Transmembrane Molecular Pump Activity of Niemann-Pick C1 Protein

Joanna P. Davies, Fannie W. Chen, Yiannis A. Ioannou*

Niemann-Pick C1 (NPC1) disease is characterized by cholesterol accumulation in lysosomes and aberrant feedback regulation of cellular cholesterol homeostasis. We provide evidence that the NPC1 protein has homology with the resistance-nodulation-division (RND) family of prokaryotic permeases and may normally function as a transmembrane efflux pump. Studies of acriflavine loading in normal and NPC1 fibroblasts indicated that NPC1 uses a proton motive force to remove accumulated acriflavine from the endosomal/lysosomal system. Expression of NPC1 in *Escherichia coli* (i) facilitated the transport of acriflavine across the plasma membrane, causing cytosolic accumulation, and (ii) resulted in transport of oleic acid but not cholesterol or cholesterol-oleate across the plasma membrane. These studies establish NPC1 as a eukaryotic member of the RND permease family.

Niemann-Pick C1 (NPC1) is a rare, panethnic, autosomal recessive disorder characterized by progressive neurodegeneration, leading to death during early childhood (*1*). Biochemical characterization of NPC1 cells revealed the accumulation of large amounts of unesterified cholesterol in the endosomal/lysosomal (E/L) compartment, a phenotype attributed to a blockade in the movement of cholesterol between the E/L system and the plasma membrane (*2*). The gene responsible for the disease, *NPC1*, encodes a protein with five transmembrane (TM) domains that share homology with the sterol-sensing domain (SSD) of two enzymes involved in cellular cholesterol homeostasis: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and sterol regulatory element binding protein cleavage-activating protein (SCAP) (*3*). In addition, NPC1 shares significant homology with the morphogen receptor Patched (*4*) and with NPC1L1, a protein identified on the basis of its high degree of homology to NPC1 (42% amino acid identity) (*5*).

To investigate the function of NPC1, we analyzed the protein sequence for additional motifs and found that it contains six copies of a prokaryotic lipid attachment site that is also found in the resistance-nodulation-division (RND) family of prokaryotic permeases. Clustal W (*6*) sequence alignments between human NPC1 and two RND permeases, *Escherichia coli* AcrB (*7*) and *Pseudomonas aeruginosa* MexD (*8*), revealed weak similarity throughout the entire sequence, with several residues completely conserved between the prokaryotic proteins and NPC1 (Fig. 1A). A potential relationship between NPC1 and members of the RND family has previously been hypothesized (*9*). To explore this relationship, we performed a Psi Blast (*10*) search. The convergence achieved after four iterations revealed that NPC1 is significantly more similar to members of the RND family ($P = 5 \times 10^{-80}$) than to mammalian SCAP ($P = 5 \times 10^{-71}$ or HMGR ($P = 6 \times 10^{-35}$), a conclusion that can be extended to Patched and NPC1L1.

The recent topological definition of both the MexD and NPC1 proteins (*11, 12*) enabled us to compare their topology and secondary structures. NPC1 consists of 13 TM domains, three large hydrophilic loops, and a short cytoplasmic tail, whereas MexD is arranged as 12 TM domains with large hydrophilic loops between TM domains 1 and 2 and between TM domains 7 and 8 (*12*). The first six TM domains and the first hydrophilic loop are repeated in the carboxyl half of MexD with relatively conserved secondary structure, suggesting an evolutionary duplication (Fig. 1B). NPC1 shares this duplicated structure precisely but contains an additional TM domain and hydrophilic loop at the amino terminus (Fig. 1B). Secondary structure prediction revealed that the second and third loops of NPC1 are similar to one another as well as to the two loops of MexD. In contrast, loop 1 of NPC1 resembles neither the latter two NPC1 loops nor the MexD loops, which is consistent with the hypothesis that this domain was acquired after the divergence of NPC1 from the ancestral RND gene. The sequence

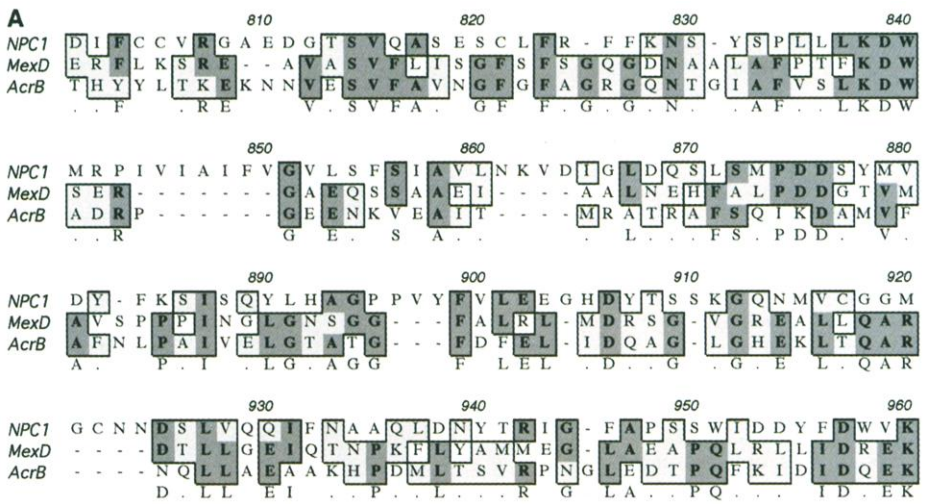

Fig. 1. Structural homology between NPC1 and members of the RND family (*25*). (**A**) Clustal W sequence alignment of human NPC1, *P. aeruginosa* MexD, and *E. coli* AcrB. (**B**) Schematic representation of the topologies of MexD and NPC1. Both proteins show a repeated domain (yellow and green) composed of six TM domains and a hydrophilic loop that reveals the internal symmetry within these proteins. Human NPC1 contains an additional TM domain with a large hydrophilic loop (red).

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and structural similarities between NPC1 and MexD suggest that NPC1 is an RND permease.

The prokaryotic RND permeases function in the efflux of lipophilic drugs, detergents, bile salts, fatty acids, metal ions, and dyes from the cytosol of gram-negative bacteria. The gene products of the *AcrAB* operon in *E. coli* (*7*) and *Salmonella typhimurum* (*13*) are among the best characterized RND proteins and are responsible for the efflux of acriflavine and other lipophilic molecules. Deletion of either gene from the *AcrAB* operon results in bacterial hypersusceptibility to bile salts and fatty acids such as decanoate (*14*). Similarly, the *MexCD* gene products have also been shown to function as efflux pumps and are responsible for multiple antibiotic resistance in *P. aeruginosa* (*8*).

To investigate whether NPC1 can function as an RND permease, we performed experiments with acriflavine, a cationic dye that can be detected by its intrinsic fluorescence. Normal fibroblasts incubated with acriflavine for 30 min exhibited fluorescence within perinuclear vesicles, presumably endosomes and lysosomes. When the fibroblasts were incubated with both acriflavine and the lysosomotropic dye lysotracker, which labels the E/L system, the two dyes completely colocalized, confirming that acriflavine accumulates in the E/L system (Fig. 2A). When cells were loaded with acriflavine for 30 min and then grown in its absence for 6 to 16 hours, the vesicular fluorescence was lost (Fig. 2B). In contrast, similarly treated NPC1$^{-/-}$ fibroblasts continued to display this vesicular pattern after 16 hours (Fig. 2B), suggesting that the efflux of acriflavine from the E/L system is blocked when NPC1 is absent.

Niemann-Pick type B fibroblasts are deficient in acid sphingomyelinase and store large amounts of sphingomyelin in their E/L system (*15*). These cells also extrude acriflavine after a 16-hour chase (Fig. 2B), indicating that acriflavine accumulation is specific to NPC1 cells and not caused by simple lysosomal engorgement with storage material. However, the membranes of the E/L system in NPC1$^{-/-}$ cells may be compromised in some way. To eliminate this possibility, we exposed cells to two dyes that specifically accumulate in the E/L system, lysotracker and acridine orange. Both normal and NPC1$^{-/-}$ cells loaded with lysotracker exhibited a bright vesicular fluorescence, and after a 6-hour chase, both cell types effectively extruded lysotracker from the E/L system (Fig. 2, C and D). In contrast, acridine orange was retained by the E/L system of both cell types even after a 16-hour chase (Fig. 2, C and D). These results demonstrate that the membrane integrity of NPC1$^{-/-}$ cells was intact and suggest that NPC1 is responsible for the specific efflux of acriflavine from the E/L system.

Because members of the RND family transport their substrates by means of a proton motive force (PMF) (*16*), we treated fibroblasts preloaded with acriflavine for 30 min with drugs that disrupt proton gradients. Incubation with PMF disrupters such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine inhibited the efflux of acriflavine out of lysosomes (Fig. 3, B and C), suggesting that its efflux requires an intact PMF. These drugs were also potent inducers of an NPC1-like phenotype, causing accumulation of unesterified cholesterol in the E/L system (Fig. 3, B and C) and thus suggesting a link between the efflux of acriflavine and cholesterol. Furthermore, drugs known to induce NPC1 phenocopies, such as U18666A and imipramine (*17*), also caused acriflavine accumulation in normal fibroblasts (Fig. 3, D and E). These results suggest that acriflavine is actively transported out of the E/L system through a PMF and that this efflux is defective in NPC1$^{-/-}$ cells.

To characterize the functional relationship of NPC1 with the prokaryotic RND permeases, we expressed human NPC1 in *E. coli*. The predicted signal peptide from NPC1 was replaced by the *E. coli* outer membrane protein A signal peptide, and this modified construct was inserted into a prokaryotic secretion vector (*18*). Another construct was also engineered with the same vector and an NPC1 cDNA containing the mutation Ile^{1061} → Thr^{1061} (I1061T) (*19*). Because this mutation appears in the juvenile rather than the infantile form of NPC1 disease, we hypothesized that it might retain partial activity.

Because the outer membrane of *E. coli* is a barrier for most hydrophobic molecules, we isolated a mutant with a permeable (leaky)

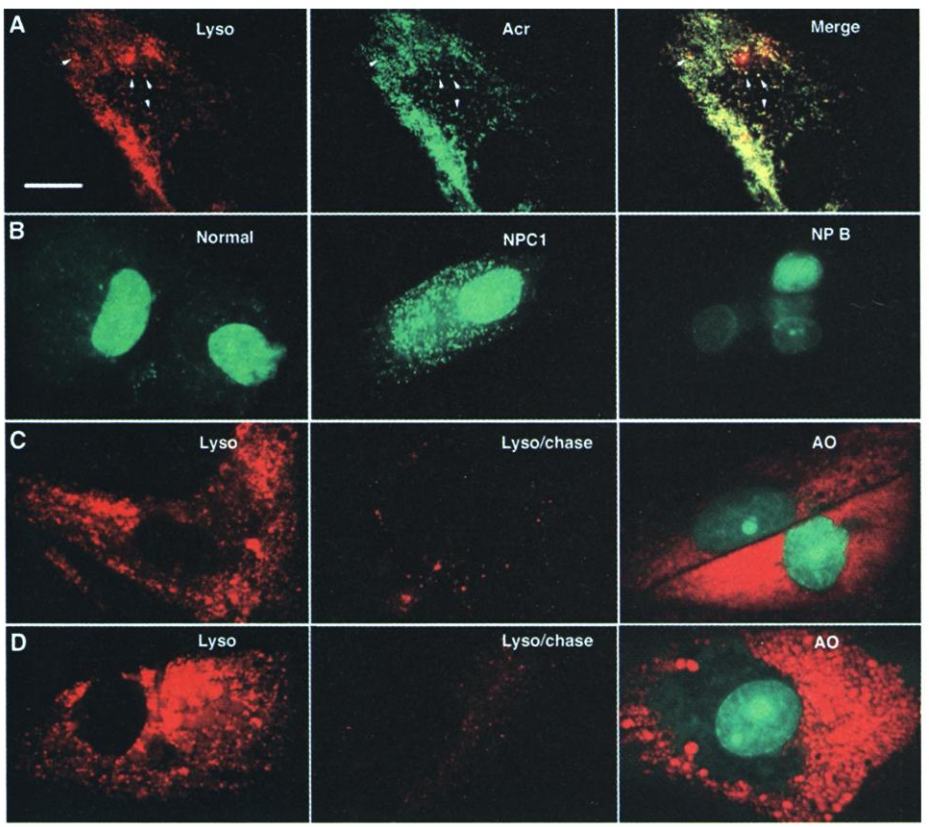

Fig. 2. The effect of acriflavine on normal and NPC1$^{-/-}$ fibroblasts. Cultures grown on cover slips in six-well dishes were labeled for 30 min with acriflavine (10 μg/ml). The dye was removed and cells were allowed to grow in normal media for 6 to 16 hours. Cells were mounted on glass slides and were viewed with a Nikon Eclipse fluorescence microscope equipped with a charge-coupled device camera and the fluorescein isothiocyanate filter set. (**A**) Fibroblasts incubated with lysotracker (Lyso) to label the E/L system and acriflavine (Acr) exhibit complete colocalization, indicating that both accumulate in the E/L system (arrowheads). Scale bar, 10 μm (bar applies to all panels). (**B**) Normal fibroblasts extrude acriflavine from the E/L system after 6 hours of growth in its absence, as shown by the lack of vesicular fluorescence. In contrast, NPC1 fibroblasts do not extrude acriflavine, as indicated by the bright vesicular staining seen even after 16 hours of growth in the absence of the dye. As an additional control, we treated fibroblasts from a Niemann-Pick type B patient (NPB) as above. No acriflavine accumulated in the E/L system after a 16-hour chase. (**C**) Normal fibroblasts labeled with lysotracker (Lyso) extruded lysotracker after a chase (Lyso/chase), whereas acridine orange (AO) remained in the E/L system even after a 16-hour chase. (**D**) NPC1 fibroblasts treated as in (C) with lysotracker or acridine orange behaved identically to normal fibroblasts under these conditions.

membrane. Bacteria were exposed to ultraviolet irradiation and a leaky mutant, 2.1.1, was identified by screening for secretion of alkaline phosphatase into the culture medium (*20, 21*). Introduction of the NPC1 construct into *E. coli* 2.1.1 cells resulted in expression of the intact NPC1 protein in the *E. coli* membrane (*21, 22*) (Fig. 4, A and B).

To determine whether NPC1 can act as a permease, we monitored its ability to transport acriflavine from the *E. coli* periplasmic space into the cytosol. Bacteria containing the wild-type (WT) NPC1 or the I1061T mutant constructs were grown, and expression of NPC1 was induced for 1 hour with 1 mM isopropyl β-D-thiogalactoside (IPTG). Acriflavine was added and cultures were incubated at 37°C for 5 to 15 min (*21, 23*). The amount of acriflavine that accumulated in these cells was determined as arbitrary fluorescence units normalized for the number of *E. coli* cells in each reaction (Fig. 4C). Upon induction with IPTG, both the WT NPC1 and the I1061T cells accumulated significantly more acriflavine over a 15-min period than did the parental 2.1.1 cells (Fig. 4C). Both WT and mutant NPC1 accumulated acriflavine in the absence of induction, suggesting that expression of NPC1 is leaky in these bacteria (*24*). As anticipated, the I1061T mutant retained partial NPC1 activity that was about 85% of the WT protein activity. In addition, these results indicate that the polarity of NPC1 is reversed in relation to that of *E. coli* AcrB, even though these proteins share the same membrane topology.

These data establish NPC1 as a mammalian member of the RND family of permeases. This is paradoxical because NPC1$^{-/-}$ mammalian cells accumulate unesterified cholesterol in their E/L system, whereas the prokaryotic RND permeases have not been shown to transport cholesterol. To determine whether NPC1 can transport cholesterol in *E. coli*, we incubated bacteria expressing NPC1 with cholesterol, cholesterol-oleate, and oleic acid (*23*). There was no significant accumulation of cholesterol or cholesterol ester in the induced NPC1 and I1061T cells (*21*); oleic acid, in contrast, showed a significant accumulation (Fig. 4D). As with acriflavine, uninduced cells accumulated more oleic acid than did control cells; however, upon induction, NPC1 cells exhibited more than a 15-fold increase in oleic acid accumulation (Fig. 4D). The I1061T NPC1 protein, which exhibited about 85% of the WT activity for acriflavine transport (Fig. 4C), had about 60% of the WT activity for oleic acid transport (Fig. 4D). These results support the notion that this mutant retains partial functional activity. Together, these results indicate that NPC1, like its prokaryotic homologs (*16*), can transport fatty acids (Fig. 4D) across a membrane. However, whether fatty acids are the normal substrates of the NPC1 permease in the mammalian E/L system, as well as how a defect in fatty acid transport could result in the cholesterol accumulation seen in NPC1 disease, remain unclear.

These data establish that NPC1 can function to transport lipophilic molecules, but not cholesterol, out of the E/L system. The fact that NPC1 can transport acriflavine and fatty acids suggests that this permease may have a "multidrug" transport function, part of which has become important for its housekeeping role in cellular cholesterol homeostasis. The topological arrangement of NPC1 is shared with a newly identified homolog, NPC1L1, and with the morphogen receptor Patched. Indeed, Patched is more related in amino acid sequence to AcrB and MexD than to NPC1, raising the possibility that Patched also functions as an RND permease. Thus, these results provide a new avenue for investigating the function of a number of proteins important in cholesterol and/or fatty acid homeostasis, morphogenesis, and tumor suppression (*3, 4*).

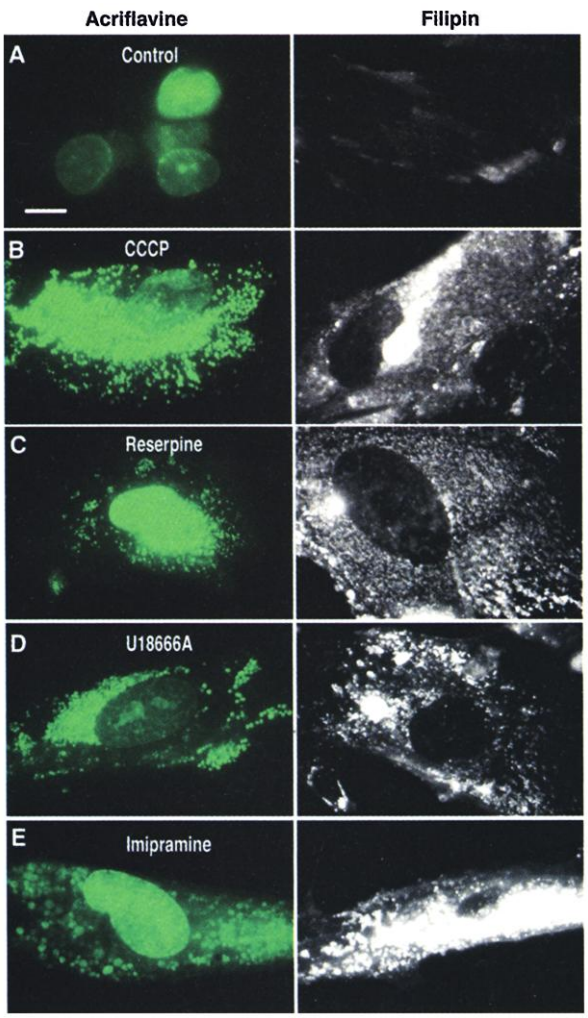

Fig. 3. Effect of PMF disrupters on acriflavine and cholesterol efflux in normal fibroblasts. Normal cells were treated as indicated and then stained with acriflavine or filipin to label unesterified cholesterol in the E/L system. (**A**) Untreated control cells extruded both acriflavine and cholesterol from their E/L system. Cells were treated with CCCP (**B**) or reserpine (**C**) to disrupt PMF gradients and then labeled with acriflavine, as in Fig. 2. Both drugs inhibited acriflavine efflux (Acriflavine panels) and cholesterol efflux (Filipin panels). Drugs known to induce NPC1 phenocopies by inhibiting cholesterol egress from the E/L system, such as U18666A (**D**) and imipramine (**E**), also inhibited acriflavine efflux in these cells. Scale bar, 10 μm.

References and Notes

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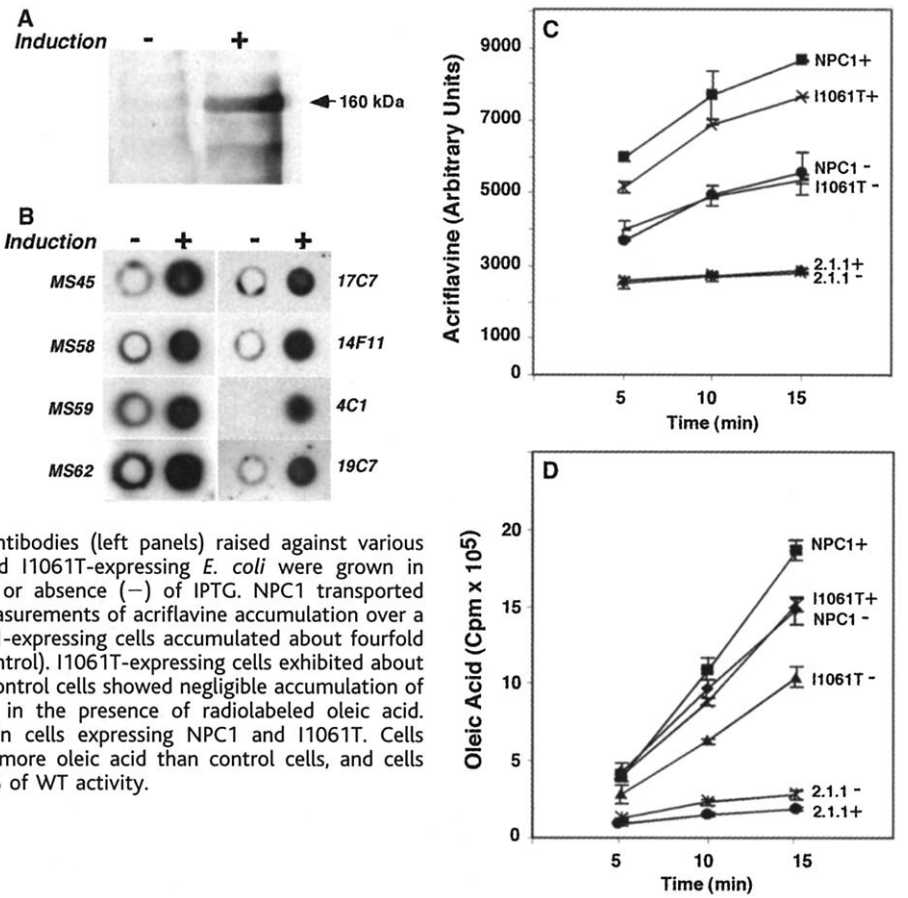

Fig. 4. Human NPC1 can transport acriflavine and fatty acids in *E. coli*. (**A**) The signal peptide from human NPC1 was replaced with a secretion signal from the *E. coli* OmpA protein. This construct was expressed in *E. coli*, 2.1.1 cells under a lactose-regulated lipoprotein promoter. Fresh cultures were induced with 1 mM IPTG for 3 hours, harvested, and lysed by sonication. After a 10-min centrifugation at 10,000*g* to remove cell debris, membranes were isolated by centrifugation at 100,000*g* for 1 hour with a fixed-angle rotor. Membrane proteins from induced and uninduced cultures were resolved by SDS–polyacrylamide gel electrophoresis through a 6% gel. Western blot analysis was carried out with a rabbit polyclonal antibody against the amino terminus of human NPC1 (amino acids 30 through 266). Human NPC1 of the expected molecular size (~160 kD) was seen in the induced *E. coli* membranes. (**B**) The integrity of the NPC1 protein was confirmed by dot-blot analysis using a group of monoclonal antibodies to NPC1 (right panels) and a group of polyclonal antibodies (left panels) raised against various fragments of the human protein. (**C**) NPC1- and I1061T-expressing *E. coli* were grown in acriflavine-containing media in the presence (+) or absence (−) of IPTG. NPC1 transported acriflavine into the *E. coli* cytosol, as shown by measurements of acriflavine accumulation over a 15-min period. In the presence of acriflavine, NPC1-expressing cells accumulated about fourfold more acriflavine than the parental 2.1.1 culture (control). I1061T-expressing cells exhibited about 85% of the activity of the WT NPC1 protein, and control cells showed negligible accumulation of acriflavine. (**D**) *Escherichia coli* cells were grown in the presence of radiolabeled oleic acid. Significant accumulation of oleic acid occurred in cells expressing NPC1 and I1061T. Cells expressing WT NPC1 accumulated about 15-fold more oleic acid than control cells, and cells expressing the I1061T mutant exhibited about 60% of WT activity.

18. The human NPC1 cDNA was cloned into the pIN III *E. coli* secretion vector [K. Nakamura, M. Inouye, *EMBO J.* **1**, 771 (1982)]. The pIN-NPC1 and pIN-I1061T plasmids were then introduced into the leaky mutant 2.1.1 (*E. coli* strain BL21-RIL; Stratagene, La Jolla, CA). Cells were grown to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 and then induced with 1 mM IPTG for 1 to 3 hours.
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22. Rabbit polyclonal and mouse monoclonal antibodies were generated against various fragments of human NPC1 expressed as fusions to the *E. coli* protein thioredoxin, containing a 6-histidine tag. Proteins were expressed and purified by metal chelation chromatography and used to immunize rabbits and mice. All antibodies were characterized for their ability to detect human NPC1 both on immunoblots and in cultured cells by immunofluorescence microscopy.
23. For acriflavine loading, cells were collected by centrifugation and 0.2 OD_{600} cells were resuspended in acriflavine loading buffer [20% sucrose, 0.3 M tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM $MgCl_2$, and acriflavine (20 μg/ml)]. After incubation at 37°C, cells were collected by centrifugation and washed [in 0.3 M tris-HCl (pH 7.5) and 0.15 M NaCl]. Pellets were resuspended in 0.1 ml H_2O and extracted with 1 ml of methanol at 42°C for 15 min. Acriflavine fluorescence was quantitated at excitation 474 and emission 511 nm. For transport studies with oleic acid, cells were resuspended in 0.1 M tris-HCl (pH 7.5), 0.15 M NaCl, 0.1 mM oleic acid, and 1 pM [^{3}H]oleic acid (5 μCi); incubated at 37°C; and washed; the pellet was dissolved in scintillation fluid.
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25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

26. We thank B. Gelb and L. Shapiro for critical reading of the manuscript. Supported by grants from the National Institute of Diabetes, Digestive, and Kidney Disease (grant R01 DK54736), the Ara Parseghian Medical Research Foundation, and the March of Dimes Foundation.

18 September 2000; accepted 10 November 2000

Identification of *HE1* as the Second Gene of Niemann-Pick C Disease

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Niemann-Pick type C2 disease (NP-C2) is a fatal hereditary disorder of unknown etiology characterized by defective egress of cholesterol from lysosomes. Here we show that the disease is caused by a deficiency in HE1, a ubiquitously expressed lysosomal protein identified previously as a cholesterol-binding protein. HE1 was undetectable in fibroblasts from NP-C2 patients but present in fibroblasts from unaffected controls and NP-C1 patients. Mutations in the *HE1* gene, which maps to chromosome 14q24.3, were found in NP-C2 patients but not in controls. Treatment of NP-C2 fibroblasts with exogenous recombinant HE1 protein ameliorated lysosomal accumulation of low density lipoprotein–derived cholesterol.

Niemann-Pick disease type C (NP-C) is an autosomal recessive lipid storage disorder characterized by progressive deterioration of the central nervous system, visceral symptoms, and premature death (*1*). The frequency of the disease is estimated at