Niemann-Pick Type C1 I1061T Mutant Encodes a Functional Protein That Is Selected for Endoplasmic Reticulum-associated Degradation Due to Protein Misfolding

Mark E. Gelthorpe, Nikola Baumann, Elizabeth Millard, Sarah E. Gale, S. Joshua Langmade, Jean E. Schaffer, and Daniel S. Ory

From the Center for Cardiovascular Research, Departments of Internal Medicine, Molecular Biology and Pharmacology, and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Over 200 disease-causing mutations have been identified in the NPC1 gene. The most prevalent mutation, NPC1I1061T, is predicted to lie within the cysteine-rich luminal domain and is associated with the classic juvenile-onset phenotype of Niemann-Pick type C disease. To gain insight into the molecular mechanism by which the NPC1I1061T mutation causes disease, we examined expression of the mutant protein in human fibroblasts homozygous for the NPC1I1061T mutation. Despite similar NPC1 mRNA levels between wild type and NPC1I1061T fibroblasts, NPC1 protein levels are decreased by 85% in NPC1I1061T cells. Metabolic labeling studies demonstrate that unlike wild type protein, which undergoes a glycosylation pattern shift from Endo H-sensitive to Endo H-resistant species, NPC1I1061T protein remains almost exclusively Endo H-sensitive and exhibits a reduced half-life ($t_{1/2}$ 42 h) versus wild type Endo H-resistant species ($t_{1/2}$ 6.5 h). Treatment with chemical chaperones, growth at permissive temperature, or inhibition of proteasomal degradation increases NPC1I1061T protein levels, indicating that the mutant protein is likely targeted for endoplasmic reticulum-associated degradation (ERAD) due to protein misfolding. Overexpression of NPC1I1061T in NPC1-deficient cells results in late endosomal localization of the mutant protein and complementation of the NPC mutant phenotype, likely due to a small proportion of the nascent NPC1I1061T protein that is able to fold correctly and escape the endoplasmic reticulum quality control checkpoints. Our findings provide the first description of an endoplasmic reticulum trafficking defect as a mechanism for human NPC disease, shedding light on the mechanism by which the NPC1I1061T mutation causes disease and suggesting novel approaches to treat NPC disease caused by the NPC1I1061T mutation.

Niemann-Pick type C (NPC) disease is a fatal neurodegenerative disease characterized by neuronal lipid storage and progressive Purkinje cell loss in the cerebellum. Mutations in the NPC1 gene are responsible for ~95% of human NPC disease (1). The human NPC1 gene encodes a 1278-amino acid polytopic protein containing 13 transmembrane domains, including a pentahelical domain that is evolutionarily and functionally related to sterol-sensing domains found in five other polytopic proteins involved in sterol interactions or sterol metabolism (2,3). The NPC1 sterol-sensing domain shares ~30% identity with the sterol-sensing domains of 3-hydroxymethylglutaryl-CoA reductase, sterol regulatory element-binding protein cleavage-activating protein (SCAP), NPC1-L1 and Patched (4). A carboxyl-terminal dileucine motif targets NPC1 to the endocytic pathway, where it localizes to a late endosomal compartment that is LAMP-2-positive, Rab7-positive, and cation-independent mannose-6-P receptor-negative (5–7).

The NPC1 protein is a key participant in intracellular sterol trafficking. Cells harboring inactivating mutations in NPC1 exhibit marked impairment of low-density lipoprotein (LDL) cholesterol esterification and mobilization of newly hydrolyzed LDL cholesterol to the plasma membrane (8–10). As a result of these trafficking defects, NPC1 mutant cells demonstrate lysosomal sequestration of LDL cholesterol, delayed down-regulation of the LDL receptor and de novo cholesterol biosynthesis, and impaired ABCA1-mediated cholesterol efflux (11–14).

Over 200 disease-causing mutations have been identified in the NPC1 gene (15–17). The most prevalent mutation, NPC1I1061T, is predicted to lie within a cysteine-rich luminal domain of the NPC1 protein and represents 15–20% of all disease alleles (4,15,18). Human fibroblasts homozgyous for the NPC1I1061T mutation exhibit markedly impaired LDL-stimulated cholesterol esterification and accumulation of unesterified cholesterol in aberrant lysosomes (15). Previous studies have shown that NPC1I1061T is expressed at lower levels and exhibits altered banding patterns on Western blotting as compared with wild type (WT) protein (19). However, the molecular mechanism through which the NPC1I1061T missense mutation results in NPC disease is poorly understood.

In the present study we examine the effect of the NPC1I1061T substitution on processing and stability of the NPC1 protein. We provide evidence that the NPC1I1061T protein is synthet-
Proteasomal Degradation of NPC1<sup>I1061T</sup>

sized but fails to advance in the secretory pathway due to recognition as a misfolded protein by the endoplasmic reticulum (ER) quality control machinery and consequent targeting for proteasomal degradation. Overexpression of NPC1<sup>I1061T</sup> led to late endosomal localization of the mutant protein and functional complementation of the NPC mutant phenotype, likely as a result of a small proportion of NPC1<sup>I1061T</sup> mutant protein that folded correctly and was thus able to escape ER quality control. Our findings provide support for use of chemical chaperones as approaches to treat NPC disease caused by the NPC1<sup>I1061T</sup> mutation.

**MATERIALS AND METHODS**

**Cell Culture and Chemicals**—Normal human skin fibroblasts NPC1<sup>I1061T</sup> mutant human fibroblast cell lines (NIH 83.16, NIH 89.79, NIH 90.39, and NIH 95.47) were generously provided by Daniel Kraft and David Marks (Mayo) (15). M12 cells are mutant CHO-K1 cells that contain a deletion of the npc1 locus (8). To generate npc1-null cells expressing WT NPC1, NPC1<sup>I1061T</sup>, and NPC1<sup>P692S</sup>, M12 cells were infected with retrovirus prepared by transient transfection of 293GPG packaging cells with the ΔU3mnp1<sup>-WT-GFP</sup>, ΔU3mnp1<sup>-I1061T-GFP</sup>, and ΔU3mnp1<sup>-P692S-GFP</sup> constructs, respectively, as previously described (3). Mouse embryonic fibroblasts were isolated from WT C57BL/6 mice. Cells were maintained in monolayer culture at 37 °C with 5% CO<sub>2</sub>. All fibroblasts cell lines were passaged in media containing Dulbecco's modified Eagle's medium with 10% (v/v) inactivated fetal bovine serum, 2 mM glutamine, Ham's F-12 medium, Lipofectamine Plus, and penicillin. CHO-derived cell lines were maintained in 1:1 Dulbecco's modified Eagle's medium:Ham's F-12 with 5% (v/v) fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. CHO-derivded cell lines were maintained in 1:1 Dulbecco's modified Eagle's medium:Ham's F-12 with 5% (v/v) fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Transfection of M12 cells was performed using Lipofectamine Plus reagent as previously described (3). Transient transfections of NPC1<sup>I1061T</sup> fibroblasts were performed using a primary cell line nucleofector kit and apparatus from Amaxa (20). Dulbecco's modified Eagle's medium, fetal bovine serum, glutamine, Ham's F-12 medium, Lipofectamine Plus, and penicillin/streptomycin were obtained from Invitrogen. Paraformaldehyde was obtained from EM Sciences. All restriction enzymes and endoglycosidases were obtained from New England Biolabs. [35S]Cys/Met (11.0 mCi/ml-EasyTag Express Labeling Kit), Western Lightning Chemiluminescent Reagent, and En<sup>3</sup>Hance were obtained from PerkinElmer Life Sciences. Complete Protease Inhibitor Mixture Tablets, phenylmethylsulfonyl fluoride, and Protein A-agarose were obtained from Roche Applied Science. The β-actin antibody, dialyzed fetal bovine serum, filipin, and glycerol were from Sigma. 4-Phenylbutyrate (sodium salt) was from EMD Biosciences. The NPC1 antibody used for immunoprecipitation and Western blotting was a rabbit anti-human NPC1 (raised against residues 1261–1278) (8). The p63 antibody was kindly provided by Jack Rohrer (21).

**Plasmids**—The ΔU3mnp1<sup>-I1061T-GFP</sup> construct was generated using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene), using the ΔU3mnp1<sup>-WT-GFP</sup> construct as a template (3). ΔU3mnp1<sup>-I1061T</sup> was generated by deletion of the COOH-terminal GFP tag. The presence of the npc1<sup>I1061T</sup> mutation and the entire mnp1 coding sequence in the expression construct was confirmed by ABI Prism automated sequencing.

**Quantification of Human NPC1 Gene Expression**—Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and reverse transcribed to cDNA using SuperScriptII RNase H<sup>-</sup> reverse transcriptase and random hexamer primers (Invitrogen). CDNA was then amplified for 40 PCR cycles using SYBR Green PCR master mixture (Applied Biosystems) and template-specific primers (50 nM) in an ABI Prism 7500 sequence detector. Primer sequences were as follows: human NPC1 (forward, 5'-cagctggaacattacaaagct-3'; reverse, 5'-tggcattccacctggaat-3'), and human glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-cagatccctctaaatc-3'; reverse, 5'-catggtacctccacatgac-3'). Relative quantification of gene expression was performed using the comparative threshold (C<sub>t</sub>) method as described by the manufacturer. Changes in mRNA expression level were calculated following normalization to glyceraldehyde-3-phosphate dehydrogenase expression.

**Protein Preparation and Western Blot Analysis**—Detergent lysates were prepared by washing cell monolayers three times with phosphate-buffered saline. Cells were then scraped in 500 μl of TNEN<sup>+</sup> (50 mM Tris, pH 8.0, 0.15 M NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1× Complete Protease Inhibitor Mixture, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. Cell lysates were collected into microcentrifuge tubes and nuclei were pelleted by centrifugation at 1000 × g for 10 minutes at 4 °C. Supernatants were transferred to new tubes. Proteins in TNEN<sup>+</sup> lysates were quantified using the bicinchoninic acid protein assay kit (Pierce). Isolation of microsomes from CHO cell lines was performed as described previously (22). Non-boiled samples were resolved by SDS-PAGE under reducing conditions. Proteins were transferred onto polyvinylidene difluoride (0.45 mm; Millipore) using a semi-dry electroblotter (Owl Scientific). Western blot analysis of NPC1 expression was performed using an affinity purified rabbit anti-human NPC1 at a dilution of 1:2000 and a peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) at 1:5000. Analysis of β-actin expression was performed using a rabbit anti-human β-actin at 1:500 and a peroxidase-conjugated donkey anti-rabbit IgG at 1:5000. Analysis of p63 expression was performed using a rabbit anti-human p63 at 1:5000 and a peroxidase-conjugated donkey anti-rabbit IgG at 1:5000. Detection was performed by chemiluminescence using Western Lightning reagents. Densitometry was performed using Quantity One software (Bio-Rad). For each of the non-peptide competition lanes, both the blot background and the region on the gel between 160 and 220 kDa in the peptide competition lanes (i.e. background bands), including the band denoted by the filled arrow, were subtracted from total NPC1 immunoreactivity.

**Metabolic Labeling and Immunoprecipitation of NPC1 Protein**—Pulse-chase labeling of NPC1 protein was achieved following a 1-h treatment in starvation media (Cys/Met-depleted) followed by a 1-h treatment in starvation media supplemented with [35S]Cys/Met (EasyTag Express Labeling Kit). After labeling, total cell lysate was collected in 500 μl of TNEN<sup>+</sup>
buffer (0.5% Nonidet P-40). The lysate was spun at 1000 × g for 10 min at 4 °C, the supernatant was collected, pre-cleared with Protein A-agarose, and incubated with NPC1 affinity purified antibody overnight at 4 °C. Protein-antibody complex was pulled down using Protein A-agarose at 4 °C for 45 min. Protein-antibody complex was removed from the Protein A-agarose using 1 × Laemmli buffer (Bio-Rad) for 10 min at 37 °C followed by vortexing. For endoglycosidase treatments, immunoprecipitations (IPs) were treated overnight at 37 °C with either Endoglycosidase H (Endo H) or peptide:N-glycosidase F (PNGase F) for 1.4–2.4-fold in NPC111061T versus WT fibroblasts (Fig. 2A), NPC1 protein levels are reduced by 85% in the NPC111061T fibroblasts (Fig. 2, B and C). Thus, the NPC111061T substitution affects steady-state levels of endogenously expressed NPC1 protein, possibly by impairing translation of the NPC1 protein or by rendering the protein unstable.

NPC111061T Missense Mutation Promotes Rapid Proteasomal Degradation of NPC1 Protein—To assess the effect of the NPC111061T mutation on stability of the NPC1 protein, we monitored the degradation of the protein in metabolic pulse-chase experiments over a 72-h period. WT and NPC111061T homozygous fibroblasts were labeled, and NPC1 protein immunoprecipitated and analyzed by autoradiography (Fig. 3, A and B). Two distinct rates for WT NPC1 protein degradation were observed. From 0–8 h post pulse labeling, WT NPC1 protein exhibited a \( t_{1/2} \) of 9 h, whereas from 8 to 72 h, the \( t_{1/2} \) was extended to 42 h (Fig. 3C). By contrast, NPC111061T was degraded with a \( t_{1/2} \) of 6.5 h, similar to that of the initial rate of degradation of WT protein (Fig. 3C). The maximal level of labeled NPC1 protein was comparable between WT and NPC111061T proteins, indicating similar rates in initiation of translation. An important difference, however, between WT and NPC111061T protein was the failure of NPC111061T to mature to higher molecular weight species (see open arrow, Fig. 3B).

NPC111061T Protein Is Sensitive to Endoglycosidase H—The maturation of the NPC1 protein in the secretory pathway was examined in metabolic pulse-chase experiments in which the sensitivity of the labeled protein to digestion by Endo H was determined. Endo H removes immature high-mannose \( N \)-linked glycans from proteins. Sensitivity to Endo H digestion indicates that the protein has not advanced beyond the ER in the secretory pathway. Conversely, resistance to Endo H digestion indicates that the glycan residues have been trimmed and/or further modified in the Golgi. Peptide:N-glycosidase F removes all \( N \)-linked glycans regardless of the glycan modification. In WT fibroblasts, the steady reduction in the level of Endo H-sensitive NPC1 protein (120-kDa band) is accompanied by a concomitant accumulation of Endo H-resistant NPC1 protein (180-kDa band) (Fig. 4A). By contrast, the NPC111061T mutant protein is present almost exclusively in the Endo H-sensitive form (Fig. 4B). As shown by the peptide competition controls, immunoreactivity detected at 180 kDa in the NPC111061T mutant blot is due to nonspecific banding, rather than accumulation of Endo H-resistant NPC1 protein. The more rapid loss of the Endo H-sensitive WT NPC1 species likely reflects the dynamic between degradation of ER-associated NPC1 protein and procession of the immature NPC1 within the secretory pathway (i.e. trafficking from ER to Golgi) to generate mature glycoprotein. The apparent molecular weight shift in NPC1 protein in the glycosidase experiments (Fig. 4 versus Fig. 3) is an artifact of the glycosidase treatment and buffer system used in the studies. Taken together, the metabolic labeling experiments demonstrate that ~50% of WT NPC1 protein, and nearly all of the NPC111061T mutant protein, is ER-retained and targeted for degradation.

![FIGURE 1. Immunofluorescence staining for NPC1 (A and C) and filipin staining for cholesterol (B and D) in WT (A and B) and NPC111061T mutant fibroblasts (C and D). Bar, 50 μm.](image-url)
NPC1I1061T Protein Is Selected for ERAD—The ER quality control pathway selects for degradation misfolded and unassembled proteins in the ER (23). To examine whether the NPC1I1061T mutant protein is a substrate for ERAD, we subjected NPC1I1061T mutant fibroblasts to treatment with either glycerol or 4-phenylbutyric acid (PBA), chemical chaperones known to stabilize misfolded proteins (24–26). Glycerol and PBA treatments resulted in 1.4- and 1.5-fold increases, respectively, in the level of the mutant protein (Fig. 5, A and B). Similarly, growth of NPC1I1061T mutant fibroblasts at the permissive temperature of 26 °C, which partially rescues the ER processing block of cystic fibrosis transmembrane conductance regulator (CFTR) Δ508 mutant (24, 27), increased NPC1I1061T protein levels 1.7-fold. The stabilizing effects of the chemical chaperones and growth at permissive temperatures on NPC1I1061T provide compelling evidence that the mutant protein is selected for ERAD due to protein misfolding. To determine the metabolic fate of the mutant protein selected for ERAD, NPC1I1061T mutant fibroblasts were treated with MG132, a proteasome inhibitor, or chloroquine, a weak base that inhibits lysosomal proteolysis. In the presence of MG132, mutant protein levels were increased 3.5-fold, implicating the proteasomal pathway in degradation of the mutant protein (Fig. 5C). By contrast, NPC1I1061T protein levels were reduced 85%
after treatment with chloroquine, clearly demonstrating that the mutant protein is not lysosomally degraded.

**Overexpression of the NPC1<sup>11061T</sup> Protein Rescues the NPC Mutant Phenotype**—Previous studies have shown that overexpression of mutant proteins that are misfolded may allow for transit of a small proportion of the mutant proteins through the Golgi (28). Heterologous overexpression of CFTR Δ508 led to escape of mutant protein from ERAD and appropriate localization at the plasma membrane (29, 30). Therefore, we investigated whether overexpression of NPC1<sup>11061T</sup> could lead to escape of NPC1<sup>11061T</sup> molecules from ERAD and proper targeting to the late endosomal compartment. In transient transfection experiments, we found that overexpression of GFP-tagged NPC1<sup>11061T</sup> in npc1-deficient CHO cells led to appropriate late endosomal localization of the mutant protein, and to clearance of lysosomal free cholesterol (Fig. 6A, top panels). By contrast, overexpression of the non-functional GFP-tagged NPC1<sup>P692S</sup> in npc1-deficient CHO cells failed to mobilize lysosomal free cholesterol (Fig. 6A, bottom panels) (3). This finding indicated that forced overexpression of an NPC1 mutant was not sufficient to complement the cholesterol accumulation phenotype. We likewise found that heterologous expression of NPC1<sup>11061T</sup> could rescue the mutant phenotype in human NPC1<sup>11061T</sup> mutant fibroblasts (Fig. 6B). To quantify the function of the NPC1<sup>11061T</sup> mutant, we transiently expressed either WT NPC1 or NPC1<sup>11061T</sup> GFP fusion proteins in the npc1-deficient CHO cells and monitored for clearance of unesterified lysosomal cholesterol by filipin staining (31). We found that the mutant protein was nearly as effective as WT protein in mobilizing lysosomal cholesterol, although at each time point examined the complementation efficiency of the npc1<sup>11061T</sup> mutant was slightly less than that of WT (Fig. 7, A–C). To exclude the possibility that the NPC1<sup>11061T</sup> mutant protein might have been artificially stabilized by the COOH-terminal GFP fusion, we compared the ability of the NPC1<sup>11061T</sup> mutant expression constructs, with and without the GFP tag, to complement npc1-deficient CHO cells. At 72 h post-transfection, we found that expression of the npc1<sup>11061T</sup>-GFP construct achieved 90.5% complementation, whereas expression of the npc1<sup>11061T</sup>-GFP protein construct achieved 87.2% complementation (Fig. 7D). Thus, the functionality and/or stability of the GFP-tagged NPC1<sup>11061T</sup> protein cannot be attributed to the presence of the GFP tag.

As an independent approach to confirm the function of the NPC1<sup>11061T</sup> mutant protein, we monitored the egress of LDL-derived cholesterol from the lysosomes using an LDL cholesterol esterification assay. For these experiments, we isolated npc1-deficient CHO cells stably re-expressing WT NPC1, NPC1<sup>11061T</sup>, or NPC1<sup>P692S</sup>. In comparison to primary mouse embryonic fibroblasts, NPC1 protein was overexpressed ~2-fold in the WT NPC1, NPC1<sup>11061T</sup>, and NPC1<sup>P692S</sup> cell lines (Fig. 8, A and B). (Because the rabbit polyclonal antibody does not efficiently recognize hamster NPC1 protein, mouse embryonic fibroblasts rather than CHO cells were used to assess relative NPC1 expression.) We found that overexpression of NPC1<sup>11061T</sup> corrected the biochemical defect to 53% of WT CHO cells (Fig. 8C). Overexpression of the NPC1<sup>P692S</sup> mutant protein, which appropriately localizes to late endosomes but is non-functional (3), failed to stimulate cholesterol esterification. Taken together, the complementation and esterification assay data demonstrate that if the NPC1<sup>11061T</sup> mutant protein can escape ER quality control, it is properly localized to late endosomes and is functional with respect to mobilization of endosomal cholesterol.

**DISCUSSION**

Protein misfolding has been implicated in the pathogenesis of over 30 human diseases, including cystic fibrosis, α<sub>1</sub>-antitrypsin deficiency, and lysosomal storage diseases (32, 33). In these disorders, mutant proteins that fail to achieve proper conformation are targeted for ERAD, resulting in loss of function and disease. In the present study, we demonstrate that the NPC1<sup>11061T</sup> missense mutation, the most prevalent NPC disease allele, disrupts NPC1 protein trafficking by promoting ER-mediated degradation of the mutant protein. Overexpression of the NPC1<sup>11061T</sup> mutant unexpectedly led to appropriate late endosomal localization of the mutant protein and correction of the mutant phenotype. We conclude that the NPC1<sup>11061T</sup> mutant protein is functional with respect to cholesterol trafficking, but is unstable because it is misfolded and targeted by...
the ER quality control machinery for proteasomal degradation. Our study provides the first description of an ER trafficking defect as a mechanism for human NPC disease, and suggests novel approaches for treatment of this progressive neurodegenerative disorder.

The function of ER quality control machinery is to prevent delivery of proteins to sites of function until they are properly folded, thereby limiting cytotoxicity of accumulated misfolded proteins (25, 34). Proteins recognized as misfolded by quality control are degraded through the actions of the ubiquitin-proteasome pathway. ER quality control not only targets mutant proteins, but also wild-type proteins that have been exposed to damaging conditions or that are slow to achieve conformation because they require extensive post-translational modifications (35). Whereas mature WT NPC1 protein that has progressed to post-ER compartments (i.e. Endo H-resistant) exhibits a half-life of 42 h, the half-life of ER-associated (i.e. Endo H-sensitive) WT NPC1 protein is only 9 h. Our data indicate that nearly half of the newly synthesized WT NPC1 protein is degraded within the ER. Similar ER-mediated degradation of immature species has been reported for other large, polytopic proteins, such as CFTR (36), and is likely due to failure of these heavily glyco-
sylated proteins to properly fold prior to arrival at the ER quality control checkpoints.

The presence of the NPC1I1061T mutation further accelerates degradation of immature NPC1 protein by the ER quality control machinery. We find that the half-life of ER-associated NPC1I1061T protein is reduced to 6.5 h, and that almost none of the endogenously expressed protein escapes the ER. The NPC1I1061T substitution could affect NPC1 protein stability by introducing structural features into the NPC1 protein that cause its recognition as a misfolded protein. The significant increase in NPC1I1061T protein levels in cells grown at permissive temperatures or in cells administered chemical chaperones (e.g. glycerol and PBA) provide evidence that the mutant form of the protein is misfolded. Alternatively, newly synthesized NPC1I1061T protein may be slower to achieve conformation, thus prolonging association with ER chaperones and increasing the likelihood of selection for ERAD.

A key finding in our study was the demonstration that overexpression of the NPC1I1061T protein rescues the mutant phenotype innpc1-null CHO cells. We show that at least a portion of the overexpressed mutant protein was correctly targeted to late endosomes, and importantly, properly functioned in mobilization of free cholesterol from this compartment, as determined by clearance of lysosomal free cholesterol and re-esterification of LDL-derived cholesterol. Overexpression of misfolded proteins leading to ER escape has been shown previously for other proteins, such as the CFTR Δ508 mutant (29). A possible explanation for "leakiness" of the mutant NPC1 protein is that enforced overexpression of the protein saturated the ER quality control machinery (37). Perhaps a more plausible explanation, based on a kinetic model, is that a small proportion of the nascent NPC1I1061T protein is able to fold correctly and therefore escape the quality control checkpoints (38). Assuming a fixed rate for leakiness for the I1061T mutant (e.g. 2–5% of the I1061T molecules achieve proper conformation), a sufficient quantity of correctly folded I1061T molecules could escape the
ER as a result of overexpression, leading to complementation of the mutant phenotype. In the present study we demonstrate that a 2-fold overexpression of NPC1I1061T protein is able to partially correct the biochemical phenotype in np1c-null cells. Assuming that the level of properly targeted NPC1 protein required for complete complementation of the cholesterol trafficking defect is ~10%, akin to the level of hydrolases required for enzymatic correction of other lysosomal disorders (39), it is reasonable to conclude that in human fibroblasts as much as 2.5–5% of the endogenous NPC1I1061T protein could exit the ER in the proper conformation. Whereas steady-state NPC1 protein levels were increased when the mutant fibroblasts were cultured at permissive temperatures or exposed to chemical chaperones that stabilize protein, the overall effect on efficiency of NPC1I1061T protein folding does not appear to be sufficient to correct the cholesterol trafficking defect (not shown). On the other hand, identification of chemical chaperones that enhance the efficiency of NPC1I1061T protein folding through high throughput small molecule screens has the potential to develop new and effective approaches for the treatment of NPC1 disease. Such an approach might not only benefit subjects with the NPC1I1061T genotype, but subjects with other missense mutations, in particular the G992W and P1007A genotypes that likewise affect the cysteine-rich luminal domain. In future studies these and other NPC1 mutants could be screened to determine whether the mutant proteins are similarly misfolded and respond to treatment with chemical chaperones. If this is indeed the case, there is the potential that a significant percentage of NPC1 subjects could benefit from small molecule-based chaperone therapy.

Acknowledgment—We are grateful for Stuart Kornfeld for critical review of the manuscript.

REFERENCES

1. Ory, D. S. (2000) Biochem. Biophys. Acta 1529, 331–339
2. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Naqle, J., Polymerropoulos, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., et al. (1997) Science 277, 228–231
3. Millard, E., Gale, S., Dudley, N., Zhang, J., Schaffer, J., and Ory, D. (2005) J. Biol. Chem. 280, 28581–28590
4. Davies, J. P., and Ioannou, Y. A. (2000) J. Biol. Chem. 275, 24367–24374
5. Frolov, A., Srivastava, K., Daphna-Iken, D., Traub, L. M., Schaffer, J. E., and Ory, D. S. (2001) J. Biol. Chem. 276, 46414–46421
6. Neufeld, E. B., Wastney, M., and Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. A., Incardona, J. P., Strauss, J. F., III, Vanier, M. T., Patterson, M. C., Brady, R. O., Pentchev, P. G., and Blanchette-Mackie, E. J. (1999) J. Biol. Chem. 274, 9627–9635
7. Zhang, M., Dwyer, N., Neufeld, E. B., Love, D. C., Cooney, A., Comly, M., Patel, S., Watari, H., Strauss, J. F., III, Pentchev, P. G., Hanover, J. A., and Blanchette-Mackie, E. J. (2001) J. Biol. Chem. 276, 3417–3425
8. Millard, E. E., Srivastava, K., Traub, L., Schaffer, J. E., and Ory, D. S. (2000) J. Biol. Chem. 275, 38445–38451
9. Neufeld, E. B., Cooney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) J. Biol. Chem. 271, 21604–21613
10. Wojtanjik, K. M., and Liscum, L. (2003) J. Biol. Chem. 278, 14850–14856
11. Liscum, L., and Faust, J. R. (1987) J. Biol. Chem. 262, 17002–17008
12. Pentchev, P. G., Comly, M. E., Kruth, H. S., Vanier, M. T., Wenger, D. A., Patel, S., and Brady, R. O. (1985) Proc. Natl. Acad. Sci. 82, 8427–8451
13. Chen, W., Sun, Y., Welch, C., Gorelik, A., Leventhal, A. R., Tabas, I., and Tall, A. R. (2001) J. Biol. Chem. 276, 43564–43569
14. Choi, H. Y., Karten, B., Chan, T., Vance, J. E., Greer, W. L., Heidenreich, R. A., Garver, W. S., and Francis, G. A. (2003) J. Biol. Chem. 278, 32569–32577
15. Park, W. D., O’Brien, J. F., Lundquist, P. A., Kraft, D. L., Vockley, C. W., Carnes, P. S., Patterson, M. C., and Snow, K. (2003) Hum. Mutat. 22, 313–325
16. Scott, C., and Ioannou, Y. A. (2004) Biochim. Biophys. Acta 1685, 8–13
17. Fernandez-Valero, E. M., Ballart, J., Paredes, J., Lluch, M., Macías, J., Vanier, M. T., Pineda, M., and Coll, M. J. (2005) Clin. Genet. 68, 245–254
18. Millard, E., Gale, S., Frolov, A., Han, X., Bickel, P. E., Cao, L., Bowcock, A., Schaffer, J. E., and Ory, D. S. (2003) J. Biol. Chem. 278, 25517–25525
19. Schweizer, A., Rohrer, J., Slot, J. W., Geuze, H. J., and Kornfeld, S. (1995) J. Cell Sci. 108, 2477–2485
20. Gale, S. E., Frolov, A., Han, X., Bickel, P. E., Cao, L., Bowcock, A., Schaffer, J. E., and Ory, D. S. (2006) J. Biol. Chem. 281, 11082–11089
21. Werner, E. D., Brodsky, J. L., and Craddock, A. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13797–13801
22. Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J., and Kopito, R. R. (1996) J. Biol. Chem. 271, 635–638
23. Shearer, A. G., and Hampton, R. Y. (2004) J. Biol. Chem. 279, 188–196
24. Rubenstein, R. C., Egan, M. E., and Zeitlin, P. L. (1997) J. Biol. Chem. 272, 2457–2465
25. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992) Nature 358, 761–764
26. Spear, E. D., and Ng, D. T. (2003) Mol. Biol. Cell 14, 2756–2767
27. Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystall, R. G., Pavirani, A., Lecocq, J. P., and Lazdunski, M. (1991) Biochem. Biophys. Res. Commun. 178, 354–356
28. Shamma, M. R., Bencchorouga, M., Hu, W., and Lukacs, G. L. (2001) J. Biol. Chem. 276, 8942–8950
29. Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Watari, M., Burd, C. G., Patel, S., Pentchev, P. G., and Strauss, J. F., III (2000) Exp. Cell Res. 259, 247–256
30. Aridor, M., Zielinski, S. E., Crowley, J. R., Dudley-Rucker, N., Schaffer, J. E., and Ory, D. S. (2003) J. Biol. Chem. 278, 25517–25525
31. Millard, E., Gale, S., Dudley, N., Zhang, J., Schaffer, J., and Ory, D. (2005) J. Biol. Chem. 280, 28581–28590