decreased further (to 40%) when fibroblasts were grown in LDL and equilibrated in the absence of serum. Although speculative, the slight increase in the relative amount of NPC1 protein compared with the amount of NPC1 mRNA for each of the culture conditions may result from a decreased rate of protein degradation. In support of this premise, although the theoretical molecular mass for the NPC1 protein has been calculated to be ~140 kDa, the NPC1 protein migrated at a molecular mass equivalent to 170 and 190 kDa using SDS-PAGE, consistent with other studies describing the NPC1 protein as being represented by two highly glycosylated isoforms (Fig. 3C). Therefore, the relative amounts of NPC1 mRNA and NPC1 protein are directly correlated, consistent with the expression of the NPC1 gene being primarily regulated at the transcriptional level. Moreover, the NPC1 protein is represented by two highly glycosylated protein isoforms that may in part serve to decrease the rate of protein degradation.

NPC1 gene promoter activity and ChIP analysis

To determine whether the m-SREBP-1c protein was capable of promoting transcription of the NPC1 gene, a human granulosa cell line was cotransfected with an

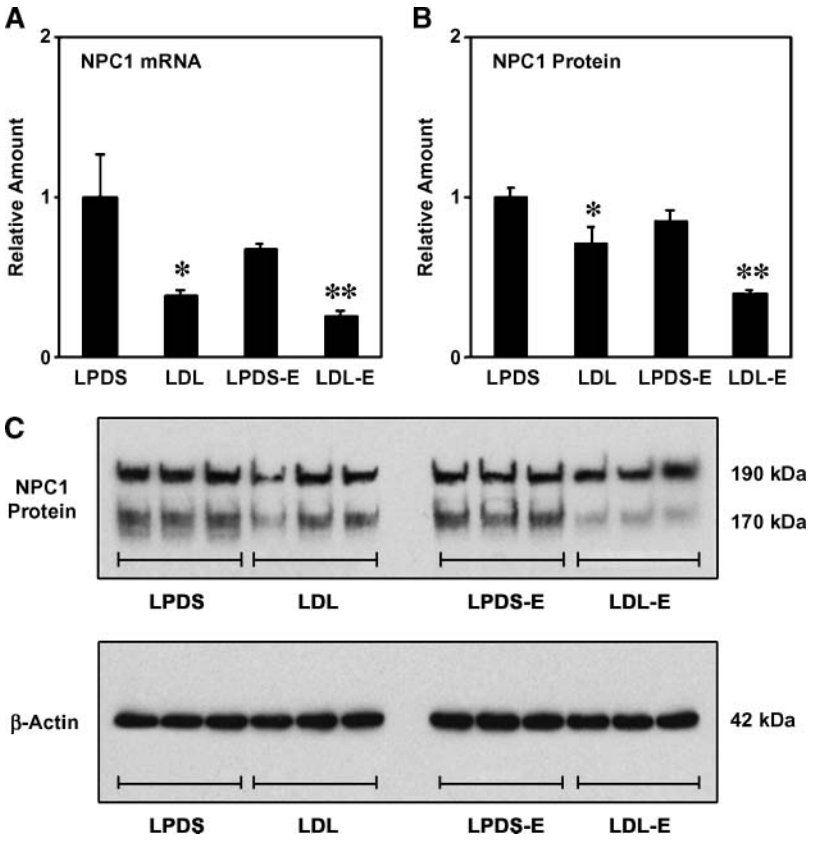

Fig. 3. Relative amounts of Niemann-Pick C1 (NPC1) mRNA and NPC1 protein. The fibroblasts were cultured and processed as described in Materials and Methods. The fibroblasts are denoted as grown in medium without LDL (LPDS) or medium with LDL (LDL), in addition to fibroblasts that were incubated in the absence of serum to promote the equilibration of cellular sterol pools after growth in medium without LDL (LPDS-E) or with LDL (LDL-E). The amounts of NPC1 mRNA were determined using quantitative RT-PCR after normalization to the corresponding amounts of 18S rRNA (internal control). The average amount of NPC1 mRNA for fibroblasts grown in medium without LDL was assigned an arbitrary value of 1.0, with the average amounts of NPC1 mRNA for fibroblasts grown using the other culture conditions expressed as fold change (A). The amounts of NPC1 protein were determined using immunoblot analysis after normalization to the corresponding amounts of β-actin (internal control). The average amount of NPC1 protein for fibroblasts grown in medium without LDL was assigned an arbitrary value of 1.0, with the average amounts of NPC1 protein for fibroblasts grown using the other culture conditions expressed as fold change (B). The values represent means ± SD. * $P \leq 0.05$ compared with fibroblasts grown in medium without LDL; ** $P \leq 0.05$ compared with fibroblasts grown in medium with LDL. Representative immunoblots of the NPC1 protein and β-actin obtained from three different plates of cells for each culture condition are provided (C).

NPC1 gene promoter-luciferase reporter construct in the absence or presence of a vector that expressed the transcriptionally active form of the SREBP-1c protein (**Fig. 4**). The results indicated that compared with control cells that did not express m-SREBP-1c protein, there was a significant increase (>20-fold) in the amount of NPC1 gene promoter activity (Fig. 4A). Moreover, to further examine whether the SREBP pathway has a role in regulating the expression of the NPC1 gene, ChIP analysis was performed on fibroblasts grown in medium without or with LDL. Companion studies have previously defined three putative SRE sequences in the first kilobase upstream from the transcriptional start site of the human NPC1 gene (Fig. 4D). To perform this study, the protein/DNA complex was immunoprecipitated with an SREBP antibody followed by amplification of a region that was 255 bp upstream from the transcriptional start site that contained the three putative SRE sequences. The results indicated a robust association between SREBP proteins and the NPC1 promoter region when fibroblasts were grown in medium without LDL (Fig. 4B, C). In contrast, this association was decreased significantly (to ~50%) in fibroblasts grown in medium with LDL, similar to the decreased amount of NPC1 mRNA that was determined when fibroblasts were grown using this culture condition. Specificity for the association between SREBP proteins and the 5′-flanking region of the NPC1 gene was confirmed by the absence of amplification for a sequence within the NPC1 gene open reading frame after immunoprecipitation. Therefore, fibroblasts grown in medium without LDL had increased interaction of SREBP protein with the NPC1 promoter region and increased transcription of the NPC1 gene compared with significantly lower interaction and transcription of the NPC1 gene when fibroblasts were grown in medium with LDL.

Cellular distribution of the NPC1 protein in relation to LAMP-1 and PDI

The cellular distribution of the NPC1 protein in relation to LAMP-1 and PDI was determined in fibroblasts grown in medium without or with LDL (**Fig. 5**). With respect to the cellular distribution of the NPC1 protein in relation to LAMP-1 (a protein marker for late endosomes/lysosomes), the results indicated two distinct distributions for the NPC1 protein (Fig. 5A–C). The major distribution of the NPC1 protein was represented by a diffuse punctate staining pattern within the perinuclear region of the cytoplasm, whereas a minor distribution was represented by colocalization with LAMP-1-positive vesicles (late endosomes/lysosomes). As described previously, fibroblasts grown in medium with LDL have an increased proportion of the NPC1 protein that colocalizes with late endosomes/lysosomes. As shown in the enlarged insets for each merged image, the NPC1 protein was found to be confined to the limiting membrane of late endosomes/lysosomes. With respect to the cellular distribution of the NPC1 protein in relation to PDI (a protein marker for the endoplasmic reticulum), the results again demonstrated

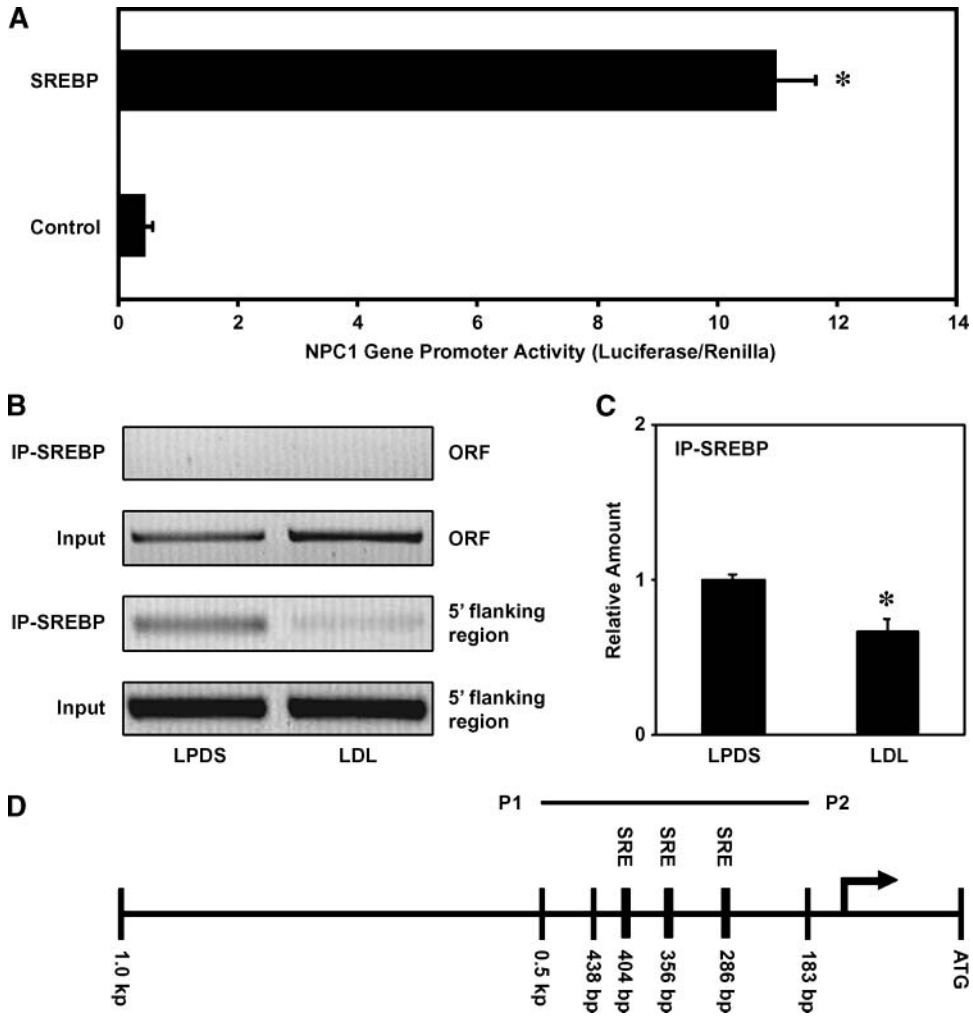

Fig. 4. NPC1 gene promoter activity and chromatin immunoprecipitation (ChIP) analysis. A fragment of the human NPC1 gene promoter region spanning 1.9 kb upstream from the transcriptional start site was cloned into the Luciferase Reporter Vector (pGL3) and transfected into a human granulosa cell line (SVG40) as described in Materials and Methods. The cells were then cotransfected with either 10 ng of an empty Renilla Luciferase Vector (pRL.SV40), which served as a control, or 10 ng of the pRL.SV40 vector capable of expressing mature (and transcriptionally active) m-SREBP-1c protein to induce NPC1 gene promoter activity (A). The amount of SREBP protein interacting with the NPC1 gene promoter region needed to induce transcription was determined using ChIP analysis. The fibroblasts were cultured and processed as described in Materials and Methods. The fibroblasts are denoted as grown in medium without LDL (LPDS) or medium with LDL (LDL). The protein/DNA complexes were immunoprecipitated with an SREBP antibody (IP-SREBP) or without an SREBP antibody using lysate (Input). After dissociation of the protein/DNA complex, the DNA was amplified using PCR primers based on a sequence that contained three sterol-regulatory element (SRE) sequences within the NPC1 gene promoter region in addition to PCR primers based on a sequence within the NPC1 gene open reading frame (ORF). Representative images of the resulting PCR products (B) and quantification by scanning densitometry of the PCR amplicons derived from the 5′-flanking region of SREBP antibody-immunoprecipitated DNA obtained from three experiments (C) are provided. The average amount of PCR product for fibroblasts grown in medium without LDL was assigned an arbitrary value of 1.0, with the average amount of PCR product for fibroblasts grown in medium with LDL expressed as fold change. The values represent means ± SD of three replicates within a typical experiment. * $P \leq 0.05$ compared with human granulosa cells not expressing mature SREBP-1c protein or human fibroblasts grown in medium without LDL. A schematic illustration of the 5′-flanking region for the human NPC1 gene promoter region indicates the three SRE sequences (numbered relative to the ATG) and the NPC1 gene promoter region that was amplified by the P1 and P2 primers (D).

two distinct distributions of the NPC1 protein (Fig. 5D–F). The major distribution of the NPC1 protein was again represented by a diffuse punctate staining pattern present within the perinuclear region of the cytoplasm, whereas another minor distribution was represented by colocalization with a reticular PDI-positive compartment (endoplasmic reticulum). As shown in the enlarged insets for each merged image, the NPC1 protein was associated with

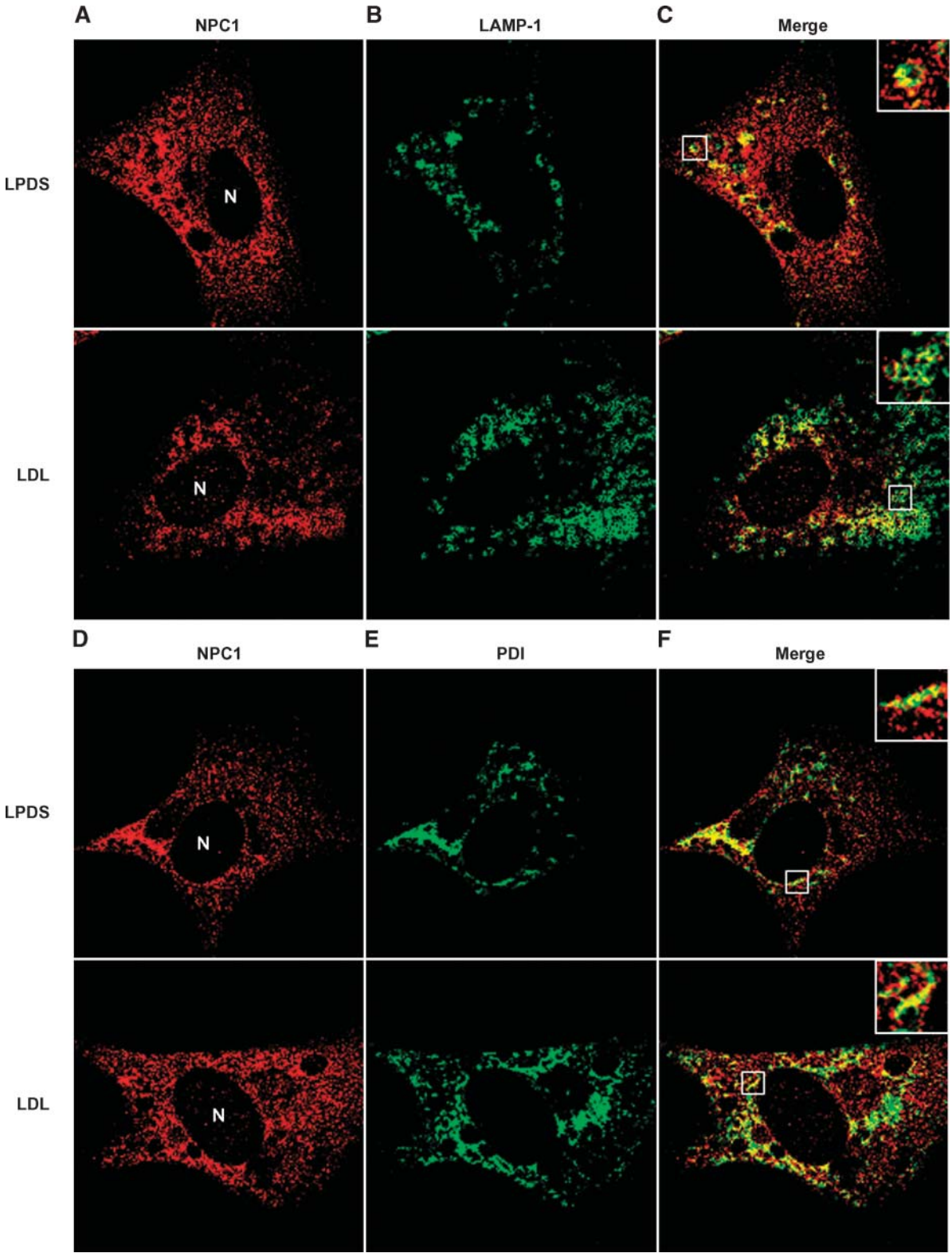

Fig. 5. Cellular distribution of the NPC1 protein in relation to lysosome-associated membrane protein-1 (LAMP-1) and protein disulfide isomerase (PDI). The fibroblasts were cultured and processed as described in Materials and Methods. The fibroblasts are denoted as grown in medium without LDL (LPDS) or medium with LDL (LDL). The distribution of the NPC1 protein in relation to LAMP-1 and PDI was determined by labeling cells for the NPC1 protein (A, D), LAMP-1, a protein marker for late endosomes/lysosomes (B), or PDI, a protein marker for the endoplasmic reticulum (E), and performing confocal fluorescence microscopy. Fluorescence images were obtained using a Bio-Rad MCR-1024 ES laser scanning confocal microscope equipped with a Nikon 100×, numerical aperture 1.4, oil-immersion objective. The corresponding merged images and an enlarged inset from each merged image are provided (C, F). The single fibroblast shown within each image is representative of other fibroblasts grown on the same coverslip. The images were acquired and processed using identical conditions. All images were derived from a single optical section estimated to be 1.0 μm thick. N, nucleus.

distinct regions of the endoplasmic reticulum. Unlike the increased colocalization of the NPC1 protein with late endosomes/lysosomes that occurred when fibroblasts were grown in medium with LDL, colocalization of the NPC1 protein with regions of the endoplasmic reticulum remained constant. Therefore, the results indicated that the NPC1 protein was localized to three different cellular compartments. Although most of the NPC1 protein was associated with LAMP-1-negative vesicles representing the unique late endosomal NPC1 compartment, fibroblasts grown in medium with LDL had an increased colocalization of the NPC1 protein with LAMP-1-positive vesicles (late endosomes/lysosomes). A smaller proportion of the NPC1 protein was associated with distinct regions of the endoplasmic reticulum, but this association was not affected when fibroblasts were grown in medium with LDL.

DISCUSSION

A number of physiological studies performed in vitro and in vivo have suggested that the NPC1 gene is constitutively expressed and not regulated in a cholesterol-dependent manner or by the SREBP pathway (31–35). Consistent with these results, initial molecular characterization of the human NPC1 gene promoter region revealed a GC-rich sequence (GC box element) and several specificity protein-1 binding sequences in the absence of a TATA sequence, which together are well-known characteristics of a housekeeping gene (31). To further examine whether the amount of cellular cholesterol might affect the expression of the NPC1 gene, the present study was performed by incubating normal human fibroblasts in medium in the absence or presence of LDL, thereby promoting the transport of LDL-derived cholesterol to the sterol-regulatory pool and feedback inhibition of the SREBP pathway. Several lines of evidence indicated that the expression of the NPC1 gene and the cellular distribution of the NPC1 protein are functions of intracellular cholesterol homeostasis. In brief, the results demonstrated the following: *i*) the defined culture conditions significantly affected both the concentration and the distribution of cellular cholesterol; *ii*) the transport of LDL-derived cholesterol to the sterol-regulatory pool, as determined by decreased processing of the p-SREBP protein and the amount of the m-SREBP protein, was correlated directly with the downregulation of the NPC1 gene; *iii*) the NPC1 gene was regulated primarily at the transcriptional level, because the relative amounts of NPC1 mRNA and NPC1 protein were correlated directly; *iv*) the m-SREBP proteins interacted with SRE sequences positioned within the NPC1 gene promoter region to increase the transcription of the NPC1 gene; and *v*) fibroblasts grown in medium with LDL enhanced the partial colocalization of the NPC1 protein with late endosomes/lysosomes, whereas the partial colocalization of the NPC1 protein with distinct regions of the endoplasmic reticulum remained constant. Together, these results indicated that the transport of LDL-derived cholesterol from late endosomes/lysosomes to the sterol-regulatory pool, known to be regulated by the NPC1 protein, is responsible for promoting feedback inhibition of the SREBP pathway and the subsequent downregulation of the NPC1 gene.

The expression of SREBP genes and the processing of the corresponding p-SREBP proteins to produce m-SREBP proteins, which serve as nuclear transcription factors capable of binding SRE sequences, have a central role in regulating intracellular lipid homeostasis (21, 43). A number of studies performed in vitro using cultured cells have determined an increased transcription of SREBP-1a and SREBP-2 genes when cellular cholesterol pools are depleted, indicating that the transcription of these genes is regulated by cholesterol-dependent feedback inhibition (44, 45). The mechanism involves SCAP and INSIG binding specifically to cholesterol and oxysterol, respectively, to inhibit the translocation and processing of p-SREBP proteins to produce m-SREBP proteins (22–26). Consistent with these results, the present study indicated that the relative amounts of SREBP mRNA and m-SREBP protein decreased when fibroblasts were grown in medium with LDL, indicating that the transport of LDL-derived cholesterol to the sterol-regulatory pool promoted feedback inhibition of the SREBP pathway.

A key finding in the present report is that the transport of LDL-derived cholesterol to the sterol-regulatory pool, as determined by decreased amounts of m-SREBP protein, was correlated directly with the downregulation of the NPC1 gene. Subsequent characterization of the NPC1 gene promoter region indicated that m-SREBP proteins were capable of interacting within a region of 0.5 kb positioned upstream from the NPC1 gene transcription start site that contained three SRE sequences. Moreover, the interaction of m-SREBP proteins with the NPC1 gene promoter region and the induced transcription of the NPC1 gene were demonstrated using both an NPC1 gene promoter-luciferase reporter construct in human granulosa cells and ChIP analysis using human fibroblasts. It is important to note that a previous report has determined that both the SREBP-1a and SREBP-2 genes are transcriptionally regulated in a cholesterol-dependent and coordinated manner in cultured cells and that both the m-SREBP-1a and m-SREBP-2 proteins are fully capable of transactivating genes containing either classic SRE (SRE-1 and SRE-3) sequences or SRE-like sequences (46). Therefore, with respect to the present study using human fibroblasts, both the m-SREBP-1a and m-SREBP-2 proteins most likely regulated transcription of the NPC1 gene.

With respect to the NPC1 protein, immunoblot analysis indicated the existence of two different immunoreactive proteins that migrated at 170 and 190 kDa using SDS-PAGE. These results are similar to those of other studies demonstrating that the NPC1 protein is posttranslationally modified by asparagine glycosylation, thereby producing two different highly glycosylated isoforms of the NPC1 protein with a molecular mass greater than would be theoretically predicted (142 kDa) from the cDNA (31, 47, 48).

These same studies also suggested that the lower molecular mass (170 kDa) NPC1 protein was less efficient in facilitating the transport of cholesterol from late endosomes/lysosomes to other cellular compartments and therefore may represent the less stable of the two NPC1 protein isoforms, possibly because of the decreased level of asparagine glycosylation. Moreover, consistent with other studies, the results presented in this report indicate that the relative amount of the NPC1 gene is primarily regulated at the transcriptional level (32, 49).

Concerning the cellular distribution of the NPC1 protein, the overall results are consistent with previous studies describing the NPC1 protein to be associated primarily with a unique LAMP-1-negative late endosomal compartment, in addition to partially colocalizing with LAMP-1-positive late endosomes/lysosomes when these compartments are enriched with LDL-derived cholesterol (7–11). However, because the NPC1 protein was confined to the limiting membrane of these compartments, the present study was unable to confirm an earlier result suggesting that the NPC1 protein actually entered into the core of the cholesterol-enriched late endosomes/lysosomes (47). As described previously, the NPC1 protein also partially colocalized with distinct regions of the endoplasmic reticulum, although this colocalization was independent of whether the fibroblasts were grown in medium with LDL (10, 50). Nonetheless, the partial colocalization of the NPC1 protein with distinct regions of the endoplasmic reticulum is intriguing, because the NPC1 protein may directly facilitate the transport of lipoprotein-derived cholesterol from late endosomes/lysosomes to the endoplasmic reticulum, as indicated in a pervious study (51).

The results in this report, describing the regulation of the NPC1 gene, are in contrast to those from other studies that used microarray analysis to identify putative SREBP target genes. In those studies, the expression of the NPC1 gene was not altered in the livers of mice fed a cholesterol-enriched diet (33, 34). Moreover, another study investigating the flow of cholesterol through cells in the mouse indicated that expression of the NPC1 gene was also not affected by feeding mice a cholesterol-enriched diet (35). These discrepancies may in part be attributed to key differences associated with the studies being conducted in vitro using human fibroblasts or in vivo using mouse livers. For instance, the fibroblasts were grown using defined culture conditions that depleted cellular cholesterol pools to maximize endocytosis and the transport of LDL-derived cholesterol to the sterol-regulatory pool, whereas mouse hepatocytes, which remain in constant contact with various plasma lipoproteins, may not be physiologically responsive to the internalization of such large amounts of lipoprotein-derived cholesterol. Moreover, once the lipoprotein-derived cholesterol is internalized within endocytic compartments, the fibroblasts are only capable of converting excess cholesterol to cholesteryl ester for storage within lipid bodies, whereas in addition to converting excess cholesterol to cholesteryl ester, mouse hepatocytes are also able to convert excess cholesterol into bile acids, secrete both bile acids and cholesterol into the bile, package sterol into lipoproteins for secretion into the blood, and promote cholesterol efflux to lipid-poor apolipoprotein A-I and HDL. Hence, it is suspected that the SREBP pathway in fibroblasts is inherently and artificially more responsive to LDL-derived cholesterol than are mouse hepatocytes after being fed a cholesterol-enriched diet. This being the case, other studies performed using human fibroblasts have reported that the relative amount of NPC1 protein remained unchanged when incubated in medium with LDL (32, 52). However, it is important to emphasize that these same studies also reported a significant increase in the amount of the NPC1 protein when fibroblasts were incubated in the presence of progesterone or U-18666A, reagents known to block the transport of cholesterol from late endosomes/lysosomes to the endoplasmic reticulum, thereby suggesting that cholesterol depletion of the sterol-regulatory pool and subsequent activation of the SREBP pathway promoted upregulation of the NPC1 gene.

In summary, the results in this report indicate that the transport of LDL-derived cholesterol from late endosomes/lysosomes to the sterol-regulatory pool, known to be regulated by the NPC1 protein, is responsible for promoting the feedback inhibition of the SREBP pathway and downregulation of the NPC1 gene. It is proposed that this downregulation of the NPC1 gene may represent a novel mechanism for inducing the sequestration of cholesterol within late endosomes/lysosomes and limiting the amount of potentially toxic cholesterol accessible to the sterol-regulatory pool.

This work was supported in part by National Institutes of Health Grant R21 DK-071544, an investigator award from the Ara Parseghian Medical Research Foundation, a grant from the Father's Day Council of Tucson, and other private donations (to W.S.G.), by Canadian Institutes of Health Research Grant MOP-79532 (to G.A.F.), and by Canadian Institutes of Health Research Grant MOP-117373 (to B.D.M.). The authors acknowledge the excellent technical assistant of Mira Dobias with the aid of Dr. Danila Campos in designing the primers used for these studies.

REFERENCES

1. Imrie, J., S. Dasgupta, G. T. N. Besley, C. Harris, L. Heptinstall, S. Knight, M. T. Vanier, A. Fensom, C. Ward, E. Jacklin, et al. 2007. The natural history of Niemann-Pick disease type C in the UK. *J. Inherit. Metab. Dis.* **30:** 51–59.
2. Garver, W. S., G. A. Francis, D. Jelinek, G. Shepherd, J. Flynn, G. Castro, C. Walsh Vockley, D. L. Coppock, K. M. Pettit, R. A. Heidenreich, et al. 2007. The national Niemann-Pick C1 disease database: report of clinical features and health problems. *Am. J. Med. Genet.* **143A:** 1204–1211.
3. Pentchev, P. G., M. E. Comly, H. S. Kruth, M. T. Vanier, D. A. Wenger, S. Patel, and R. O. Brady. 1985. A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. *Proc. Natl. Acad. Sci. USA.* **82:** 8247–8251.
4. Liscum, L., and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-(diethylamino)ethoxy]-androst-5-en-17-one. *J. Biol. Chem.* **264:** 11796–11806.

5. Xie, C., S. D. Turley, and J. M. Dietschy. 1999. Cholesterol accumulation in tissues of the Niemann-Pick type C mouse is determined by the rate of lipoprotein-cholesterol uptake through the coated-pit pathway in each organ. *Proc. Natl. Acad. Sci. USA.* **96:** 11992–11997.
6. Reid, P. C., S. Sugii, and T-Y. Chang. 2003. Trafficking defects in endogenously synthesized cholesterol in fibroblasts, macrophages, hepatocytes, and glial cells from Niemann-Pick type C1 mice. *J. Lipid Res.* **44:** 1010–1019.
7. Neufeld, E. B., M. Wastney, S. Patel, S. Suresh, A. M. Conney, N. K. Dwyer, C. F. Roff, K. Ohno, J. A. Morris, E. D. Carstea, et al. 1999. The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J. Biol. Chem.* **274:** 9627–9635.
8. Higgins, M. E., J. P. Davies, F. W. Chen, and Y. A. Ioannou. 1999. Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol. Genet. Metab.* **68:** 1–13.
9. Garver, W. S., R. A. Heidenreich, R. P. Erickson, M. A. Thomas, and J. M. Wilson. 2000. Localization of the murine Niemann-Pick C1 protein to two distinct intracellular compartments. *J. Lipid Res.* **41:** 673–687.
10. Garver, W. S., K. Somers, K. Krishnan, T. Mitchell, R. A. Heidenreich, and M. A. Thrall. 2002. The Niemann-Pick C1 protein in feline fibroblasts. *Mol. Genet. Metab.* **76:** 31–36.
11. Blom, T. S., M. D. Linder, K. Snow, H. Pihko, M. W. Hess, E. Jokitalo, V. Veckman, A. Syvanen, and E. Ikonen. 2003. Defective endocytic trafficking of NPC1 and NPC2 underlying infantile Niemann-Pick type C disease. *Hum. Mol. Genet.* **12:** 257–272.
12. Blanchette-Mackie, E. J., N. K. Dwyer, L. M. Amende, H. S. Kruth, J. D. Butler, J. Sokol, M. E. Comly, M. T. Vanier, J. T. August, R. O. Brady, et al. 1988. Type C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. *J. Biol. Chem.* **85:** 8022–8026.
13. Liscum, L., R. M. Ruggiero, and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts. *J. Cell Biol.* **108:** 1625–1636.
14. Garver, W. S., K. Krishnan, J. R. Gallagos, M. Michikawa, G. A. Francis, and R. A. Heidenreich. 2002. Niemann-Pick C1 protein regulates cholesterol transport to the *trans*-Golgi network and plasma membrane caveolae. *J. Lipid Res.* **43:** 579–589.
15. Wojtanik, K. M., and L. Liscum. 2003. The transport of low density lipoprotein-derived cholesterol to the plasma membrane is defective in NPC1 cells. *J. Biol. Chem.* **278:** 14850–14856.
16. Frolov, A., S. E. Zielinski, J. R. Crowley, N. Dudley-Rucker, J. E. Schaffer, and D. S. Ory. 2003. NPC1 and NPC2 regulate cellular cholesterol homeostasis through generation of low density lipoprotein cholesterol-derived oxysterols. *J. Biol. Chem.* **278:** 25517–25525.
17. Choi, H. Y., B. Karten, T. Chan, J. E. Vance, W. L. Greer, R. A. Heidenreich, W. S. Garver, and G. A. Francis. 2003. Impaired ABCA1-dependent lipid efflux and hypoalphalipoproteinemia in human Niemann-Pick type C disease. *J. Biol. Chem.* **278:** 32569–32577.
18. Pentchev, P. G., H. S. Kruth, M. E. Comly, J. D. Butler, M. T. Vanier, D. A. Wenger, and S. Patel. 1986. Type C Niemann-Pick disease. A parallel loss of regulatory responses in both the uptake and esterification of low density lipoprotein-derived cholesterol in cultured fibroblasts. *J. Biol. Chem.* **261:** 16775–16780.
19. Liscum, L., and J. R. Faust. 1987. Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts. *J. Biol. Chem.* **262:** 17002–17008.
20. Argoff, C. E., M. E. Comly, J. Blanchette-Mackie, H. S. Kruth, H. T. Pye, E. Goldin, C. Kaneski, M. T. Vanier, R. O. Brady, and P. G. Pentchev. 1991. Type C Niemann-Pick disease: cellular uncoupling of cholesterol homeostasis is linked to the severity of disruption in the intracellular transport of exogenously derived cholesterol. *Biochim. Biophys. Acta.* **1096:** 319–327.
21. Brown, M. S., and J. L. Goldstein. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell.* **89:** 331–340.
22. Nohturfft, A., R. A. DeBose-Boyd, S. Scheek, J. L. Goldstein, and M. S. Brown. 1999. Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. *Proc. Natl. Acad. Sci. USA.* **96:** 11235–11240.
23. Nohturfft, A., D. Yabe, J. L. Goldstein, M. S. Brown, and P. J. Espenshade. 2000. Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes. *Cell.* **102:** 315–323.
24. Yang, T., P. J. Espenshade, M. E. Wright, D. Yabe, Y. Gong, R. Aebersold, J. L. Goldstein, and M. S. Brown. 2002. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to Insig-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell.* **110:** 489–500.
25. Radhakrishnan, A., L. P. Sun, H. J. Kwon, M. S. Brown, and J. L. Goldstein. 2004. Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain. *Mol. Cell.* **15:** 259–268.
26. Radhakrishnan, A., Y. Ikeda, H. J. Kwon, M. S. Brown, and J. L. Goldstein. 2007. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc. Natl. Acad. Sci. USA.* **104:** 6511–6518.
27. Edwards, P. A., D. Tabor, H. R. Kast, and A. Venkateswaran. 2000. Regulation of gene expression by SREBP and SCAP. *Biochim. Biophys. Acta.* **1529:** 103–113.
28. Weber, L. W., M. Boll, and A. Stampfl. 2004. Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins. *World J. Gastroenterol.* **10:** 3081–3087.
29. Beltroy, E. P., J. A. Richardson, J. D. Horton, S. D. Turley, and J. M. Dietschy. 2005. Cholesterol accumulation and liver cell death in mice with Niemann-Pick type C disease. *Hepatology.* **42:** 886–893.
30. Garver, W. S., D. Jelinek, J. N. Oyarzo, J. Flynn, M. Zuckerman, K. Krishnan, B. H. Chung, and R. A. Heidenreich. 2007. Characterization of liver disease and lipid metabolism in the Niemann-Pick C1 mouse. *J. Cell. Biochem.* **101:** 498–516.
31. Watari, H., E. J. Blanchette-Mackie, N. K. Dwyer, M. Watari, C. G. Burd, S. Patel, P. G. Pentchev, and J. F. Strauss, III. 2000. Determinants of NPC1 expression and action: key promoter regions, posttranscriptional control, and the importance of a "cysteine-rich" loop. *Exp. Cell Res.* **259:** 247–256.
32. Zhang, M., N. K. Dwyer, E. B. Neufeld, D. C. Love, A. Cooney, M. Comly, S. Patel, H. Watari, J. F. Strauss, III, P. G. Pentchev, et al. 2001. Sterol-modulated glycolipid sorting occurs in Niemann-Pick C1 late endosomes. *J. Biol. Chem.* **276:** 3417–3425.
33. Maxwell, K. N., R. E. Soccio, E. M. Duncan, E. Sehayek, and J. L. Breslow. 2003. Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *J. Lipid Res.* **44:** 2109–2119.
34. Horton, J. D., N. A. Shah, N. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA.* **100:** 12027–12032.
35. Garver, W. S., C. Xie, J. J. Repa, S. D. Turley, and J. M. Dietschy. 2005. Niemann-Pick C1 expression is not regulated by the amount of cholesterol flowing through cells in the mouse. *J. Lipid Res.* **46:** 1745–1754.
36. Gevry, N., D. Lacroix, J. H. Song, N. Pescador, M. Dobias, and B. D. Murphy. 2002. Porcine Niemann-Pick C1 protein is expressed in steroidogenic tissues and modulated by cAMP. *Endocrinology.* **143:** 708–716.
37. Gevry, N. Y., E. Lalli, P. Sassone-Corsi, and B. D. Murphy. 2003. Regulation of Niemann-Pick C1 gene expression by the 3′5′-cyclic adenosine monophosphate pathway in steroidogenic cells. *Mol. Endocrinol.* **17:** 704–715.
38. Chung, B. H., T. Wilkinson, J. C. Greer, and J. P. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
39. Brown, M. S., and J. L. Goldstein. 1975. Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. *Cell.* **6:** 307–316.
40. Oram, J. F., J. J. Albers, and E. L. Bierman. 1980. Rapid regulation of the activity of the low density lipoprotein receptor of cultured human fibroblasts. *J. Biol. Chem.* **255:** 475–485.
41. Heider, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* **19:** 514–518.
42. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
43. Brown, M. S., and J. L. Goldstein. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. USA.* **96:** 11041–11048.
44. Shimomura, I., J. D. Shimano, J. D. Horton, J. L. Goldstein, and M. S. Brown. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J. Clin. Invest.* **99:** 838–845.

45. Sato, R., J. Inoue, Y. Kawabe, T. Kodama, T. Takano, and M. Maeda. 1996. Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2. *J. Biol. Chem.* **271:** 26461–26464.
46. Amemiya-Kudo, M., H. Shimano, A. H. Hasty, N. Yahagi, T. Yoshikawa, T. Matsuzaka, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, et al. 2002. Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. *J. Lipid Res.* **43:** 1220–1235.
47. Watari, H., E. J. Blanchette-Mackie, N. K. Dwyer, J. M. Glick, S. Patel, E. B. Neufeld, R. O. Brady, P. G. Pentchev, and J. F. Strauss, III. 1999. Niemann-Pick C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. *Proc. Natl. Acad. Sci. USA.* **96:** 805–810.
48. Watari, H., E. J. Blanchette-Mackie, N. K. Dwyer, M. Watari, E. B. Neufeld, S. Patel, P. G. Pentchev, and J. F. Strauss, III. 1999. Mutations in the leucine zipper motif and sterol-sensing domain inactivate the Niemann-Pick C1 glycoprotein. *J. Biol. Chem.* **274:** 21861–21866.
49. Ohsaki, Y., Y. Sugimoto, M. Suzuki, H. Hosokawa, T. Yoshimori, J. P. Davies, Y. A. Ioannou, M. T. Vanier, K. Ohno, and H. Ninomiya. 2006. Cholesterol depletion facilitates ubiquitylation of NPC1 and its association with SKD1/Vps4. *J. Cell Sci.* **119:** 2643–2653.
50. Ko, D. C., M. D. Gordon, J. Y. Jin, and M. P. Scott. 2001. Dynamic movements of organelles containing Niemann-Pick C1 protein: NPC1 involvement in late endocytic events. *Mol. Biol. Cell.* **12:** 601–614.
51. Underwood, K. W., N. L. Jacobs, A. Howley, and L. Liscum. 1998. Evidence for a cholesterol transport pathway from lysosomes to endoplasmic reticulum that is independent of the plasma membrane. *J. Biol. Chem.* **273:** 4266–4274.
52. Patel, S. C., S. Suresh, U. Kumar, C. Y. Hu, A. Cooney, E. J. Blanchette-Mackie, E. B. Neufeld, R. C. Patel, R. O. Brady, Y. C. Patel, et al. 1999. Localization of Niemann-Pick C1 protein in astrocytes: implications for neuronal degeneration in Niemann-Pick type C disease. *Proc. Natl. Acad. Sci. USA.* **96:** 1657–1662.