Amino acid substitution in NPC1 that abolishes cholesterol binding reproduces phenotype of complete NPC1 deficiency in mice

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Substitution mutations in adjacent amino acids of the N-terminal domain of NPC1, a lysosomal membrane protein, abolish its cholesterol binding activity and impair its ability to export cholesterol from lysosomes of cultured cells lacking *npc1* [Kwon HJ, et al. (2009) *Cell* 137:1213–1224]. Here, we show that the same two mutations (proline-202 and phenylalanine-203, both changed to alanine) reproduce the phenotype of complete NPC1 deficiency when knocked into the mouse *npc1* gene by homologous recombination. Homozygous *npc1*^{pf/pf} mice exhibited neurodegeneration beginning at day 49 and died at a median age of 84 d, as previously reported for mice that lack *npc1*. Liver and other organs of the *npc1*^{pf/pf} mice accumulated excess cholesterol in lysosomes. In liver, mRNAs encoding several lysosomal proteins were elevated, including NPC1 and NPC2 and several digestive enzymes (acid lipase, β -glucuronidase, and cathepsins B and D). Weekly treatment with hydroxypropyl- β -cyclodextrin (HPCD) beginning at 7 wk reduced hepatic cholesterol accumulation and diminished the lysosomal mRNAs. We conclude that the cholesterol binding site in the N-terminal domain of NPC1 is essential for cholesterol export from lysosomes in living animals as it is in cultured cells. The HPCD-mediated reduction of excess lysosomal enzymes may contribute to the ability of this drug to delay the progression of NPC disease in mice.

cholesterol binding domain | cyclodextrin | knockin mutation | lysosomal storage disease | Niemann-Pick disease

In Neimann-Pick C disease, a mutation in either of two lysosomal proteins, NPC1 or NPC2, causes cholesterol and other lipids to accumulate in lysosomes throughout the body, resulting in death from multiorgan failure (1). To explain this dual requirement, we proposed a handoff model in which NPC2 and NPC1 act sequentially to remove cholesterol from lysosomes (2, 3). Malfunction of either protein causes a primary accumulation of cholesterol with secondary accumulation of other lipids, and this leads to cell death, especially in liver, lung, and cerebellum.

The sequential handoff model for NPC action is supported by biochemical and ultrastructural studies. Cholesterol enters lysosomes as a result of the receptor-mediated endocytosis of LDL. Lysosomal acid lipase hydrolyzes the cholesteryl esters of LDL, and the resulting insoluble cholesterol is postulated to be scavenged by NPC2 before it has a chance to form crystals. NPC2 is a soluble protein of 132 amino acids that is localized to the lysosomal lumen. Sterol binding studies (4) and X-ray crystallography (5) have demonstrated that NPC2 binds cholesterol in a specific orientation, with the isoctyl side chain buried and the 3β -hydroxyl group exposed. The model postulates that NPC2 carries cholesterol to the lysosomal lining membrane, but it is prevented from accessing the membrane because the membrane is coated by a dense glycocalyx (2). At this point NPC2 transfers its cholesterol to NPC1, a 1,278-amino acid protein with 13 transmembrane helices that is embedded in the lysosomal membrane (6, 7). The cholesterol acceptor site on NPC1 is contained in the N-terminal domain, a sequence of 240 amino acids that is postulated to project into the lumen of the lysosome (2, 4).

The truncated N-terminal domain of NPC1, referred to as NPC1(NTD), can be expressed and purified as a soluble protein (4). Crystallographic studies show that this domain forms a stable structure that is largely α -helical (2). Cholesterol binds to this domain in an orientation opposite to its orientation in NPC2 (i.e., the 3β -hydroxyl group is buried in the protein, where it makes specific contacts with certain amino acids, whereas the isoctyl side chain projects to the surface). When NPC1(NTD) is incubated with cholesterol in solution containing ethanol and dilute detergent, the cholesterol binds to the protein very slowly at 4 °C (8). Slow binding is attributed to the presence of several α -helices that partially occlude the sterol binding pocket (2). Binding is accelerated by orders of magnitude if the cholesterol is first bound to NPC2 and then transferred to NPC1(NTD) (8). We speculate that NPC2 interacts with NPC1(NTD) so as to move aside the obstructing helices, allowing cholesterol to slide from NPC2 to NPC1 without having to enter the water phase. The sliding model is supported by crystallography showing the opposite orientation of cholesterol in the two binding sites (2, 3).

The cholesterol handoff model is supported by alanine-scanning mutagenesis of NPC2 and NPC1(NTD) (2, 3). These mutations revealed two classes of defects. Replacement of amino acids in either of the sterol binding pockets led to a loss of cholesterol binding. The other class of mutations localized to patches on the surface of both proteins that are not required for cholesterol binding but are required for cholesterol transfer from NPC2 to NPC1(NTD). In modeling studies, these patches could be aligned so as to bring the two cholesterol binding sites into close proximity, in such a way that the plane of the cholesterol would be held constant as the sterol slid from NPC2 to NPC1(NTD) (3).

The physiologic relevance of the identified amino acids required for binding and transfer was confirmed by introducing the mutations into plasmids encoding NPC2 or full-length NPC1. Proteins encoded by these mutant plasmids failed to rescue defective sterol transport in cultured cells deficient in NPC2 or NPC1, respectively (2, 3).

In the present studies, we have sought to confirm the physiologic relevance of the cholesterol binding site on NPC1(NTD) in organs of living animals. For this purpose, we chose a mutation that replaces two adjacent amino acids in the cholesterol binding pocket (proline-202 and phenylalanine-203) with alanines. In previous studies, NPC1(NTD) containing these mutations failed to bind cholesterol when tested in vitro, and full-length NPC1 containing these mutations failed to complement the defect in NPC1-deficient CHO cells (2). Here, we show that knockin mice homozygous for the P202A/F203A mutation (designated as *npc1*^{pf/pf} mice) exhibit a clinical and pathologic syndrome indistinguishable from that

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Fig. 2. NPC1 and NPC2 levels in livers of WT and *npc1^{pf/pf}* mice. Nine-week-old male WT and *npc1^{pf/pf}* littermates were fed an ad libitum chow diet before study. (A) mRNA analysis. Relative amounts of NPC1 and NPC2 mRNAs in livers were determined by quantitative real-time PCR with apoB as the invariant control. Values represent the amount of mRNA relative to that in WT littermates, which is arbitrarily defined as 1. Each bar represents the mean $\pm$ SEM of data from 4 mice. Asterisks denote the level of statistical significance (Student *t* test) between the WT and *npc1^{pf/pf}* mice. ***P* < 0.01. The number of PCR cycles (cycle threshold, *C_t*) required to reach the threshold line of 0.15 is shown inside the bar graph. (B) Immunoblot analysis. For each genotype, whole-cell lysates were prepared from four mouse livers. Aliquots of the pooled lysates (40 μ g protein) were subjected to 8% SDS/PAGE and immunoblot analysis. β -Tubulin was used as a loading control. (C) Glycosidase digestion. For each genotype, membrane fractions from four mouse livers were pooled. Aliquots of the pooled fractions (40 μ g and 20 μ g for WT and *npc1^{pf/pf}*, respectively) were incubated in the absence or presence of the indicated glycosidase and subjected to SDS/PAGE and immunoblot analysis.

Discussion

The studies in this article demonstrate that a knockin mutation (P202A/F203A) that abolishes cholesterol binding to the N-ter-

minal domain of NPC1 reproduces the lethal phenotype of complete NPC1 deficiency in mice. This observation provides strong evidence that the cholesterol binding site on NPC1(NTD) that was demonstrated previously to be essential for NPC1 function in cultured cells (2, 4) is also essential for the function of the protein in the whole animal.

The *npc1^{pf/pf}* mice harboring the knockin P202A/F203A mutation accumulated cholesterol in various organs in a pattern that resembles that seen in NPC1-deficient *npc1^{nih/nih}* mice, the standard model in the field (9). This accumulation led to organ dysfunction and death at the same age as previously observed in the *npc1^{nih/nih}* mice. Like the *npc1^{nih/nih}* mice (10–12, 15), the *npc1^{pf/pf}* mice showed dramatic improvement when treated with the cholesterol solubilizer HPCD. Weekly injections caused a marked fall in hepatic cholesterol content (Fig. 7A and B).

In important quantitative studies, Dietschy and coworkers observed that weekly injections of HPCD, beginning at 7 d of age, markedly prolonged the lifespan of *npc1^{nih/nih}* mice (15). This improvement was associated with a marked reduction in the content of unesterified cholesterol in liver. In another study they showed that a single injection of HPCD at 49 d of age lowered tissue cholesterol to a much lesser extent (19). Our results indicate that a reduction in liver cholesterol can be attained when the initial injection is given at 7 wk of age and repeated twice at weekly intervals. We have not yet studied the *npc1^{pf/pf}* mice to determine whether lifespan can be extended under these circumstances.

We considered the possibility that cholesterol accumulation in the *npc1^{pf/pf}* mice was caused by impaired folding of the mutant NPC1, thereby precluding its exit from the ER and its transport to the lysosome. Two observations argued against this possibility. First, we used endoglycosidase H treatment to show that the *N*-linked carbohydrates of the mutant NPC1 were processed in the Golgi to the same extent as wild-type NPC1, indicating that the protein left the ER (Fig. 2C). Second, when an epitope-tagged version of the mutant NPC1 was transfected into cultured cells, the protein colocalized with the lysosome marker LAMP1 (Fig. 3).

A unique observation in the current studies was the elevation in the hepatic content of mRNAs encoding a subset of lysosomal proteins. A previous study showed that brains of *npc1^{nih/nih}* mice had elevations in two lysosomal enzymes, cathepsins B and D (18). In livers of the *npc1^{pf/pf}* mice, we found elevations in the mRNAs encoding these two enzymes as well as the mRNAs encoding other lysosomal enzymes, such as β -glucuronidase, cathepsin L1, lysosomal acid lipase, neuraminidase, and acid β -glycosidase. However, not all lysosomal mRNAs were elevated. Most importantly, there was no elevation in the mRNA encoding acid sphingomyelinase. Cultured cells lacking NPC1 are reported

Fig. 3. Colocalization of transfected NPC1(P202A/F203A) with endogenous LAMP1. SV-589 fibroblasts were transfected with TK-driven plasmids expressing Flag-tagged versions of either WT NPC1 (A–C) or mutant NPC1 (P202A/F203A) (D–F) as described in *Materials and Methods*. Twenty-four hours after transfection, the cells were fixed and immunostained for Flag-tagged NPC1 (red) and endogenous LAMP1 (green). Nuclei were stained blue with DAPI. Fluorescence images were viewed and processed by confocal microscopy as described in *Materials and Methods*. Each image represents a maximum intensity projection of a confocal z-stack of 32 images. (Scale bars, 10 μ m; magnification: 190 $\times$.)

Fig. 4. Growth and survival of WT and *npc1^{pf/pf}* mice. (A) Littermate WT ($n = 30$, 18 male and 12 female) and *npc1^{pf/pf}* ($n = 30$, 18 male and 12 female) mice were weighed weekly beginning at 5 wk of age. (B) The same cohort of WT ($n = 30$) and *npc1^{pf/pf}* ($n = 30$) mice were followed up to 112 d of age, and the percent survival of each group was plotted as a function of time. For comparison, the reported survival curve for *npc1^{nih/nih}* mice (14) was replotted in red.

to have reduced acid sphingomyelinase activity despite normal levels of the enzyme protein (20, 21). Reduced sphingomyelinase activity is likely responsible for the elevated sphingomyelin levels in NPC1-deficient lysosomes (1, 22).

In addition to the elevations in certain lysosomal enzyme mRNAs, we also found elevations in the mRNAs encoding both NPC1 and NPC2 (Figs. 2A and 7C). The elevations in NPC1 and NPC2 protein were at least as profound as the mRNA elevations (Fig. 2B). These data raise the possibility that the accumulation of cholesterol triggers a compensatory response designed to up-regulate the proteins that might enhance cholesterol removal from lysosomes. Consistent with this hypothesis was the observation that all of the elevated lysosomal mRNAs were restored toward normal when cholesterol was depleted from the lysosomes by HPCD treatment (Fig. 7C).

Amritraj et al. (18) provided evidence that the up-regulation of cathepsins B and D in cerebellum of NPC1-deficient mice was

associated with a maldistribution of the enzyme from lysosomes to the cytoplasm. They postulated that these proteolytic enzymes initiated a series of reactions leading to neuronal death. Considered together with their data, our observation that cathepsin mRNA levels are reduced by HPCD treatment raises the possibility that the improvement in lifespan in HPCD-treated mice is related to the lowering of cathepsin levels that occurs when lysosomal cholesterol is reduced. This hypothesis can be tested in future experiments in the mouse model.

Materials and Methods

Generation of *npc1* Knockin Mice. The targeting vector used to generate *npc1* knockin mice was constructed in pJB1, a gene replacement vector with a loxP-flanked *pgkneopA* cassette and two copies of the HSV-TK gene as selection markers (23). The two regions of homology were generated by PCR using genomic DNA derived from SM-1 ES cells (derived from 129SvEv blastocysts). PCR products were cloned into pJB1. For the short-arm region containing exon 5 of *npc1*, the codons for a proline (CCA) and a phenylalanine (UUU) at adjacent residues 202 and 203 of NPC1 were mutated to alanine codons (GCA and GCU, respectively) by site-directed mutagenesis (Stratagene QuikChange Site-Directed Mutagenesis Kit). The integrity of all constructs was confirmed by restriction analysis and DNA sequencing.

SM-1 ES cells were cultured, transfected with the linearized targeting vector, and selected with 250 $\mu\text{g}/\text{mL}$ G418 as described previously (24). Three independent ES clones that had undergone homologous recombination as determined by PCR and Southern blotting were injected into C57BL/6J blastocysts to generate chimeric mice. When crossed to C57BL/6J females, male chimeras from all three clones gave offspring that carried the *npc1* knockin allele through the germ line.

Animal Studies. All experiments were carried out with WT and *npc1* knockin (*npc1^{pf/pf}*) littermates obtained from intercrosses of *npc1^{+/pf}* heterozygous male and female mice, all of which are hybrids of C57BL/6J and 129SvEv strains. The *npc1^{nih/nih}* mice used in Fig. 6 (obtained from Dr. John Dietschy, University of Texas Southwestern Medical Center, Dallas, TX) were on a pure BALB/c background (9, 11). All mice were housed in colony cages with a 12-h light/12-h dark cycle and fed ad libitum with Harlan Teklad Global Diet 2018 (Harlan Laboratories) and killed in the nonfasting state during the early phase of the light cycle. For the growth and survival study, the WT and *npc1^{pf/pf}* littermates were weighed weekly, and their general appearance was monitored daily. Starting at 7 wk of age when the *npc1^{pf/pf}* mice started to show tremor and ataxia, food pellets were placed directly in the cage. When a mouse exhibited visible difficulty in ambulating to reach water or take food, it was killed, and this was noted as the day of death. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center at Dallas.

Cyclodextrin Treatment. HPCD (CTD Holdings, catalog no. THPB-EC) in a 20% (wt/vol) saline solution was administered to mice at a dose of 4,000 mg/kg body weight by s.c. injection as described previously (11). Male WT and *npc1^{pf/pf}* littermates were injected with HPCD or saline at 7, 8, and 9 wk of

Fig. 5. Histology of various tissues from WT and *npc1^{pf/pf}* mice. Tissues from 9-wk-old female WT (A–C) and *npc1^{pf/pf}* (D–F) littermates were fixed, sectioned, and stained with H&E as described in *Materials and Methods*. Arrows (A) denote Purkinje cells that are present in cerebellum of WT mice but absent from *npc1^{pf/pf}* mice. (Scale bars, 50 μm ; magnification: 40 $\times$.)

Fig. 6. Accumulation of cholesterol and gangliosides in organs of *npc1^{pf/pf}* mice. (A) Total cholesterol content in various organs of 9-wk-old male WT and *npc1^{pf/pf}* littermates was determined as described in *Materials and Methods*. Each bar represents mean $\pm$ SEM of data from four mice. Asterisks denote level of statistical significance (Student *t* test) between the WT and *npc1^{pf/pf}* mice. ***P* < 0.01. (B) Gangliosides from tissues of 9-wk-old male *npc1^{pf/pf}*, *npc1^{nih/nih}*, and WT mice were subjected to TLC and visualized by orcinol staining as described in *Materials and Methods*. For each genotype, equal weights of liver or brain tissue from four mice were pooled. Each lane corresponds to the ganglioside fraction isolated from 3 mg of brain or 1.5 mg of liver (wet weight). Position of migration of standards for GM1, -2, and -3 are indicated.

age. Three days after the final injection the mice were killed to obtain plasma and tissues for various analyses.

Quantitative Real-Time PCR Analysis. Total RNA was prepared from mouse liver using RNA STAT-60 (Tel-Test) and subjected to quantitative real-time PCR as described previously (24). All reactions were carried out in triplicate, and the relative amounts of mRNAs were calculated using the comparative *C_t* method with apolipoprotein B (apoB) as the invariant control. Primers for apoB were described previously (24). Primers not previously described can be found in Table S1.

Glycosidase Treatment and Immunoblot Analysis. Membrane proteins were prepared from frozen tissues as described previously (25). For glycosidase treatment, the 100,000 $\times$ g membrane pellet from 50 mg liver or brain was resuspended in 100 μ L buffer containing 10 mM Tris-chloride at pH 6.8, 100 mM NaCl, and 0.5% (wt/vol) SDS and shaken for 30 min at room temperature. Equal amounts of solubilized membrane protein from the liver or brain tissues of four mice were then pooled. Aliquots of the pooled membrane protein were incubated in the absence or presence of 2,500 U Endo H or 2,500 U PNGase F (New England Biolabs) and subjected to 8% SDS/PAGE and immunoblot

Fig. 7. Cyclodextrin treatment of WT and *npc1^{pf/pf}* mice. Male WT and *npc1^{pf/pf}* littermates were treated with three weekly injections of saline or HPCD beginning at 7 wk of age as described in *Materials and Methods*. At 9.5 wk of age (3 d after the final injection), the mice were killed to obtain plasma and organs for analysis. (A) Content of total and free cholesterol in livers of WT and *npc1^{pf/pf}* mice injected with saline or HPCD. Each bar represents the mean $\pm$ SEM of data from five mice. (B) Histology of representative livers from WT and *npc1^{pf/pf}* mice injected with saline or HPCD. Livers were fixed, sectioned, and stained with H&E. (Scale bars, 40 μ m; magnification: 40 $\times$.) (C) Relative amounts of mRNAs in livers of the indicated groups were determined by real-time PCR, with apoB as the invariant control. Values represent the amount of mRNA relative to that in WT littermates injected with saline, which is arbitrarily defined as 1 and denoted by the dotted line. Each bar represents the mean $\pm$ SEM of data from five mice. Statistical analysis (A and B) was performed with two-tailed Student *t* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

analysis as described previously (3). The primary antibodies for immunoblot analyses were 0.5 μ g/mL rabbit polyclonal antibody directed against a synthetic peptide corresponding to C-terminal region of human NPC1 (catalog no. 36983, Abcam), 1 μ g/mL rabbit polyclonal anti-NPC2 (3), and 1:1,000 dilution of rabbit monoclonal anti- β -tubulin (catalog no. 2128, Cell Signaling). Bound antibodies were visualized by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) using a 1:3,000 dilution of anti-rabbit IgG (GE Healthcare) conjugated to horseradish peroxidase.

Histology. For histological analysis, anesthetized mice were transcardially perfused with normal saline and then with 10% (vol/vol) formalin. After perfusion, the tissues were fixed in 10% formalin overnight at 4 °C. The fixed tissues were embedded in paraffin, sectioned at 5 μ m, and stained with H&E.

Immunofluorescence. Human SV-589 fibroblasts were grown at 37 °C in 5% CO₂ in DMEM containing 100 U/mL penicillin, 100 μ g/mL streptomycin sulfate, and 10% FCS. On day 0, the cells were set up at 65×10^3 cells/35-mm glass-bottom dish (MatTek, catalog no. P35G-1.5-14-C). On day 1, the cells were transfected with 1 μ g pTK-NPC1-His8-Flag3 or pTK-NPC1(P202A/F203A)-His8-Flag3 using FuGENE 6 transfection reagent (Roche Applied Science) as described previously (2). Twenty-four hours after transfection, the cells were fixed for 10 min at room temperature in 4% (wt/vol) paraformaldehyde, washed with PBS (three times; 1 min per wash), and treated with 0.1% (vol/vol) Triton X-100 in PBS for 90 s. After washing 3 times with PBS, the cells were incubated for 30 min in a blocking solution containing 10% (vol/vol) normal goat serum (Invitrogen, catalog no. 50-062Z). Thereafter, the cells were incubated in the same blocking solution for 16 h at 4 °C with 1 μ g/mL mouse monoclonal anti-LAMP1 (Abcam, catalog no. ab25630) and 0.5 μ g/mL rabbit polyclonal anti-Flag (Sigma-Aldrich, catalog no. F7425) to detect endogenous LAMP1 and the transfected Flag-tagged NPC1 proteins, respectively. After washing three times with PBS, the cells were incubated for 1 h at room temperature with 5 μ g/mL Alexa Fluor 488 (green) donkey anti-mouse IgG (Invitrogen, catalog no. A-21202) and 5 μ g/mL Alexa Fluor 568 (red) goat anti-rabbit IgG (Invitrogen, catalog no. A-11036) anti-

bodies. After three washes with PBS, the cells were incubated with 300 nM DAPI (Invitrogen, catalog no. D3571) in PBS for 10 min to stain the nuclei. Fluorescence was viewed with a Leica TCS SP5 laser scanning confocal microscope using an Argon Laser with excitation wavelength of 488 nm for Alexa Fluor 488 (green) and a HeNe1 laser with excitation wavelength of 561 nm for Alexa Fluor 568 (red). DAPI (blue) fluorescence was viewed using a HeNe1 laser with excitation wavelength of 405 nm. Colocalization analysis was done using the Imaris software (version 7.2.3; www.bitplane.com).

Lipid Measurements. The content of total and free cholesterol in tissues was measured using Wako kits (catalog no. 439-17501 and 435-35801, respectively). Extraction and analysis of gangliosides were carried out as previously described (26). Briefly, total lipids from brain and liver tissues were extracted in chloroform:methanol:PBS (1:1:0.9, vol/vol/vol) by the method of Bligh and Dyer (27). Equal amounts (based on wet tissue weight) of total lipid extracts from four mice per group were pooled, and aliquots of the pooled samples were then separated into neutral and anionic lipid fractions using DEAE Sephadex A-25 columns (GE Healthcare). The anionic lipid fractions (containing gangliosides) were desalted with PD-10 Sephadex G-25 columns (GE Healthcare). Aliquots of the ganglioside fraction corresponding to 3 mg brain or 1.5 mg liver tissue were analyzed by TLC using high-performance TLC plates (catalog no. 818140, Macherey-Nagel). The plates were developed in a chloroform:methanol:0.2% CaCl₂ (60:35:8, vol/vol/vol) solution. Gangliosides were visualized by staining with 0.5% (wt/vol) orcinol dissolved in 3 M sulfuric acid. Aliquots (4 μ g each) of GM1, GM2, and GM3 standards (Avanti) were loaded on the same TLC plate.

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