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Isolation of NPC1-Deficient Chinese Hamster Ovary Cell Mutants by Gene Trap Mutagenesis[1]

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Chinese hamster ovary cell mutants defective in the *NPC1* gene (NPC1-trap) were generated by retrovirus-mediated gene trap mutagenesis from a parental cell line JP17 expressing an ecotropic retrovirus receptor. Insertion of the gene trap vector in the *NPC1* gene and the absence of the gene product were verified by 5′RACE and immunological analyses, respectively. NPC1-trap cells showed intracellular accumulation of low-density lipoprotein (LDL)–derived cholesterol and had an increased level of unesterified cellular cholesterol. Cholesterol biosynthesis through the mevalonate pathway was upregulated in the mutant cells as assessed by [^{14}C]acetate incorporation into cellular sterols. When JP17 cells were depleted of lipoproteins and then loaded with LDL, cell surface LDL receptors were promptly downregulated and the mature form of the sterol regulatory element–binding protein–1 disappeared from the nucleus. These responses to LDL were obviously retarded in NPC1-trap cells, suggesting an impaired response of the cholesterol-regulatory system to LDL. NPC1-trap cells will be a useful tool to study the regulation of cellular cholesterol homeostasis and the pathogenesis of Niemann-Pick disease type C.

Key words: CHO, cholesterol, LDL, Niemann-Pick C, SREBP.

Niemann-Pick disease type C (NPC) is an autosomal recessive lipid storage disorder characterized by the abnormal accumulation of unesterified cholesterol in late endosomes/lysosomes (*1*). The majority of the cases of NPC are caused by mutations in the *NPC1* gene (*2*). Studies using fibroblasts isolated from patent skin have shown delays in the down-regulation of both cell surface low-density lipoprotein (LDL) receptor and *de novo* cholesterol synthesis in response to LDL (*3, 4*). In mammalian cells, intracellular cholesterol homeostasis is maintained by transcriptional feedback-regulation by sterol regulatory element binding proteins (SREBPs) (*5*). Although it is anticipated that feedback-regulation by SREBP is impaired in NPC cells, this has not been directly proven.

The somatic cell genetic approach has helped to define critical steps of intracellular cholesterol metabolism (*6*). Chinese hamster ovary (CHO) cells have often been used in this approach, because they have both of the two known cholesterol synthetic pathways, the LDL-derived and mevalonate-derived pathways (*7, 8*) and also because they are functionally haploid in many genetic loci and a single integration of a mutation causes a loss of gene function (*9, 10*). Chang and colleagues isolated several mutant CHO cell lines with defects in the *NPC1* gene (*11, 12*). These cells were isolated from a mutant cell line, 25-RA, that contains a gain of function mutation in the SREBP-cleavage activating protein (SCAP) gene (*13*). Because SREBP is constitutively active in cells derived from 25-RA due to this mutation, feedback-regulation by SREBP can not be assessed in their NPC1-deficient mutants.

In the present study, we isolated new lines of NPC1-defecient CHO mutants by retrovirus-mediated gene trap mutagenesis. The mutant cells exhibit a phenotype characteristic of NPC1-deficient cells and showed impairment in feedback-regulation by SREBP in response to LDL.

MATERIALS AND METHODS

Gene Trap Mutagenesis—CHO cells were maintained in Ham's F-12/10% fetal calf serum (FCS). An ecotropic retrovirus packaging cell line GP+E86 was maintained in

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Abbreviations: 25-HC, 25-hydroxycholesterol; 5′-RACE, 5′-rapid amplification of cDNA ends; CHO, Chinese hamster ovary; FCS, fetal calf serum; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; SCAP, SREBP cleavage activating protein; SREBP, sterol-responsive element binding protein; NPC, Niemann-Pick disease type C; TLC, thin layer chromatography.

DMEM/10% FCS. A CHO cell line expressing the murine ecotropic retrovirus receptor (JP17) was established by standard methods (*14*) and used as a parental cell line for gene trap mutagenesis. The supernatant containing gene trap retroviruses was harvested from GP+E86 cells transfected with a retrovirus gene trap construct ROSA β-geo (*15*). JP17 cells were exposed to the freshly prepared supernatant supplemented with 8 μg/ml polybrane for 3 h and then cultured in normal medium for 48 h. Thereafter, the infected cells went through sequential phases of selection, first with 0.5 mg/ml G418 for 7 days and second with 5 μg/ml filipin for another 7 days. G418/filipin-resistant colonies were isolated by colony-lifting and expanded in nonselective medium. The insertion site of the trap vector in each clone was determined by a 5′-RACE PCR analysis using a primer specific for the trap vector as described (*16*).

Western Blotting, Immunocytochemistry, and Filipin Staining—Antibodies against the NPC1 C-terminus and SREBP-1 N-terminus (Santa Cruz) were used for Western blotting and immunocytochemistry as described (*17, 18*). For Western blotting, cells were scraped, collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1 mM AEBSF, and 1 μg/ml aprotinin) and lysed by sonication. After centrifugation at 100,000 ×*g* for 30 min, the pellet was resuspended in lysis buffer to give membrane preparations. Nuclear fractions were prepared as described (*13*). Western blotting was done as described (*17*) and the blot was developed with an ECL system (Amersham). The cells were immunostained as described (*18*) except that the bound antibodies were detected with an Alexa488-conjugated secondary antibody and images were obtained with a Bio-Rad MRC-1024 confocal microscope. Filipin staining was performed as described (*19*) and images were obtained with a Nikon fluorescence microscope.

Electron Microscopy—Cells were cultured on a plastic-sheet (Wako Pure Chemical Industries) and stained with filipin. The cells were post-fixed with 2.5% glutaraldehyde in PBS and embedded in epoxy resin. Sections with a thickness of 80–100 nm were cut and images were obtained with a Hitachi HU-12A electron microscope.

Thin Layer Chromatography (TLC)—Extraction of total lipids and TLC analysis were done as described (*20*). In brief, lipids were extracted from cell pellets in chloroform/methanol (2:1 v/v) with or without saponification and separated by TLC on Silica Gel 60 (Merck) in petroleum ether: diethyl ether:acetic acid (60:40:1 v/v/v) for total sterols or in hexan:diethyl ether:acetic acid (90:10:1 v/v/v) for non-saponified samples. TLC sheets were dried and developed by charring with $CuSO_4$ and heating at 180°C.

Incorporation of [^{14}C]Acetate into Lipids—Cells were incubated in F12/10% FCS supplemented with [^{14}C]acetate (1 μCi/ml, American Radiolabeled Chemicals) for 10 or 20 h. Lipids were extracted and separated by TLC as described above and the autoradiograph was developed with BAS 2000 (Fujitsu, Tokyo).

DiI-LDL Uptake—Lipoprotein-deficient serum (LPDS) was prepared from FCS as described (*21*). The cells were cultured in F12/10% LPDS for 48 h. On the day of experiment, the cells were incubated in fresh F12/10% LPDS supplemented with 100 μg/ml LDL (Sigma) or 1 μg/ml 25-hydroxycholesterol (25-HC, Sigma). At the time indicated, the cells were exposed to 10 μg/ml DiI-labeled LDL (Molecular Probes) for 10 min, washed with PBS and fixed. The fluorescent images were obtained with a confocal microscope. 25-HC was dissolved in ethanol to give a final concentration of 0.1% ethanol in the culture. At this concentration, ethanol had no effect on either cellular DiI-LDL uptake or the intracellular localization of SREBP-1 (data not shown).

RESULTS

Isolation of NPC1-Deficient CHO Mutants—Because CHO cells are resistant to retrovirus infection (*22*), we established a subline of CHO cells, JP17, that expressed an ecotropic retrovirus receptor, and used it as a parental strain for ROSA β-geo–mediated gene trap mutagenesis. Infecting a total of 1.5×10^5 cells, we obtained 1,858 cell clones that were doubly resistant to G418 and filipin. By staining individual clones with filipin, we isolated 61 cell clones that had an abnormal intracellular accumulation of free cholesterol. Cell fusion studies using human and mouse NPC1-deficient fibroblasts (*23*) revealed that all of these mutants were allelic for *NPC1* gene mutations (data not shown). Disruption of the hamster *NPC1* gene in a representative clone was verified by 5′-RACE that revealed a trap vector insertion between nucleotides 306 and 436 in exon 4. This clone (NPC1-trap) was used in subsequent experiments.

Absence of the *NPC1* gene product in NPC1-trap cells was verified by anti-NPC1 staining of fixed cells and Western blotting of membrane preparations. No staining of the perinuclear vesicular structures in JP17 cells was detected in NPC1-trap cells (Fig. 1A). Likewise, no staining of bands

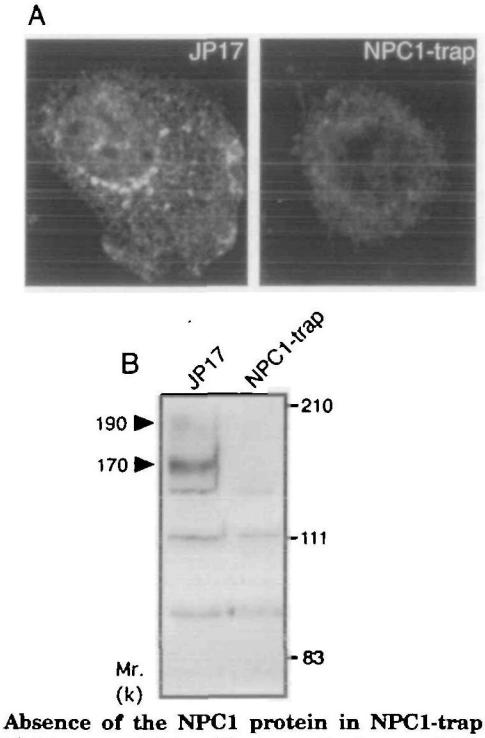

Fig. 1. **Absence of the NPC1 protein in NPC1-trap cells.** A: Anti-NPC-1 immunostaining. Shown are the images obtained with a confocal microscope. B: Western blotting of membrane preparations with the same antibody. Proteins were separated in a 7.5% polyacrylamide gel. The positions of the 170 and 190 kDa NPC1 bands are indicated. Molecular masses are given on the right (kDa).

with molecular masses of 170 or 190 kDa in JP17 cell preparations was detected in NPC1-trap cells (Fig. 1B).

Accumulation of Free Cholesterol in NPC1-Trap Cells—Filipin staining of NPC1-trap cells showed dense staining of the perinuclear vesicles, presumably representing late endosomes/lysosomes (Fig. 2A). This staining was lost when the cells were cultured in F12/10% LPDS for 48 h and was also abolished by the transient expression of human NPC1 (data not shown). Electron microscopic analyses of the cells stained with filipin showed multi-concentric lamellar-like structures in the perinuclear vesicles (Fig. 2B) characteristic of intracellular accumulations in NPC1-deficient cells (*24*). TLC analyses of cellular lipids revealed an increased level of both total (a 1.3 ± 0.06-fold, $n = 3$) and free (a 1.27 ± 0.10-fold, $n = 3$) cholesterol in NPC1-trap cells, most likely reflecting the accumulation of free cholesterol in endocytic vesicles (Fig. 2, C, D, E, and F).

Upregulation of Cholesterol Synthesis through the Mevalonate Pathway in NPC1-Trap Cells—To assess cellular cholesterol synthesis through the mevalonate pathway, we measured the incorporation of [^{14}C]acetate into cellular lipids. TLC separation of labeled lipids showed the incorporation of [^{14}C]acetate into cholesterol, squalane, lanosterol and two unidentified intermediate forms of sterols. While this labeling pattern was indistinguishable between JP17 and NPC1-trap cells, the level of incorporation into cholesterol was obviously higher in NPC1-trap cells at both the 10- and 20-h labeling periods than in JP-17 cells (Fig. 3).

Impaired Downregulation of Cell Surface LDL Receptor and SREBP-1 in Response to LDL in NPC1-Trap Cells—Besides the accumulation of free cholesterol and the upregulation of cholesterol synthesis, skin fibroblasts from NPC patients are distinguished by their delayed downregulation of cell surface LDL receptors in response to LDL (*25*). We examined whether this happens in our mutant by measuring the uptake of DiI-labeled LDL. When cultured in F12/10% LPDS for 48 h, both JP17 and NPC1-trap cells actively incorporated DiI-LDL into their cytoplasmic granules in a 10-min exposure period. Subsequent loading of LDL promptly suppressed this incorporation in JP17 but not in NPC1-trap cells, indicating the sustained presence of cell surface LDL receptors in NPC1-trap cells (Fig. 4).

Because the LDL-induced downregulation of LDL receptors is mediated by a transcription factor, SREBP (*5*), we examined the cellular localization of SREBP-1 by immunostaining. In cells cultured in the standard medium, SREBP-1 was localized in the cytoplasm, presumably in the endoplasmic reticulum (*13*), whereas in cells depleted of lipoproteins, SREBP-1 was localized mainly in the nucleus (Fig. 5A). While this nuclear translocation of SREBP-1 occurred in both JP17 and NPC1-trap cells, the subsequent responses to LDL-loading were quite different between the two cell clones; upon the addition of LDL, SREBP-1 disappeared from the nucleus within 3 h in JP17 but not at all in

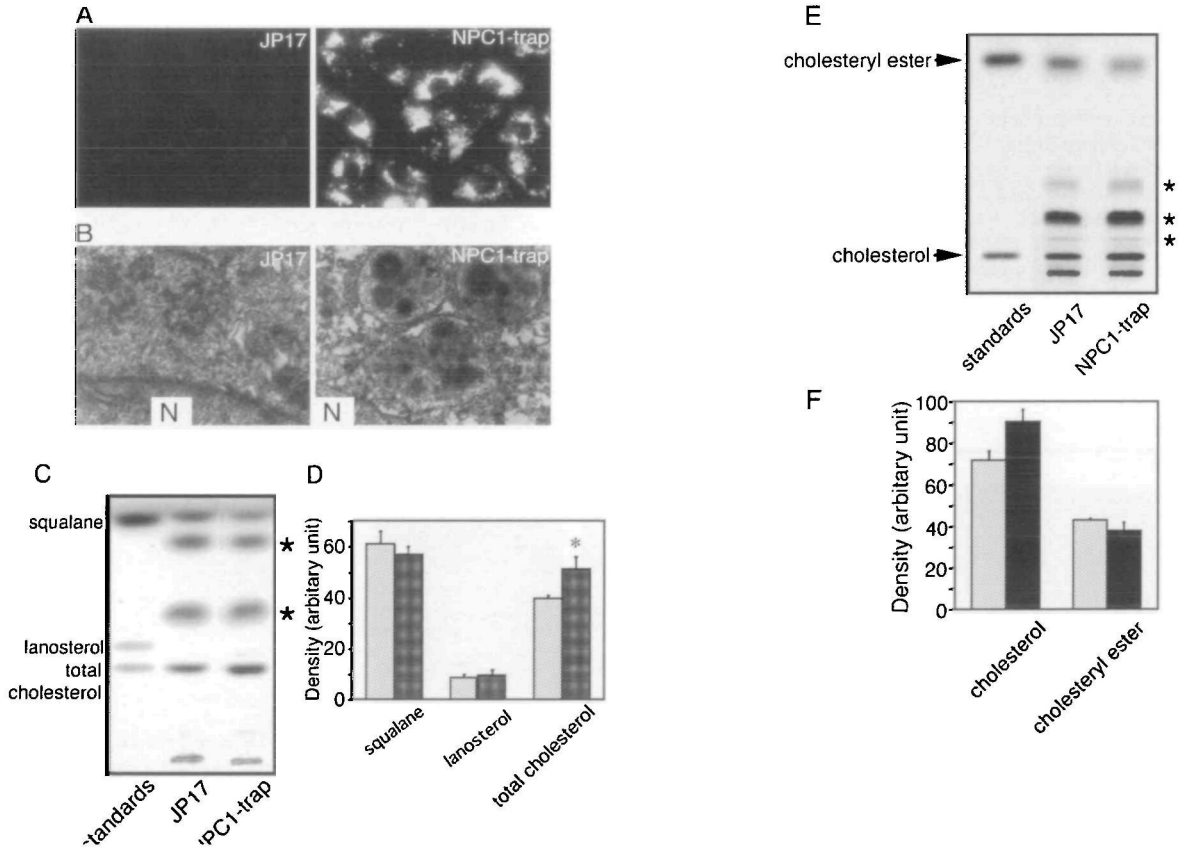

Fig. 2. **Free cholesterol accumulation in NPC1-trap cells.** A and B: Cells were stained with filipin and images were obtained with a fluorescence microscope (A) or an electron microscope (B, ×40,000). N, nucleus; C and E, TLC analyses of cellular lipids with (C) or without (E) saponification. The asterisks indicate unidentified lipids. D and F, densitometry. Densities of the bands on TLC were quantified using an NIH image software. Each value represents the mean ± SEM of three independent determinations. *$p < 0.01$, significantly different from the values of JP17 cells. Hatched bars, JP17; gray bars, NPC1-trap.

NPC1-trap cells (Fig. 5A). In accordance with these results, anti–SREBP-1 Western blotting revealed the sustained presence of the mature form (~70 kDa) of SREBP-1 in the nuclear fraction of NPC1-trap cells (Fig. 5B). This analysis also revealed the absence of the precursor form (~125 kDa) of SREBP-1 in the nuclear fraction of NPC1-trap cells, suggesting an altered intracellular distribution of the precursor form that was not apparent in the immunostaining.

Like LDL-derived cholesterol, oxidized sterols such as 7-kerocholesterol and 25-hydroxycholesterol (25-HC) can regulate SREBP (*26*), but unlike LDL-derived cholesterol, the intracellular transport of oxidized sterols does not depend on vesicular transport involving NPC1 (*18, 27*). Therefore, we tested the effects of 25-HC on DiI-LDL uptake and SREBP-1 localization as controls. As expected, 25-HC caused the downregulation of DiI-LDL uptake (Fig. 4) and the disappearance of nuclear SREBP (Fig. 5A) in cells depleted of lipoproteins, and these responses to 25-HC were indistinguishable between JP17 and NPC1-trap cells.

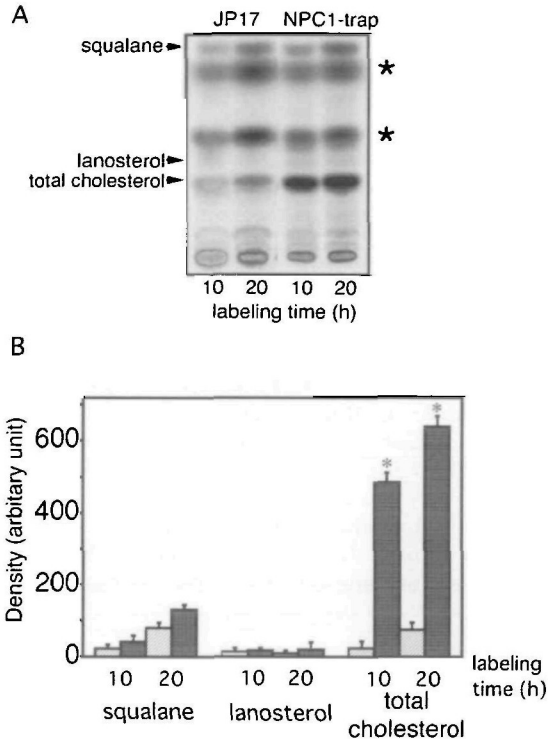

Fig. 3. Increased incorporation of [^{14}C]acetate into cholesterol in NPC1-trap cells. A: Cells were incubated in F12/10% FCS supplemented with [^{14}C]acetate (1 μCi/ml) for 10 or 20 h and total cellular lipids were separated by TLC. Shown is a representative autoradiograph of the TLC plate. The asterisks indicate unidentified lipids. B: Densitometry. Densities of the bands on the autoradiograph were quantified using NIH image software. Each value represents the mean ± SEM of three independent determinations. *p < 0.01, significantly different from the values of JP17 cells. Hatched bars, JP17; gray bars, NPC1-trap.

DISCUSSION

In this study, we established NPC1-deficient CHO mutants using gene trap mutagenesis. Insertion of the retrovirus gene trap vector is believed to occur at a single site in the whole genome (*15*). Therefore, our successful isolation of NPC1-trap cells is consistent with the recent report that only one allele of the *NPC1* gene is transcribed in CHO cells (*12*). There are two complementation groups in human NPC, NPC1, and NPC2 (*23*), and we failed in the present study to isolate a CHO mutant that corresponds to the NPC2 complementation group. With regard to the functional haploidity of the genes, we presume that both alleles of the *NPC2* gene are active in CHO cells, and that a single gene trap event is not enough to disrupt the expression of NPC2.

NPC1-deficient mutants were selected based on their reduced sensitivity to the cytotoxicity of filipin. Filipin is a polyene antibiotic that binds to plasma membrane cholesterol and has strong cytotoxic effects (*28*). Although it has been known for some time that NPC1-deficient cells are resistant to the cytotoxicity of filipin (*29*), how this happens remains unknown. One possible explanation is the reduced plasma membrane cholesterol in NPC1-deficient cells secondary to impaired utilization of LDL-derived cholesterol.

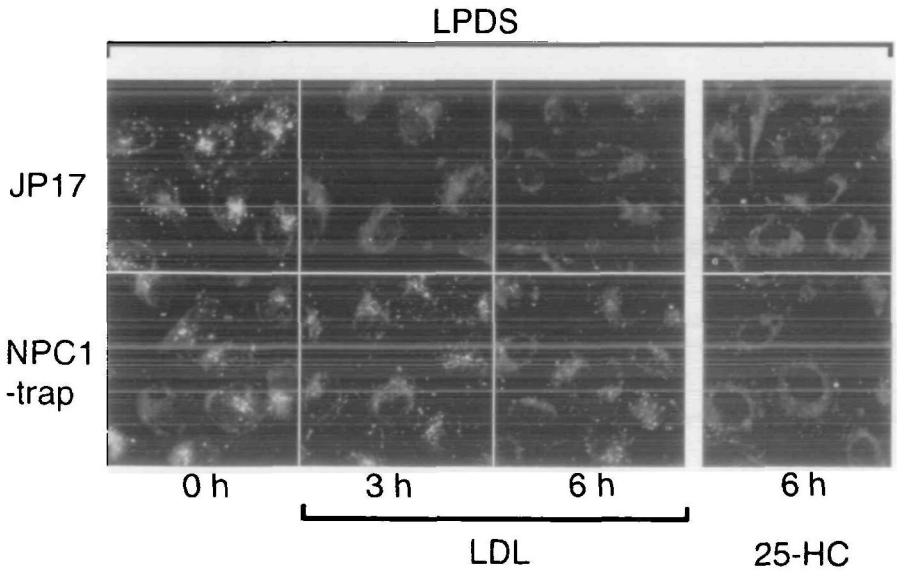

Fig. 4. **Delayed downregulation of DiI-LDL uptake in response to LDL in NPC1-trap cells.** Cells were cultured in F12/10% LPDS for 48 h and then incubated in fresh F12/10% LPDS supplemented with LDL (100 μg/ml) or 25-HC (1 μg/ml) for the time indicated. The cells were then exposed to DiI-LDL (10 μg/ml) for 10 min, fixed, and fluorescent images were obtained with a confocal microscope.

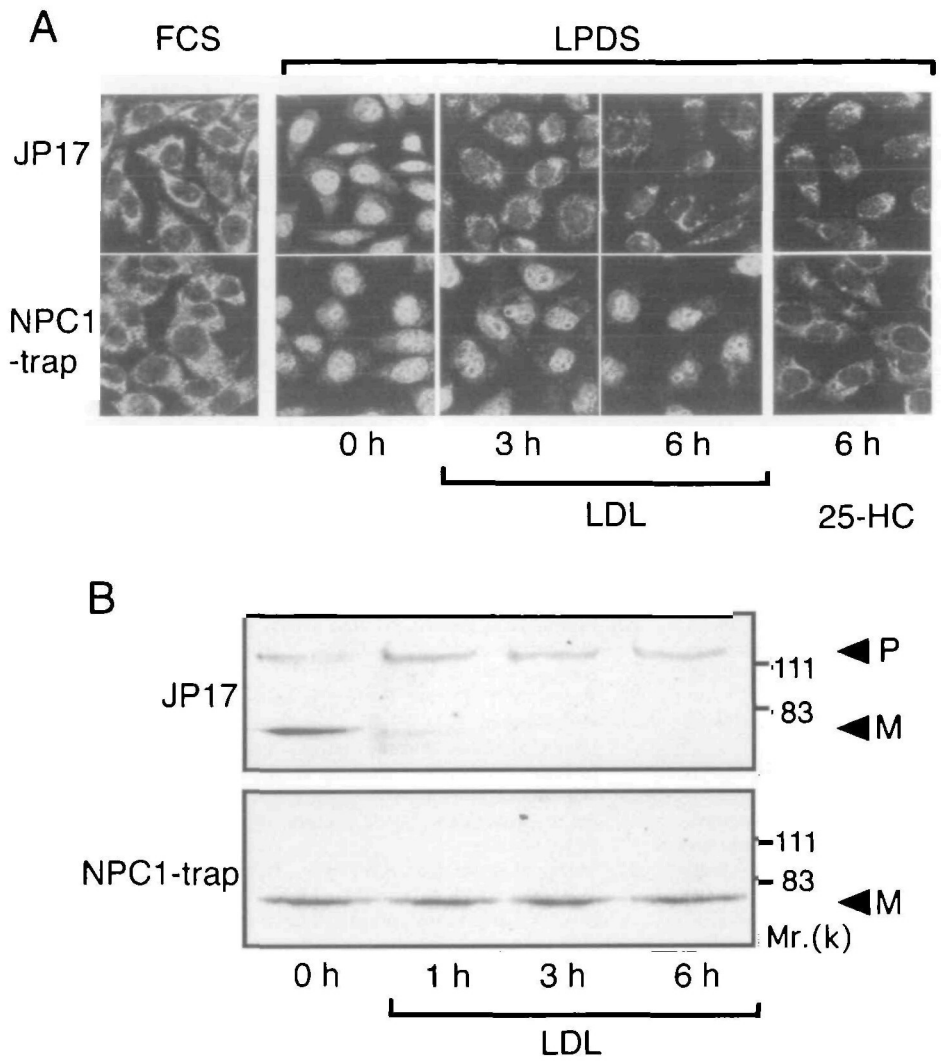

Fig. 5. **Delayed downregulation of SREBP-1 in response to LDL in NPC1-trap cells.** Cells were cultured in F12/10% FCS or 10% LPDS for 48 h. Those cultured in LPDS were then incubated in fresh F12/10% LPDS supplemented with LDL (100 μg/ml) or 25-HC (1 μg/ml) for the time indicated. A: Anti-SREBP1 immunostaining. Shown are the images obtained with a confocal microscope. B: Western blotting of nuclear fractions with the same antibody. Proteins were separated in a 10% polyacrylamide gel. Molecular masses are given on the right. M, the mature form (~70 kDa); P, the precursor form (~125 kDa).

Alternatively, Lange *et al.* proposed that filipin is sequestrated in the large intracellular cholesterol pools in NPC1-deficient cells and fails to reach the plasma membrane where it causes the cytotoxic effect (*30*). When intracellular cholesterol accumulation is diminished by culturing the cells in LPDS, NPC1-trap cells showed a normal sensitivity to filipin as compared with JP17 cells (data not shown), a finding consistent with the idea of Lange *et al.*

Studies using skin fibroblasts from NPC patients have described various phenotypic abnormalities of NPC1-deficient cells that fall into two categories, the accumulation of free cholesterol and other lipids in endosomes/lysosomes, and a delay in various cellular responses to LDL (*24*). In an attempt to establish an *in vitro* model for the disease, NPC1-deficient CHO cells were established from 25-RA cells (*11*) and have been used for functional analyses of the NPC1 protein (*17, 18, 27*). Although 25-RA–derived mutants have intracellular accumulations of free cholesterol that can be abolished by the expression of human and hamster NPC1 (*12*), these cells could not be used to assess the involvement of NPC1 in the LDL-induced feed-back regulation of cholesterol metabolism because of the presence of a gain of the function mutation in the SCAP gene (*13*). We have shown in the present study that, in addition to free cholesterol accumulation, our NPC1-trap cells have clear defects in both the LDL-induced downregulation of cell surface LDL receptors as well as those of SREBP-1. Thus, our cells provide a useful *in vitro* system in which to study the role of NPC1 in the regulation of cellular cholesterol homeostasis, and, further, to clarify the pathogenesis of NPC.

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