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Niemann-Pick C1 protein: Obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization

HIDEMICHI WATARI*, E. JOAN BLANCHETTE-MACKIE†, NANCY K. DWYER†, JANE M. GLICK‡, SHUTISH PATEL§, EDWARD B. NEUFELD†, ROSCOE O. BRADY¶, PETER G. PENTCHEV¶, AND JEROME F. STRAUSS III*‖

*Center for Research on Reproduction and Women's Health, Departments of Obstetrics and Gynecology and ‡Cellular and Molecular Engineering, University of Pennsylvania Medical Center, Philadelphia, PA 19104; †Section of Lipid Cell Biology, National Institutes of Diabetes and Digestive and Kidney Diseases, and ¶Developmental and Metabolic Neurology Branch, National Institutes of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892; and §Neurology Research Laboratory, Veterans Administration Connecticut Healthcare System, Newington, CT 06111

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ABSTRACT Niemann-Pick type C (NPC) disease is an inherited lipid storage disorder that affects the viscera and central nervous system. A characteristic feature of NPC cells is the lysosomal accumulation of low density lipoprotein-derived cholesterol. To elucidate important structural features of the recently identified *NPC1* gene product defective in NPC disease, we examined the ability of wild-type NPC1 and NPC1 mutants to correct the excessive lysosomal storage of low density lipoprotein-derived cholesterol in a model cell line displaying the NPC cholesterol-trafficking defect (CT60 Chinese hamster ovary cells). CT60 cells transfected with human wild-type NPC1 contained immunoreactive proteins of 170 and 190 kDa localized to the lysosomal/endosomal compartment. Wild-type NPC1 protein corrected the NPC cholesterol-trafficking defect in the CT60 cells. Mutation of conserved cysteine residues in the NPC1 N terminus to serine residues resulted in proteins targeted to lysosomal membranes encircling cholesterol-laden cores, whereas deletion of the C-terminal 4-aa residues containing the LLNF lysosome-targeting motif resulted in the expression of protein localized to the endoplasmic reticulum. None of these mutant NPC1 proteins corrected the NPC cholesterol-trafficking defect in CT60 cells. We conclude that transport of the NPC1 protein to the cholesterol-laden lysosomal compartment is essential for expression of its biological activity and that domains in the N terminus of the NPC1 protein are critical for mobilization of cholesterol from lysosomes.

Niemann-Pick type C (NPC) disease is an autosomal recessive neuroviceral lipid storage disorder (1, 2). One of the most pronounced abnormalities in affected cells is the accumulation of free cholesterol derived from low density lipoproteins (LDL) in lysosomes and the Golgi apparatus (3–7). This biochemical phenotype is displayed by cells from NPC patients (3–7), cells from mice homozygous for the spontaneously occurring C57BLKS/J*spm* and BALB/c *npcnih* mutations (8), in addition to Chinese hamster ovary cell mutants generated in the laboratory, including the CT60 line (9–12).

A gene that is mutated in human NPC disease, named *NPC1,* recently was identified by positional cloning (13). This nomenclature recognizes that mutations in at least one other distinct gene can cause the disorder. The human *NPC1* cDNA sequence was isolated from a yeast artificial chromosome that corrected the cholesterol-trafficking defect in human NPC fibroblasts, cells derived from mice homozygous for the BALB/c *npcnih* mutation, and CT60 cells (13, 14). The gene mutated in BALB/c *npcnih* mice was shown to be the murine homolog of the human *NPC1* gene (15).

The human *NPC1* cDNA sequence predicts a protein of 1,278 aa with an estimated molecular mass of 142 kDa (13). A region between amino acid residues 55 and 165, which is free of transmembrane domains, is highly conserved between the human and murine *NPC1* sequences as well as orthologs in *Caenorhabditis elegans* and *Saccharomyces cervisiae* (13, 15). This NPC domain, consisting of 112 aa, contains eight cysteine residues with conserved spacing in all *NPC1* orthologs. Beyond this N-terminal sequence lies up to 16 putative transmembrane domains and a sequence with strong homology to the sterol-sensing domains of 3-hydroxy-3-methylglutaryl CoA reductase, the sterol response element binding protein cleavage activating protein (SCAP), and the Hedgehog signaling protein, Patched (13, 15). The C terminus of NPC1 contains a dileucine motif that serves as a lysosomal targeting sequence for a lysosomal integrated membrane protein, LIMPII (16–18). In the present study, we undertook experiments to identify structural features of the human NPC1 protein that are critical for its actions on intracellular cholesterol trafficking.

MATERIALS AND METHODS

***NPC1* cDNA Expression Constructs.** To produce the template plasmid DNA for site-directed mutagenesis, wild-type *NPC1* cDNA (13) was subcloned into the pSV-SPORT-1 vector (GIBCO). The C63S, C74S/C75S, and C97S mutations and the deletion of the C-terminal 4-aa residues (C-4) were produced by site-directed mutagenesis using reagents purchased from CLONTECH. Each construct was sequenced to confirm that the desired mutation(s) had been introduced into the cDNA as described (19).

Cell Culture and Transfection. The Chinese hamster ovary CT60 cells were generously provided by T. Y. Chang (Dartmouth University, Hanover, NH). The cells were cultured as described (11). In other studies, a line of H-ras and temperature-dependent simian virus 40 large T antigen-transformed granulosa cells derived from BALB/c *npcnih* were studied (14). These cells display the characteristic NPC sterol-trafficking defect.

For transfection, cells were cultured in 6-well plastic culture plate to 50–80% confluence. CT60 cells were transfected by using LipofectAMINE PLUS reagent (Life Technologies, Grand Island, NY) with 2.0 μg/ml of either an empty pSV-SPORT-1 vector, the wild-type, or the mutant *NPC1* cDNAs in pSV-SPORT-1 with or without 0.2 μg/ml of the plasmid expressing an enhanced green fluorescent protein (EGFP) mutant carrying a nuclear targeting sequence (pEGFP). pEGFP was used to identify transfected cells for analysis of

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Abbreviations: NPC, Niemann-Pick type C; LDL, low density lipoprotein; EGFP, enhanced green fluorescent protein.

‖To whom reprint requests should be addressed at: 778 Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104. e-mail: jfs3@mail.med.upenn.edu.

filipin staining. The media were changed to the regular growth media or to media supplemented with 10% lipoprotein-deficient serum for Western blot analysis and for filipin staining and immunocytochemistry, respectively, after 24 h.

Western Blotting. Cells were scraped from the dishes into lysis buffer consisting of 100 mM Tris·HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml of phenylmethylsulfonyl fluoride, and 1 μg/ml of aprotinin and centrifuged at 10,000 $\times$ g for 2 min at 4°C, and supernatants were subjected to SDS/PAGE and then Western blotting (19). A polyclonal antibody (NPC1-C antibody) raised against a peptide corresponding to 19-aa residues (NKAKSCATEERYKGTERER) in the C terminus of human NPC1 (amino acid residues 1256–1274) was used to detect NPC1 (S.P., unpublished work).

Filipin Staining. Transiently transfected cells were fixed with 3% paraformaldehyde in PBS for 30 min. Cells were washed in PBS (three times for 5 min), quenched with 1.5 mg/ml glycine in PBS for 10 min, and stained with 0.05 mg/ml of filipin (Polysciences) in PBS for 30 min. After washing in PBS (three times for 5 min), slides were mounted with coverslips in phenylenediamine/glycerol. For quantitative analysis, 100 EGFP positive cells were selected and viewed with 25$\times$ objective. Intense filipin fluorescence staining of cholesterol in large perinuclear granules is characteristic of the lysosomal cholesterol accumulation in NPC cells (3–5, 13, 14). Cells with markedly reduced filipin-fluorescence were scored as corrected. The corrected cells contained fewer and smaller filipin-positive granules. Cells with some larger filipin-positive granules, representing an intermediate pattern between uncorrected and corrected were scored as uncorrected in our analysis. Values presented are means ± SE from three separate experiments. ANOVA and the Tukey-Kramer test were used to determine significant differences between empty vector control and wild type or mutants. $P < 0.05$ was used as the level of significance.

Immunocytochemical Staining of NPC1. CT60 cells grown on uncoated glass coverslips were transfected as described above. At the end of the culture period, cells were fixed with 3% paraformaldehyde in PBS for 30 min. Cells were immunostained with an indirect procedure as described (6). Immunostaining for NPC1 was done with rabbit affinity purified anti-peptide NPC1-C antibodies and for lysosomal membrane glycoprotein, mouse monoclonal anti-lgp95 (20). Secondary, fluorescently labeled goat anti-rabbit and anti-mouse IgG was from Jackson Immunoresearch. Stained cells were examined with a Zeiss LSM 410, confocal microscope equipped with a UV laser.

RESULTS

Wild-Type NPC1 Is Transported to Lysosomes and Corrects the NPC Cholesterol-Trafficking Defect. To investigate the biological function of wild-type NPC1, we transfected CT60 cells with expression plasmids and performed filipin staining to detect free cholesterol in the lysosomal compartment. The cholesterol-loaded granules were identified as lysosomes based on previous studies of normal and NPC fibroblasts that established that the mass of endocytically derived cholesterol is accumulated in lysosomes during the first 24 h of incubation

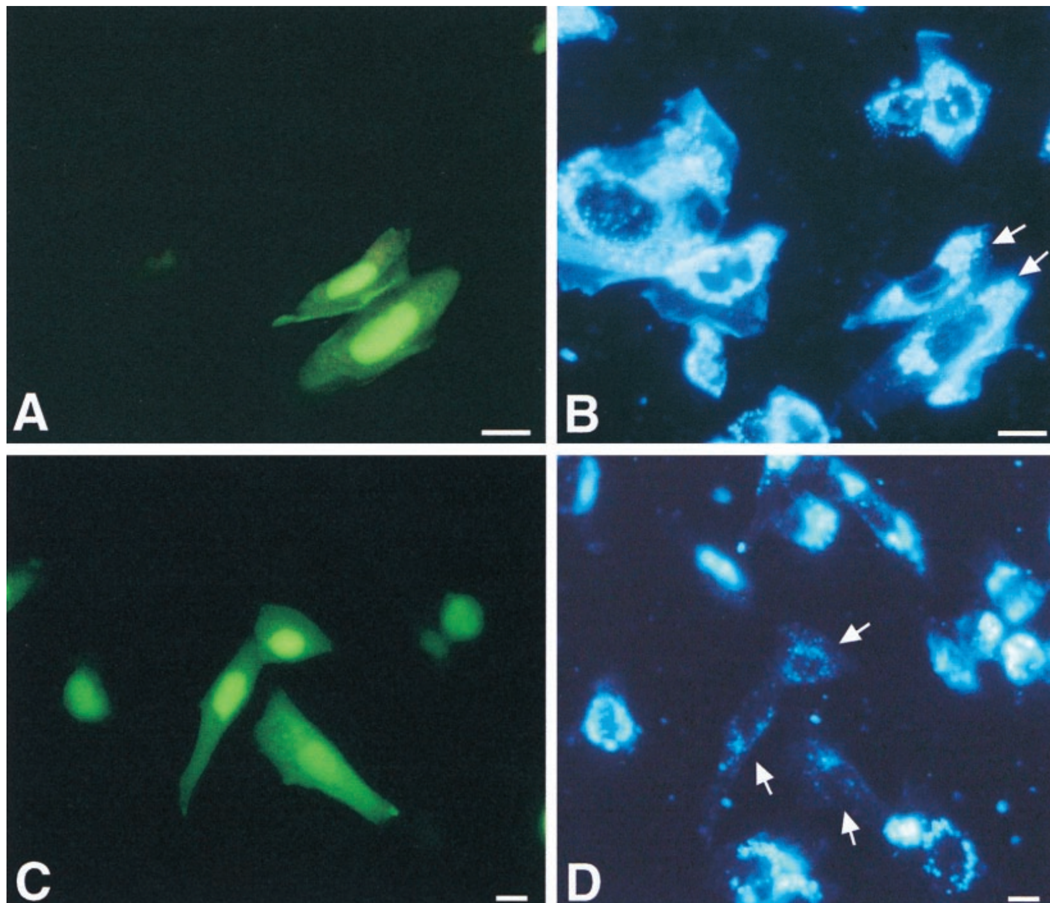

FIG. 1. Photomicrographs of filipin-stained CT60 cells transfected with empty vector or wild-type NPC1 expression plasmid. (*A*) EGFP expression by CT60 cells transfected with pEGFP and empty vector. (*B*) Filipin staining. Arrows indicate EGFP-expressing cells. (*C*) EGFP expression by CT60 cells transfected with pEGFP and wild-type NPC1 expression plasmid. (*D*) Filipin staining. Arrows indicate EGFP-expressing cells. (Bar = 10 μm.)

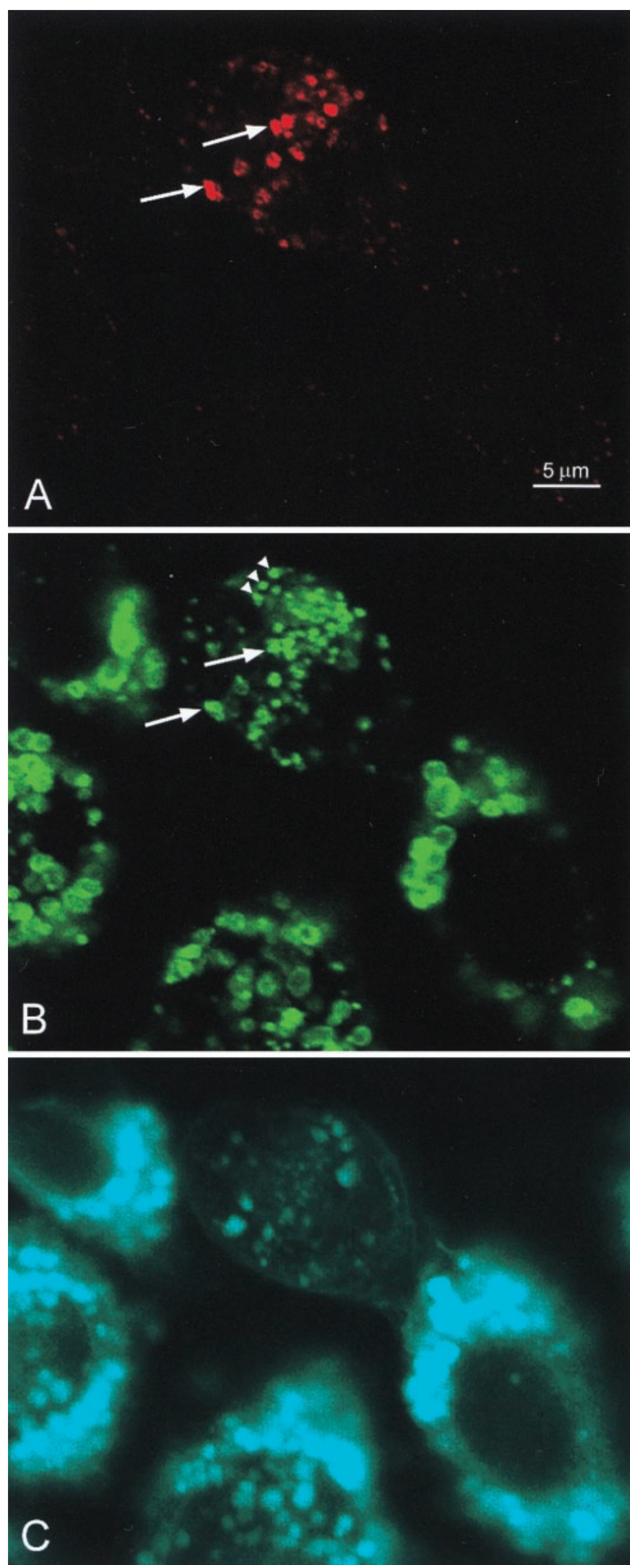

FIG. 2. CT60 cell transfected with wild-type NPC1 immunostained for (*A*) NPC1 protein and (*B*) lysosomal membrane glycoprotein (lgp95) and (*C*) stained with filipin for cholesterol localization. (*A*) NPC1 is present in the transfected cell in granular structures that colocalize with lgp95 (in *B* at arrows). There are additional lgp95 containing organelles (in *B* at arrowheads) that do not contain NPC1 protein. Thus NPC1 protein is in a subset of the lgp95-containing organelles. (*C*) The transfected CT60 cell expressing NPC1 protein does not contain accumulations of intracellular cholesterol in lgp95 containing lysosomes as seen in the four adjacent cells not expressing NPC1 protein. (Bar = 5 μm.)

with LDL. We used pEGFP to identify the transiently transfected cells. EGFP-positive CT60 cells transfected with empty vector and cultured in the presence of LDL showed intense filipin staining in perinuclear granules reflective of lysosomal cholesterol accumulation (Fig. 1 *A* and *B*), whereas EGFP-positive CT60 cells transfected with wild-type NPC1 typically had many fewer and smaller filipin-stained granules, implying correction of the NPC cholesterol-trafficking defect (Figs. 1 *C* and *D* and 2, and Table 1). Sixty-seven percent of the CT60 cells transfected with wild-type NPC1 protein were corrected, whereas less than 8% of the CT60 cells transfected with empty vector showed reduced filipin staining. Because partially corrected cells were scored as uncorrected in our analysis, the 67% correction rate undoubtedly underestimates the impact of NPC1 expression in the CT60 cells.

We also transfected a line of granulosa cells derived from the BALB/c *npcnih* mice with the human NPC1 expression plasmid and found that transfected cells had reduced filipin staining, whereas cells transfected with empty vector did not (data not shown). Because the transfection efficiency of the granulosa cells was quite low, all further experiments were carried out with CT60 cells.

To assess the subcellular localization of wild-type NPC1 protein, immunocytochemical studies were carried out on CT60 cells transfected with empty vector or wild-type NPC1 expression vector. The antibody used for these studies recognized human NPC1 protein in Western blots of extracts of CT60 cells transfected with NPC1 expression plasmid, but CT60 cells transfected with empty vector contained no detectable NPC1 protein (Fig. 3). CT60 cells transfected with wild-type NPC1 displayed a granular pattern of NPC1 immunostaining in 1.0-μm vesicles that were also positive for lysosomal membrane glycoprotein (lgp95), establishing that the NPC1-positive organelles were components of the endosomal/lysosomal pathway (Fig. 2). However, the NPC1 protein was present in some, but not all, of the lgp95-positive vesicles. Cells containing large filipin-positive granules did not stain for NPC1 protein, but did contain lgp95-positive vesicles (Fig. 2). Preimmune serum gave no staining of CT60 cells transfected with wild-type NPC1 expression plasmid and CT60 cells transfected with empty vector did not show NPC1 immunostaining when reacted with immune serum (data reviewed but not presented).

Mutant NPC1 Proteins That Do Not Localize to the Core of Cholesterol-Loaded Lysosomes Do Not Correct the NPC Cholesterol-Trafficking Defect. To elucidate the structural features of human NPC1 protein that are required for its subcellular localization and biological function, we introduced point mutation(s) into the human *NPC1* cDNA coding sequence and analyzed the subcellular localization and biological activities of the resulting mutant proteins. We focused attention on the N

Table 1. Correction of the NPC sterol trafficking defect in CT60 cells by expression of NPC1

Transfected plasmid	% Corrected cells
Empty vector	7.9 ± 1.0
Wild-type NPC1	67.8 ± 4.6*
C63S	8.1 ± 1.8
C74S/C75S	5.5 ± 1.0
C97S	8.3 ± 2.9
C-4	7.5 ± 1.0

CT60 cells were transfected with the indicated plasmid and the pEGFP expression plasmid. Cells were incubated with LDL and then fixed for filipin staining as described in the text. One hundred EGFP-stained cells were analyzed for filipin staining in each treatment. The values presented are the percentage of EGFP-expressing cells showing a marked reduction in perinuclear filipin-positive granules. Values are means ±SE from three separate experiments. *Different from all other groups, $P < 0.001$.

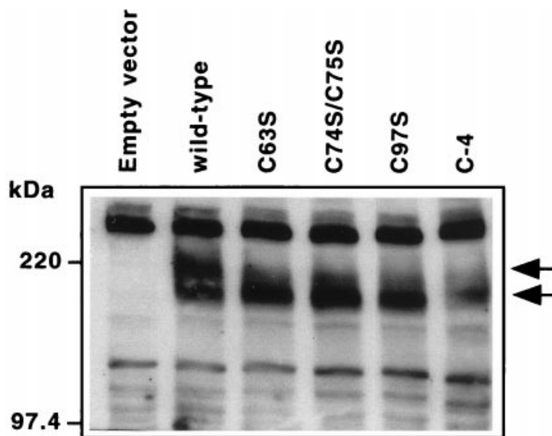

FIG. 3. Expression of wild-type NPC1 and NPC1 mutants in CT60 cells. CT60 cells were transfected with the indicated plasmids, and Western blot analysis was performed on cell extracts as described in *Materials and Methods*. Each lane contained 100 μg of protein. Arrows indicate the NPC1 bands.

and C termini of the NPC1 protein because the N terminus is highly conserved, suggesting functional importance, and the C terminus contains the dileucine motif (LLNF), which is a potential lysosomal targeting sequence. We mutated conserved cysteine residues in the N terminus (C63, C74/C75, and C97) to serine residues and introduced a stop codon (TGA) just before the dileucine motif (C-4) to delete the putative lysosomal targeting sequence.

Each of the mutant proteins was detectable in the transfected CT60 cells by Western blot analysis (Fig. 3). However, in contrast to the wild-type NPC1 protein that yielded proteins of 170 and 190 kDa, the mutant NPC1 expression plasmids each yielded a major immunoreactive protein of 170 kDa. These findings were confirmed with a polyclonal antiserum generated against a recombinant protein representing amino acid residues 25–266 of the human NPC1 N terminus (data not shown).

Immunocytochemical studies revealed that N-terminal mutant proteins, C63S, C74S/C75S, and C97S, were localized at the surface of lysosomes. Fig. 4 shows representative immunostaining of the C97S mutant. These NPC1 domain mutant proteins were visualized as rings (Fig. 4*B*) surrounding filipin-stained spheres (Fig. 4 *A* and *D*). The mutant NPC1 protein (Fig. 4*B*) colocalized with lgp95 (Fig. 4*C*), indicating that it was restricted to the membrane surface of lysosomes whose cores were loaded with free cholesterol. A similar pattern of immunostaining was obtained with the C63S and C74S/C75S mutants (data not shown).

The C-4 mutant lacking the lysosomal targeting sequence was localized to intracellular membranes displaying a reticular pattern characteristic of the endoplasmic reticulum and in the nuclear envelope (Fig. 5 *A* and *B*). None of the N-terminal mutant NPC1 proteins or the C-4 mutant lacking the dileucine motif corrected the cholesterol-trafficking defect in the CT60 cells identified by filipin staining (Table 1).

DISCUSSION

In the present study we have shown that wild-type NPC1 protein is localized in a subset of vesicles containing lgp95, a marker for both late endosomes and lysosomes in Chinese hamster ovary cells (20). In human fibroblasts, we found a similar localization of NPC1 protein in a subset of vesicles containing LAMP2 (21), a marker for late endosomes and lysosomes (22). The LAMP2-positive, NPC1-containing vesicles, linked to a prelysosomal compartment in fibroblasts by kinetic studies on uptake of [^{14}C] sucrose, are distinct from LAMP2-positive lysosomes that accumulate cholesterol derived from LDL. The fibroblast study also suggested that wild-type NPC1-containing vesicles interact transiently with the cholesterol-laden lysosomes, and that under certain experimental conditions, NPC1 protein can enter the core of these organelles (21). Thus, NPC1 appears to move through prelysosomal vesicles before its entry into the lysosomal compartment. In the CT60 cells transfected with wild-type NPC1 expression vector, the lgp95-positive, NPC1-containing vesicles may be prelysosomal vesicles, similar to those that we

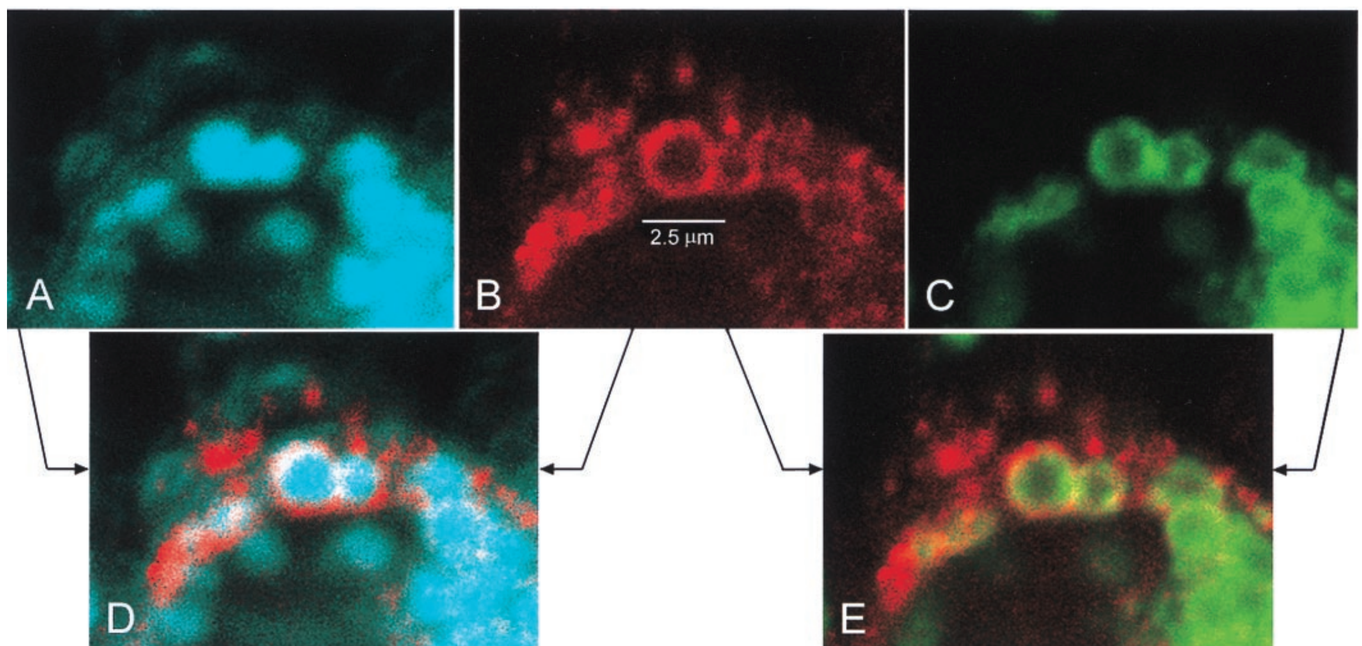

FIG. 4. CT60 cell transfected with the C97S NPC1 mutant. (*A*) Filipin staining showed transfected cells did not clear of cholesterol and that the NPC1 protein (*B*) was present in rings at the surface of the cholesterol-laden cores of lysosomes that are lgp95 positive (*C*). The lgp95 immunostaining that marks protein in the lysosomal membrane colocalizes with the mutant NPC1 protein, indicating that the mutant NPC1 is at the surface of lysosomes. *D* is the merged image of *A* and *B*; and *E* is the merged image of *B* and *C*. (Bar = 2.5 μm.)

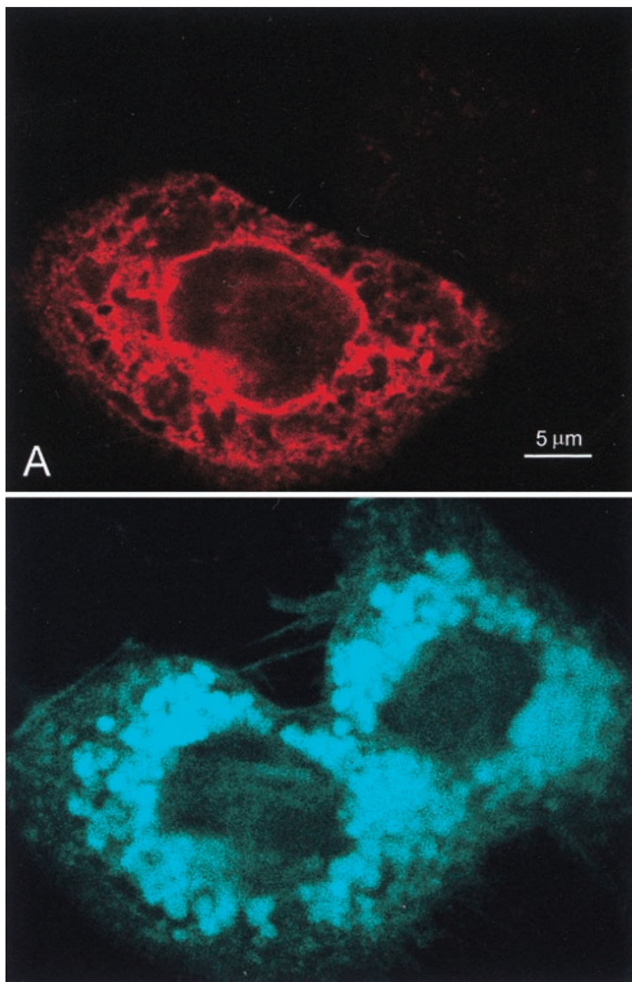

FIG. 5. CT60 cell transfected with the C-4 NPC1 mutant lacking the dileucine motif. The mutant NPC1 protein (*A*) is present in a reticular pattern characteristic of the endoplasmic reticulum and in the nuclear envelope (*B*). The cell expressing mutant NPC1 has not cleared of cholesterol evidenced by its accumulation of filipin-positive lysosomes similar to the adjacent cell, not expressing mutant NPC1 protein. (Bar = 5 μm.)

observed in fibroblasts, or they may represent lysosomes that have been cleared of cholesterol. In contrast, the nonfunctional N-terminal mutant NPC1 proteins appear to be retained on the surface of the cholesterol-laden lysosomes.

The NPC1 C terminus contains a dileucine motif (LLNF). Related dileucine motifs mediate lysosomal targeting and internalization of proteins from the cell surface (16–18). Deletion of the dileucine motif from the NPC1 C terminus resulted in a protein that accumulated in the endoplasmic reticulum and was incapable of correcting the NPC sterol trafficking abnormality. The N terminus of NPC1 contains 13 hydrophobic amino acid residues typical of signal peptides that target proteins to the endoplasmic reticulum. Thus, newly synthesized NPC1 protein may be directed to the endoplasmic reticulum first, and then sorted through a prelysosomal compartment and ultimately to lysosomes by the dileucine motif. The C-terminal mutant lacking the lysosomal targeting dileucine motif may not be able to escape from the endoplasmic reticulum, accounting for the immunocytochemical distribution that we observed with the C-4 mutant. It is also possible that the NPC1 dileucine motif is essential for protein function once NPC1 enters the lysosomal compartment. However, this possibility cannot be evaluated in our experimental system.

Mutations in the conserved cysteine residues in the NPC1 domain also caused loss of biological activity despite targeting of the mutant proteins to the surface membranes of lysosomes. This localization to membranes encircling cholesterol-loaded spheres raises the possibility that NPC1 must enter into the lysosome core, which is compacted with cholesterol-enriched multilamellar structures (23), to function. This notion is consistent with our observations on human fibroblasts where NPC1 could be found in the core of cholesterol-laden lysosomes (21). That all three N-terminal cysteine residue mutants lost biological activity suggests that the NPC1 domain is functionally important. The correct formation of intra- or interchain disulfide bonds may be crucial for the proper assembly of NPC1 proteins, which in turn could influence maintenance of an active confirmation. A misfolded N-terminal domain might prevent movement of the NPC1 protein to its site of action within the lysosomal core.

The human *NPC1* cDNA sequence predicts a protein with an estimated molecular mass of 142 kDa. However, two proteins of larger size, 170 and 190 kDa, were detected in cells transfected with the wild-type NPC1 expression plasmid. These immunoreactive proteins presumably represent post-translationally modified NPC1. There are 14 potential N-glycosylation sites conserved in human and mouse NPC1 (13, 15). In studies to be reported elsewhere, we found that treatment of extracts of COS-1 cells transfected with NPC1 expression plasmid with *N*-glycosidase F resulted in an 18-kDa reduction in the apparent molecular mass of a tryptic peptide recognized by a polyclonal antiserum raised against residues 25–266 of the NPC1 N terminus (H.W. and J.F.S., unpublished observations). This finding confirms that NPC1 is glycosylated. Interestingly, all of the biologically inactive mutant constructs yielded a major 170-kDa product in the CT60 cells, suggesting that a posttranslational modification resulting in the formation of the 190-kDa immunoreactive species may be essential for NPC1's functional activity, or alternatively, that the NPC defect influences protein glycosylation. The latter idea is consonant with the recent observations of Nohturfft *et al.* (24) revealing sterol regulation of the processing of the carbohydrate chains of SCAP, a protein that shares structural features with NPC1.

Although we examined the ability of wild-type NPC1 and NPC1 mutants to correct the excessive lysosomal accumulation of LDL-derived free cholesterol in the present studies, it also is known that NPC cells show enrichment of cholesterol in trans-cisternal Golgi compartments and delayed relocation of cholesterol to and from the plasma membrane (6, 25). Further studies are needed to determine whether the wild-type and mutant NPC1 proteins can correct these alterations in intracellular cholesterol movement. Such studies could provide important insight into the complexities of intracellular cholesterol transport (26–29).

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