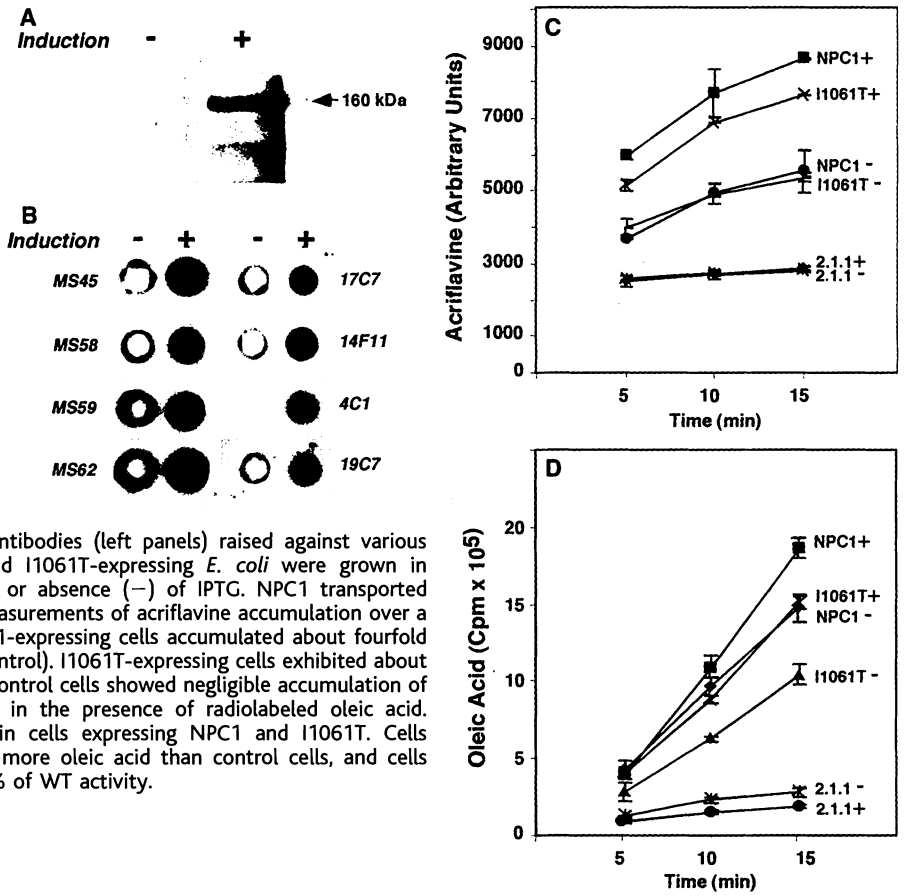

Fig. 4. Human NPC1 can transport acriflavine and fatty acids in *E. coli*. (**A**) The signal peptide from human NPC1 was replaced with a secretion signal from the *E. coli* OmpA protein. This construct was expressed in *E. coli*, 2.1.1 cells under a lactose-regulated lipoprotein promoter. Fresh cultures 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.

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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

about 1 in 10^5 live births. At the cellular level, the most prominent feature of the NP-C lesion is lysosomal sequestration of low density lipoprotein (LDL)–derived cholesterol, resulting in downstream effects on cholesterol homeostasis (*2*, *3*). Somatic cell hybridization experiments with skin fibroblast cultures from unrelated NP-C patients demonstrated the existence of a major complementation group comprising ~95% of cases, designated NP-C1, and a minor complementation group, designated NP-C2 (*4*–*6*). The NP-C1 gene has been identified (*7*) and mapped to chromosome 18q11, and the defect in NP-C2 has been excluded from this region by linkage analysis (*6*).

We have identified the molecular defect in NP-C2 as part of an ongoing study directed at characterizing the lysosome proteome (*8*, *9*). Our approach is based on the fact that many soluble lysosomal proteins acquire a posttranslational modification that distinguishes them from most other types of proteins, the mannose 6-phosphate (Man6-P) marker. This modification is recognized by Man6-P receptors (MPRs), which divert newly synthesized lysosomal enzymes from the secretory pathway to the endolysosomal system (*10*). Purified MPR derivatives typically bind phosphorylated lysosomal proteins with subnanomolar affinity and can be used to detect and purify Man6-P glycoproteins (*9*, *11*).

A two-dimensional gel map of MPR affinity-purified proteins from human brain contained a group of proteins sharing the same NH_2-terminal sequence, as determined by Edman degradation (longest sequence, EPVQFKDXGSVDGVIK) (*12*), which are likely to represent differentially glycosylated isoforms of the same protein (Fig. 1A). This sequence perfectly matches the processed NH_2-terminus of HE1 (*13*, *14*), a 151–amino acid glycoprotein containing a 19–amino acid signal that, along with homologs from numerous mammalian species, represents a major secretory component of epididymal fluid (*15*–*18*). Western blotting with polyclonal antibodies against recombinant HE1 confirmed the identity of the proteins (Fig. 1B), and probing with radiolabeled MPR verified that HE1 contained the Man6-P modification (Fig. 1C).

Analysis of *HE1* mRNA by Northern blotting revealed a single transcript of 0.9 kb in all tissues examined, with highest levels in testis, kidney, and liver and lowest levels in lung and muscle (Fig. 1D). This wide distribution is consistent with the

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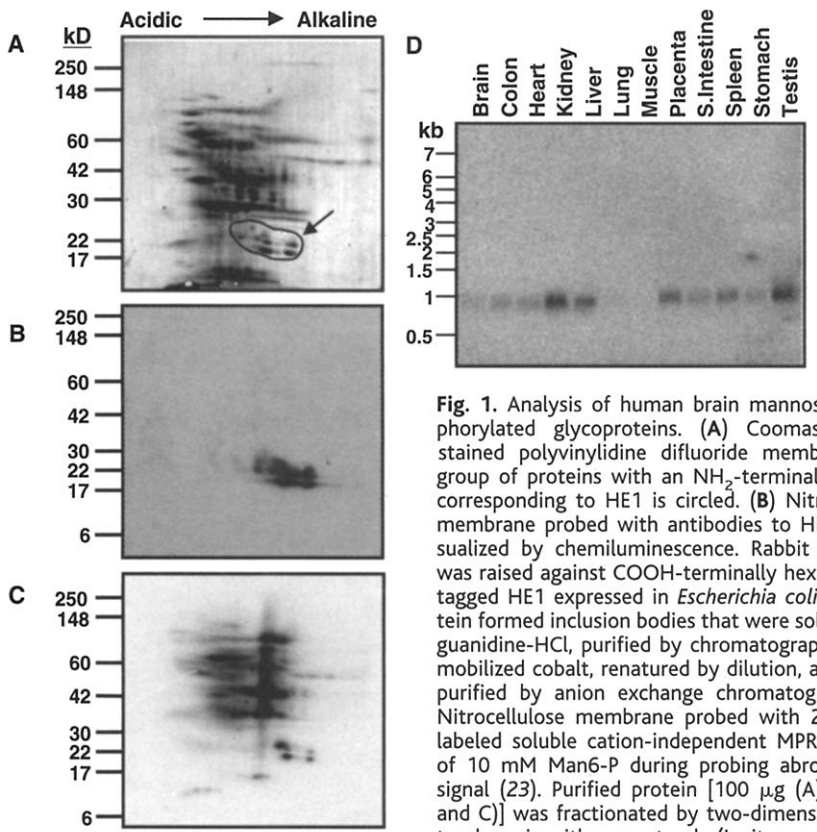

Fig. 1. Analysis of human brain mannose 6-phosphorylated glycoproteins. (**A**) Coomassie blue–stained polyvinylidine difluoride membrane. The group of proteins with an NH_2-terminal sequence corresponding to HE1 is circled. (**B**) Nitrocellulose membrane probed with antibodies to HE1 and visualized by chemiluminescence. Rabbit antiserum was raised against COOH-terminally hexahistidine-tagged HE1 expressed in *Escherichia coli*. The protein formed inclusion bodies that were solubilized in guanidine-HCl, purified by chromatography on immobilized cobalt, renatured by dilution, and further purified by anion exchange chromatography. (**C**) Nitrocellulose membrane probed with 2 nM ^{125}I-labeled soluble cation-independent MPR. Inclusion of 10 mM Man6-P during probing abrogated the signal (*23*). Purified protein [100 μg (A); 5 μg (B and C)] was fractionated by two-dimensional electrophoresis with precast gels (Invitrogen, Carlsbad, California) (first dimension, isoelectric focusing on pH 3 to 10 gels without prior sample reduction; second dimension, reducing SDS-PAGE on 10 to 20% acrylamide gels). (**D**) HE1 expression in various human tissues. A membrane containing 2 μg of the indicated polyadenylated human RNA in each lane (Origene Technologies, Rockville, Maryland) was probed with a ^{32}P-labeled DNA fragment corresponding to the entire coding region of *HE1*.

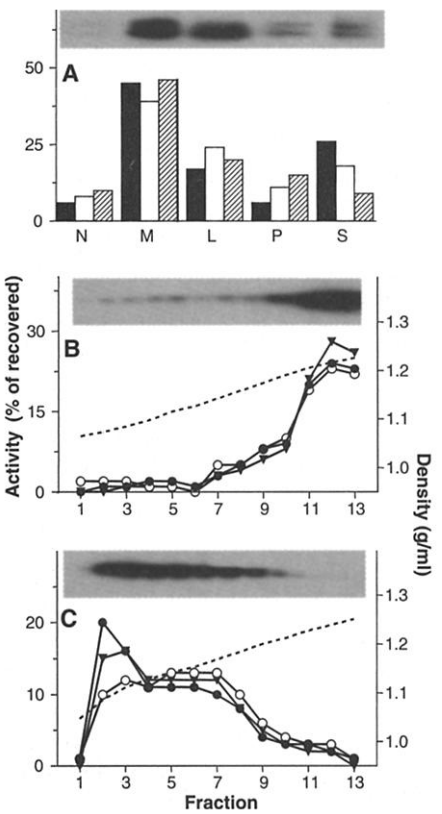

Fig. 2. Subcellular distribution of HE1 homolog in rat liver. (**A**) Differential centrifugation was performed as described in (*31*). N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, soluble fraction. Fractions were analyzed by Western blotting with rabbit antibodies to HE1 (inset) and for lysosomal enzyme activities (β-galactosidase, filled bars and circles; tripeptidyl peptidase I, open bars and circles; β-hexosaminidase, hatched bars and triangles) as described in (*9*, *32*). (**B** and **C**) Sucrose density gradients (dashed lines) on the pooled M+L fraction were prepared from control rats (B) or rats injected with the nonionic detergent Triton WR1339 (C). Isopycnic centrifugations were performed as described (*33*).

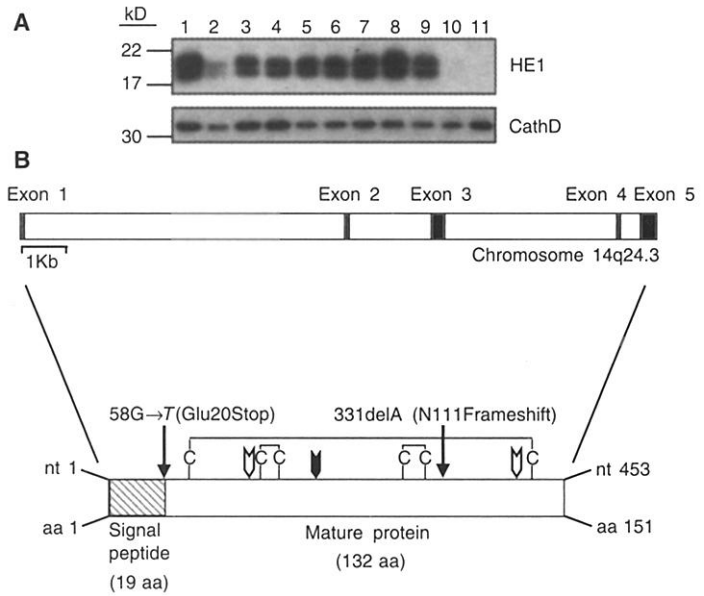

Fig. 3. (**A**) HE1 and cathepsin D protein levels in control and mutant fibroblasts. Lanes 1 to 3, unaffected controls (GM06556, GM05757B, and GM03652F); lanes 4 and 5, sea blue histiocyte disease (GM01912 and GM00843); lanes 6 to 9, NP-C1 disease (GM11095, GM03123A, GM00110B, and NPC1 90.48); and lanes 10 and 11, NP-C2 disease (NPC2 93.10 and NPC2 99.04). Cells cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum were lysed in 0.1% Triton X-100 and 150 mM NaCl. Extracts were centrifuged 20 min at 13,000*g* and soluble protein (7 μg) separated by SDS–polyacrylamide gel electrophoresis. The blot was first probed with rabbit antibodies to HE1 and chemiluminescence and, as a loading control, subsequently reprobed with rabbit antibodies to cathepsin D (Calbiochem, La Jolla, California) and chemiluminescence. Fibroblasts originated from the Coriell collection (GM numbering) and from Peter Pentchev (NPC numbering). (**B**) Schematic of the HE1 gene and protein. Mutations are indicated on the protein schematic (arrows) with nt 1 representing the first nucleotide of the initiation codon. Potential N-linked glycosylation sites are indicated by arrowheads, the filled arrowhead indicating the site that is conserved among mammalian HE1 orthologs. The predicted disulfide pairing of the cysteines is assigned by homology to equivalent cysteines in an apparently related dust mite protein (*34*). The genomic structure of *HE1* was determined by sequence alignments between the HE1 cDNA (Q15668) and genomic DNA sequence (AC005479). The chromosomal localization of *HE1* (14q24.3) was determined by identifying mapped clones in the Sequence Tagged Sites (STS) database that aligned to AC005479 (e.g., STS clones G38283, G38146, and G38077) and was confirmed by radiation hybrid panel mapping to chromosome 14 with the Coriell monochromosomal somatic hybrid panel. aa, amino acid.

presence of *HE1* sequences in numerous cDNA and SAGE libraries [see UniGene cluster Hs.119529 (*19*)]. In addition, a bovine HE1 homolog is present in milk (*20*), and bovine and murine *HE1* mRNAs have been detected in several tissues (*20*, *21*). These observations suggest that, in addition to its postulated specialized role in sperm maturation (*16*, *22*), HE1 may have a more global function.

We demonstrated that HE1 is a lysosomal protein by subcellular fractionation of rat liver. After differential centrifugation of the homogenate, HE1 and the lysosomal markers were found mainly in the heavy mitochondrial fraction (M) and the light mitochondrial fraction (L) (Fig. 2A). When the pooled M and L fractions were analyzed by isopycnic centrifugation in density gradients made of sucrose (Fig. 2B) or Nycodenz (*23*), we observed a clear codistribution of HE1 and lysosomal enzymes, suggesting that they reside in the same compartment. We also monitored HE1 distribution in rats injected with Triton WR1339, a nonionic detergent that accumulates in lysosomes and induces a striking and specific shift of these organelles in sucrose density gradients (*24*). The redistribution of HE1 after such treatment (compare Fig. 2, B and C) indicates that most, if not all, of this protein is located in lysosomes. The abundance of HE1 in epididymal fluid is not inconsistent with it being a lysosomal protein; epididymal fluid has an acidic milieu (*25*) and is an abundant source of several other lysosomal proteins (*15*, *26*, *27*).

It has recently been reported that the porcine homolog of HE1 specifically binds cholesterol (*16*). Given its lysosomal location and the lysosomal cholesterol accumulation in NP-C2 disease cells, we hypothesized that HE1 might be involved in NP-C2. Western blotting revealed that HE1 was undetectable in fibroblasts from two independent NP-C2 patients (Fig. 3A, lanes 10 and 11). In contrast, the protein was detectable in fibroblasts from unaffected controls, patients with sea blue histiocyte disease, and patients with NP-C1 disease (Fig. 3A, lanes 1 to 9).

The availability of the complete sequence of the human *HE1* gene (GenBank accession number AC005479) allowed us to design polymerase chain reaction primers and readily amplify the entire coding region. Sequence analysis revealed the presence of mutations in two unrelated NP-C2 patients. One patient (NPC2 99.04) was homozygous for transversion of G to T in exon 1 that results in conversion of Glu^{20} to a termination codon (Fig. 3B). As Glu^{20} corresponds to the NH_2-terminus of the mature protein, this represents a null mutation. The other patient (NPC2 93.10) was compound heterozygous for the Glu20Stop mutation and a single nucleotide deletion in exon 2 that shifts the reading frame and generates a stop codon four codons downstream. This severe truncation also affects the predicted disulfide pairing of the protein and is likely to represent a null allele (Fig. 3B). No mutations were detected in sequence analysis of DNA from eight individuals who represented either unaffected controls, patients with NP-C1, or patients with other diseases. Thus, mutations in *HE1* are specifically associated with NP-C2.

Lysosomal cholesterol storage in NP-C2 fibroblasts is demonstrated by the bright punctate fluorescence after probing with filipin, a cholesterol-binding antibiotic (Fig. 4A) (*4*, *6*). Given our finding that NP-C2 is due to a deficiency in a soluble lysosomal protein, we reasoned that it should be possible to reverse this phenotype by supplying the HE1 protein in trans. A Chinese hamster ovary (CHO) cell line stably transfected with a human *HE1* expression construct secreted large amounts of the protein into the media compared with control CHO cells (Fig. 4D). Cultivation of NP-C2 cells in normal medium supplemented with small amounts (0.3% vol/vol) of this HE1-conditioned medium diminished cholesterol accumulation compared with controls (naïve medium or equivalent amounts of conditioned medium from untransfected CHO cells) (Fig. 4, A to C and E). Cultivation of NP-C2 cells with large amounts (>10%) of conditioned media from untransfected CHO cells partially reversed cholesterol accumulation, presumably reflecting the presence of low amounts of endogenous HE1 homolog secreted by the CHO cells (*23*). Addition of Man6-P to the HE1-conditioned media prevented the reduction in cholesterol accumulation, indicating that uptake occurred through MPR-mediated endocytosis (*23*). In contrast to the results obtained with NP-C2 cells, comparable experiments showed that the HE1-conditioned medium had no effect

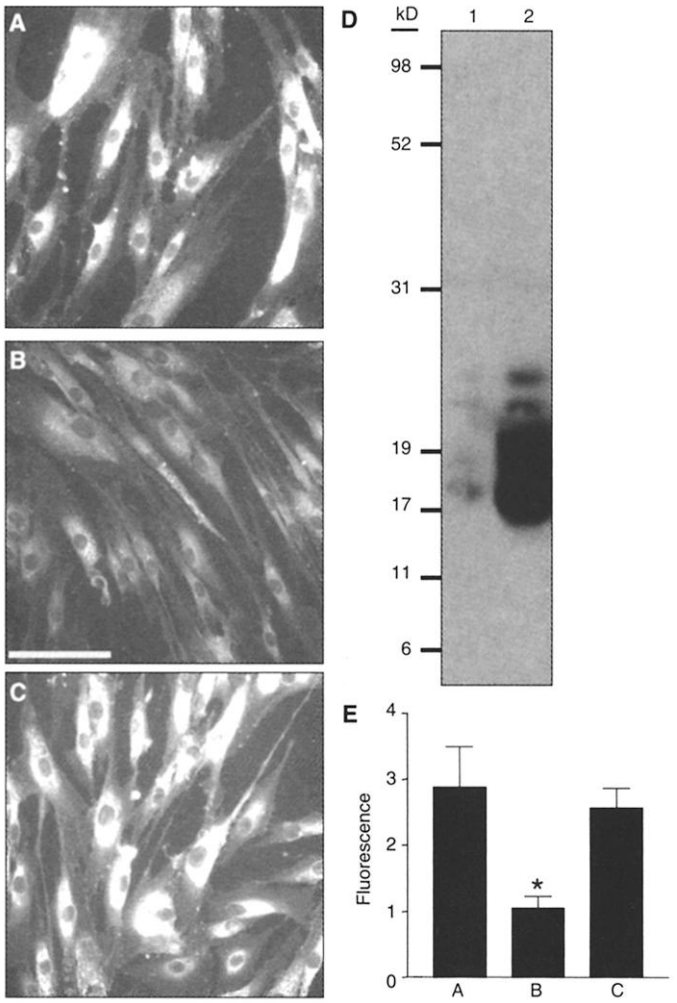

Fig. 4. Correction of cholesterol accumulation in NPC2 fibroblasts. NP-C2 fibroblasts were cultured for 4 days in complete medium (RPMI 1640 plus 15% fetal bovine serum) containing the following supplements: (**A**) no supplement, (**B**) 0.3% conditioned medium from a CHO cell line producing recombinant human HE1, and (**C**) 0.3% conditioned medium from untransfected CHO cells. The scale bar in (B) represents 100 μm. Fluorescent micrographs in (A) to (C) are of subconfluent cells. (**D**) The Western blot shows the relative levels of HE1 in medium from control (lane 1) or HE1-transfected (lane 2) CHO cells. Cells were fixed with Bouin's fixative and stained with filipin. (**E**) For quantitation, cells were grown to confluence, and fields containing ~200 cells were selected under bright-field illumination to eliminate operator bias. Fluorescence measurements were then collected with a charge-coupled device camera (*35*). For each condition, the average pixel intensity of five fields was used for the analysis. Data were corrected for background staining obtained with unaffected control fibroblasts (477 ± 15, mean ± standard error). The asterisk indicates that the difference in staining intensity of the HE1-treated fibroblasts compared with the two control groups is statistically significant ($P < 0.05$).

on reducing cholesterol accumulation in NP-C1 fibroblasts (*23*), thus further demonstrating the specificity of the defect in NP-C2.

The finding that NP-C2 is due to a deficiency in a soluble lysosomal protein is consistent with earlier observations that cocultivation of mononuclear NP-C1 and NP-C2 fibroblasts partially reversed cholesterol accumulation in a subset of the cells (*5*). It may also explain why fibroblasts from patients with I-cell disease, which lack the enzyme that normally generates the Man6-P lysosomal targeting signal and have low intracellular levels of multiple lysosomal proteins, accumulate LDL-derived cholesterol (*28*).

Although the pathway for delivery of LDL and generation of free cholesterol in the lumen of the lysosome is well established, it is not clear how this cholesterol travels to the plasma membrane, endoplasmic reticulum, and other cellular sites. *NPC1* encodes a protein with multiple transmembrane domains and a putative sterol sensing domain that resides in an endolysosomal compartment (*29*, *30*). However, despite extensive investigation, the precise function of NPC1 remains elusive. NP-C patients from both complementation groups demonstrate similar clinical and biochemical phenotypes, suggesting that NPC1 and HE1 may interact or function sequentially in a common metabolic pathway. One intriguing possibility is that HE1 functions as a carrier that prevents unregulated intercalation of cholesterol into lipid bilayers, perhaps selectively transferring sterols to a transmembrane protein such as NPC1. The identification of the molecular basis for NP-C2 should facilitate genetic, biochemical, and physical studies to elucidate basic mechanisms for cholesterol transport.

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