Production and characterization of recombinant human CLN2 protein for enzyme-replacement therapy in late infantile neuronal ceroid lipofuscinosis

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Late infantile neuronal ceroid lipofuscinosis (LINCL) is a fatal recessive childhood disease caused by mutations in the *CLN2* gene, which encodes the lysosomal enzyme tripeptidyl peptidase I. As a step towards understanding the protein and developing therapeutics for the disease, we have produced and characterized recombinant human CLN2 (ceroid lipofuscinosis, neuronal 2) protein from Chinese-hamster ovary cells engineered to secrete high levels of the enzyme. The protein was secreted as an inactive soluble proenzyme of ≈ 65 kDa that appears as a monomer by gel filtration. Upon acidification, the protein is processed to mature form and acquires activity. The enzyme is efficiently delivered to the lysosomes of LINCL fibroblasts by mannose

6-phosphate-receptor-mediated endocytosis ($EC_{50} \approx 2$ nM), where it remains active for long periods of time ($t_{1/2} \approx 12$ days). In addition, the enzyme is taken up by rat cerebellar granule neurons by mannose 6-phosphate-dependent and -independent mechanisms. Treatment of LINCL fibroblasts with recombinant CLN2 protein restores normal enzyme activity and ameliorates accumulation of the major storage protein, mitochondrial ATP synthase subunit c.

Key words: endocytosis, lysosomal storage, overexpression, tripeptidyl peptidase I.

INTRODUCTION

Late infantile neuronal ceroid lipofuscinosis [LINCL; OMIM (Online Mendelian Inheritance in Man) 204500] is an autosomal recessive neurodegenerative disease caused by mutations in the *CLN2* gene [1]. *CLN2* encodes a lysosomal protease [1] that was later found to be identical with lysosomal tripeptidyl peptidase I (TPP-I, EC 3.4.14.9) [2]. At the cellular level, LINCL is characterized by lysosomal accumulation of autofluorescent storage material [3] whose major identifiable component is mitochondrial ATP synthase subunit c (subunit c) [4] in neurons and other cell types. Affected individuals usually develop normally until about age 3 years, at which point they exhibit symptoms such as ataxia and seizures. The disease is associated with progressive loss of neurons and photoreceptors, and, within several years, LINCL patients become blind, mute, bedridden and demented. Currently, there is no effective treatment for the disease and death typically occurs between ages 6 and 15 [5]. However, one possibility for LINCL is enzyme-replacement therapy, in which recombinant enzyme is administered to the affected cells to correct the metabolic defect, an approach that has proven successful for Gaucher's disease [6,7]. As a first step towards developing a therapy, we have characterized recombinant human CLN2 (ceroid lipofuscinosis, neuronal 2) protein (CLN2p) produced in a Chinese-hamster ovary (CHO) cell line that overexpresses this protein, and have investigated its uptake, lysosomal targeting and function in cultured cells.

EXPERIMENTAL

Plasmid construction

A fragment corresponding to nt 1–1707 of the human CLN2 cDNA (GenBank® accession number AF017456, Arg¹⁷⁵ variant)

flanked by *Xho*I sites was generated using standard PCR-based methods and subcloned into *Xho*I-digested expression vector pMSXND1 [8]. After restriction mapping, correctly oriented constructs were sequenced to confirm absence of unwanted changes in the coding region. Plasmid DNA was linearized with *Pvu*II before transfection.

Cell transfection, selection and methotrexate (MTX)-based gene amplification

CHO cells were maintained in Dulbecco's modified Eagle's (DME)/F12 medium (Sigma) supplemented with 10% (v/v) fetal-bovine serum (FBS) and were transfected using LIPOFECTAMINE™ (GIBCO). Cells were selected with G418 and resistant clones isolated using cloning cylinders. Subclones were analysed for secretion of CLN2p by measuring TPP-I activity after acidification to activate the proenzyme [9]. The clone generating conditioned medium with the highest activity was grown in nucleotide-deficient α -minimum essential medium (GIBCO) containing 0.2 μ M MTX. When resistant cells emerged, they were expanded, aliquots frozen, and the remaining cells challenged with ≈ 2 -fold higher concentration of MTX. This process was repeated for a total of 12 cycles, reaching a final MTX concentration of 400 μ M.

Purification and characterization of recombinant CLN2 protein

For production of recombinant protein, adherent CHO cells that overexpress CLN2p were seeded into P150 dishes and grown in DME/F12 medium supplemented with 10% FBS. When monolayers appeared confluent, cells were washed with protein-free

Abbreviations used: LINCL, late infantile neuronal ceroid lipofuscinosis; TPP-I, tripeptidyl peptidase I; CLN2p, CLN2 (ceroid lipofuscinosis, neuronal 2) protein; LAMP, lysosome-associated membrane protein; 4-MU, 4-methylumbelliferyl; MTX, methotrexate; Man-6-P, mannose 6-phosphate; MPR, Man-6-P receptor; subunit c, mitochondrial ATP synthase subunit c; CHO, Chinese-hamster ovary; DME, Dulbecco's modified Eagle's; FBS, fetal-bovine serum.

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DME/F12 and cultured in this medium for 8 days. Typically, media from 12 dishes were pooled (total volume ≈ 1 litre), centrifuged at 200 *g* to remove floating cells, and the supernatant concentrated and buffer exchanged using a stirred cell equipped with a YM-30 membrane (Amicon). This and subsequent chromatography steps were conducted at $< 5^\circ\text{C}$. The concentrate (final volume of 40 ml in 50 mM NaCl/20 mM Tris, pH 8.0) was loaded on to an Uno Q12 (Bio-Rad) anion-exchange column at a flow rate of 2 ml/min. The column was washed with 50 mM NaCl/20 mM Tris, pH 8.0 (120 ml) and recombinant CLN2p eluted with a linear gradient of 50–525 mM NaCl in 20 mM Tris, pH 8.0 (480 ml total volume). Fractions were analysed for TPP-I enzyme activity after pre-activation at pH 3.5 [10].

Enzyme assays

The TPP-I assay was performed as described using the end-point method [9] after acidification to activate proenzyme [10] unless noted otherwise. β -Galactosidase, β -glucuronidase and β -hexosaminidase activities were measured using 4-methylumbelliferyl (4-MU) substrates (4-MU galactoside, 4-MU glucuronide and 4-MU *N*-acetylglucosaminide) as described by Sleat et al. [11]. Briefly, 40 μl of 1 mM substrate in 100 mM acetate buffer (pH 4.0)/0.1% Triton X-100/150 mM NaCl was mixed with 10 μl of samples in replicate, incubated at 37°C for 2 h and the reaction terminated by the addition of 100 μl of 0.5 M glycine, pH 10.5. Substrate solutions mixed with buffer were used as blanks. Fluorescent reaction products were measured using a CytoFluor II fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, MA, U.S.A.) with excitation at 360 nm and emission at 460 nm. Enzyme activities were linear with respect to input sample.

Cell culture

LINCL fibroblasts (CABM011) [12] were from a patient who was compound heterozygous for the Arg²⁰⁸stop and Arg¹²⁷stop alleles and had no detectable TPP-I activity. Unaffected control fibroblasts GM05665 were from the Coriell Institute for Medical Research (Camden, NJ, U.S.A.). Fibroblasts were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS and maintained at 37°C in a humidified atmosphere containing 5% CO_2 . Typically, experiments were conducted in 48-well plates using duplicate wells for each condition. When cells were confluent, media were replaced with fresh media containing the indicated concentrations of purified recombinant human CLN2p (0.5 ml). Immediately before processing, cells were washed three times with PBS (0.5 ml) at room temperature and then rapidly cooled by placing dishes in an ice/water bath. The cells were lysed by adding 1% Nonidet P40/10 mM Tris (pH 7.5)/150 mM NaCl (0.2 ml/well) and incubated for 1 h at 4°C on a rocking platform. The lysates were transferred to Microfuge tubes and centrifuged for 20 min at 13000 *g*. The supernatants were used for enzyme activity and protein [13] assays. For microscopy, cells were seeded into 16-well glass chamber slides (Nalge Nunc) and processed as described below.

Cerebellar granule neurons were prepared from postnatal-day-8 Sprague–Dawley rat pups as described in [14]. The cells were plated into 48-well plates at a density of 150 000 cells/cm² and processed as described above.

Microscopy

Cells were rinsed with PBS at room temperature and fixed for 1 h at 4°C using Bouin's fixative supplemented with 0.2% glutaraldehyde. All subsequent steps were conducted at room tem-

perature. Cells were washed (three times, 5 min each time) with PBS, permeabilized for 10 min with 0.5% Triton X-100/PBS, and incubated for 30 min in blocking buffer (PBS supplemented with 3% BSA/0.2% Tween 20/500 mM NaCl). The cells were then incubated for 1 h with blocking buffer containing a 1:100 dilution of rabbit antiserum R72-5 raised against hexahistidine-tagged CLN2p produced in insect cells [10] and a 1:100 dilution of anti-[human lysosome-associated membrane protein (LAMP)-1] mouse hybridoma tissue-culture supernatant (H4A3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.) for 1 h at room temperature. Cells were washed (four times, 5 min each time) with 0.5% Triton X-100/PBS and once for 5 min with blocking solution. Bound antibodies were detected using goat anti-rabbit antibody–FITC conjugate (Sigma) and goat anti-mouse antibody–Cy5 conjugate (Biological Detection Systems, Inc, Pittsburgh, PA, U.S.A.). Images were obtained using a Zeiss LSM-410 confocal laser-scanning microscope equipped with a 63 plan-apochromat objective.

RESULTS

CHO-cell production system

Full-length human CLN2 cDNA was cloned into pMSXND1 vector, yielding a construct that contains a CLN2 expression cassette, a neomycin-resistance cassette for G418 selection and a dihydrofolate reductase expression cassette for MTX-based selection. CHO cells were transfected and selected with G418. A resistant clone that secreted mannose 6-phosphorylated CLN2p (results not shown) was subjected to multiple cycles of selection with increasing concentrations of MTX. Cells resistant to 400 μM MTX were subcloned and the clone that secreted the highest level of CLN2p (LL404) chosen for subsequent analysis.

Figure 1 Enzyme activities in CHO cells

The parental (untransfected), G418-selected (transfected), and MTX-selected (amplified) CHO cells were grown in 12-well plates to confluency in DME/F12 containing 10% FBS. Media were replaced with 2 ml of serum-free DME/F12 on day 0, and cells (open bars) and media (tinted bars) assayed for the indicated enzyme activity on day 4 as described in the Experimental section. Values are means $\pm$ S.E.M. for duplicate determinations.

Figure 2 Anion-exchange chromatography of recombinant CLN2p

Left panel: Uno Q12 ion-exchange chromatography on concentrated material from 1 litre of CHO-cell conditioned medium. Right panel: SDS/PAGE on 7.5 μ l of either concentrated CHO-cell conditioned medium ('Load') or the peak fraction after anion exchange chromatography ('AEX'). Proteins were revealed by Coomassie Brilliant Blue staining.

Analysis of the G418-selected clone revealed that total levels of cellular and secreted CLN2p increased by a factor of five compared with the parental CHO cells (Figure 1A). This level of expression had little apparent effect on the intracellular targeting of CLN2p as inferred from measurements of TPP-I activity associated with cells and conditioned media. Here, for both cell lines, the amount of enzyme recovered in the media after 4 days was $\approx 1/10$ of the steady-state level in the cells. MTX selection resulted in a large increase in CLN2p production, with the activity found in the media increasing disproportionately compared with that associated with the cells (Figure 1A). Pulse-chase experiments on the MTX-amplified cells indicated that a large proportion of the newly synthesized CLN2 is secreted directly into the media (results not shown). Taken together, this suggests that high-level expression of CLN2p alters its intracellular targeting.

The high-level expression of CLN2p also appeared to affect the biosynthesis of other lysosomal enzymes. The relative specific activity of β -galactosidase, β -glucuronidase and β -hexosaminidase activities in cell extracts actually decreased after MTX selection, whereas the amount secreted increased 0.3–1.8-fold (Figures 1B–1D). The decrease in total amount of these other enzymes upon CLN2p overexpression may reflect mistargeting combined with instability of these enzymes in the culture medium, a decrease in their stability in the lysosome due to the increased levels of the CLN2p protease, or a general effect on protein synthesis.

Purification and characterization of recombinant CLN2 protein

The MTX-selected cells adapted well to growth in standard protein-free media formulations (e.g. DME/F12), and the resulting conditioned medium represents a rich source of CLN2p, with its 66 kDa proenzyme [10] representing the major protein constituent (Figure 2). Most of the contaminating proteins could be removed after a single anion-exchange-chromatography step conducted at pH 8.0 (Figure 2, right panel). Essentially all of the applied TPP-I activity was recovered and the pooled fractions eluting from 200–225 ml contained 17 mg of CLN2p as

Figure 3 Comparison of proform and mature CLN2p

Anion-exchange purified recombinant human CLN2p diluted into either PBS, pH 7.4, or 150 mM NaCl/0.1% Triton X-100/50 mM formate, pH 3.5, buffer and incubated for 16 h at 37 °C. Left panel: Coomassie Brilliant Blue-stained SDS/PAGE gel. Right panel: kinetic assay of TPP-I activity [9] conducted at pH 4.5 on the samples preincubated at either pH 7.4 (open circles) or pH 3.5 (closed circles). Results are means $\pm$ S.E.M. for duplicate determinations.

calculated from the A_{280} using an absorption coefficient of 1.313 ml/mg $\cdot$ cm.

When TPP-I activity was measured at pH 4.5 without any pre-treatment, there was essentially no detectable activity (Figure 3, right panel). In contrast, if the protein was preincubated at pH 3.5, the protein was converted into a 46 kDa form (Figure 3, left panel) and acquired enzyme activity (Figure 3, right panel). These results indicate that the CLN2p secreted from the CHO cells represents inactive proenzyme. The CLN2 transcript encodes a 563-amino-acid protein, and Edman degradation revealed that the N-terminus of the purified protein corresponded to residue 20 before activation and residue 196 after activation (results not shown). Identical results have been obtained with hexahistidine-tagged protein produced in an insect-cell expression system [10].

Figure 4 Uptake of recombinant CLN2p by LINCL fibroblasts

Confluent LINCL fibroblasts in 48-well plates were incubated with 1 ml of RPMI 1640/10% FBS containing the indicated concentrations of recombinant CLN2p purified by anion exchange and gel filtration. Where indicated, the medium also contained 10 mM Man-6-P. At the indicated times, cells were processed and assayed for TPP-I activity as described in the Experimental section. Activity was normalized to wells plated with normal control fibroblasts. Data were fitted to sigmoidal dose-response (left panel) and one-site-binding (right panel) models using Prism 3.0 (GraphPad Software). Results are means $\pm$ S.E.M. for duplicate determinations.

To investigate the quaternary structure of the purified CLN2p, we analysed its behaviour following gel filtration on a Superose 12 column run at neutral pH. The protein is eluted as a single peak (results not shown) that, in comparison with globular-protein standards, has an apparent molecular mass of 65 kDa. Given the apparent size determined after denaturing SDS/PAGE (Figure 2, right panel), this indicates that the CLN2p precursor exists as a monomer at neutral pH.

CLN2p was first identified as a mannose 6-phosphorylated glycoprotein isolated from human brain [1]. To investigate the phosphorylation state of the proenzyme secreted from CHO cells, we applied purified protein to an affinity column of immobilized soluble cation-independent mannose 6-phosphate (Man-6-P) receptor (MPR). Although a small amount of the protein was recovered in flow-through and wash fractions, most of the protein (92%) was retained on the column and was specifically eluted with Man-6-P (results not shown). This demonstrates that the vast majority of the recombinant CLN2p was mannose 6-phosphorylated.

Lysosomal targeting, stability and function of recombinant CLN2p in LINCL fibroblasts

To evaluate the ability of the recombinant proenzyme to be endocytosed, we cultured LINCL fibroblasts with increasing concentrations of CLN2p for various periods of time and then analysed intracellular TPP-I activity. In the absence of recombinant enzyme there was essentially no endogenous activity. In contrast, after exposure of the cells to ≥ 10 nM CLN2p for 1 day, the intracellular activity was comparable with that of unaffected control fibroblasts (Figure 4, left panel).

The uptake of recombinant CLN2p was time- and concentration-dependent (Figure 4) and occurred via MPR-mediated endocytosis as demonstrated by its inhibition by Man-6-P (Figure 4, left panel). The binding of CLN2p to receptor was of relatively high affinity as demonstrated by the EC_{50} of the dose-response curves (Figure 4, left panel). The calculated apparent EC_{50} for uptake was 1.5 nM for the 1-day time point and shifted slightly to the right with increasing time (apparent $EC_{50} \approx 1.7, 2.9$ and 4.0 for day 2, 4 and 8 respectively). Note that these EC_{50} are based on the concentration of CLN2p initially present at day 0 and the apparent shift may reflect enzyme depletion. However, at higher CLN2p concentrations, uptake

increased steadily with increasing time (Figure 4, right panel), indicating that the recombinant CLN2p was stable in the culture media for long periods of time and retained the Man-6-P modification.

We used double-label immunofluorescence and confocal microscopy to determine the intracellular location of the CLN2p endocytosed by LINCL cells. The specificity of the CLN2p antibody, raised against recombinant CLN2p [10], was confirmed by the lack of staining in LINCL fibroblasts (Figure 5, top panels). In contrast, the normal control showed punctate cytoplasmic staining (Figure 5, bottom panels), as did the LINCL cells incubated with recombinant CLN2p (Figure 5, middle panels). The staining patterns of CLN2p and the lysosomal marker LAMP-1 were very similar, demonstrating colocalization of these two proteins (Figure 5, right panels). Consistent with this, kinetic enzyme-activity measurements conducted using conditions designed to distinguish mature and proenzyme (see below) indicated that essentially all endocytosed CLN2p was processed into mature form, presumably reflecting targeting to an acidic intracellular compartment (results not shown). Taken together, these results demonstrate that the recombinant CLN2p is correctly delivered to the lysosome of LINCL cells.

To investigate the stability of the endocytosed enzyme, LINCL cells were grown for 1 day in complete medium containing recombinant CLN2p and analysed for intracellular TPP-I activity, either immediately (day 0) or at various times after removal of the exogenous CLN2p. This analysis revealed that the endocytosed protein was quite stable, having a calculated half-life of ≈ 11.5 days (Figure 6).

The results presented here indicate that the recombinant CLN2p is efficiently endocytosed by LINCL fibroblasts and targeted to the lysosome. However, when considering enzyme replacement as a potential therapy for LINCL, it is important to demonstrate that the endocytosed enzyme is functionally equivalent to the native enzyme and can correct the biochemical phenotype of the disease. A biochemical hallmark for LINCL is accumulation of subunit c in lysosomes. We cultured LINCL fibroblasts in the presence or absence of CLN2p and determined the relative levels of subunit c by Western blotting (Figure 7). Enzyme treatment dramatically reduced subunit c to levels approaching that of unaffected control fibroblasts (Figure 7), suggesting that recombinant CLN2p can correct the metabolic defect in LINCL cells.

Figure 5 Localization of CLN2p in normal and LINCL fibroblasts

Where indicated, LINCL fibroblasts were incubated with 30 nM recombinant CLN2p for 1 day and then chased with complete media for 1 day. Cells were stained with anti-CLN2 antibodies (left panels) and anti-LAMP-1 antibodies (centre panels). The yellow colour in the merged images (right panels) shows co-localization of the CLN2p (green) and LAMP-1 (red) immunofluorescence.

Uptake of CLN2p by cultured neurons

To determine the ability of the recombinant enzyme to be delivered to neurons, we cultured rat cerebellar granule neurons with increasing concentrations of CLN2p for 1 day and analysed intracellular TPP-I activity using the kinetic assay. Depending on how the samples were processed (see the legend to Figure 8), the TPP-I activity reflects either the mature CLN2p (triangles) or both precursor and mature CLN2p (circles) present within the neurons (Figure 8). At concentrations of recombinant CLN2p where there was a significant increase of TPP-I activity over

endogenous levels (> 3 nM in the absence of Man-6-*P* and > 10 nM in the presence of Man-6-*P*), thus allowing reliable estimation of the endocytosed protein, $\approx 80\%$ of the endocytosed CLN2p was in the mature form (Figure 8). This indicates that the enzyme is targeted to an acidic intracellular compartment, but that this process is slower or less efficient than in fibroblasts. Also, unlike fibroblasts, the uptake did not saturate at high CLN2p concentrations (compare Figures 4 and 8). However, the Man-6-*P*-inhibitable uptake was saturable (Figure 8, inset; EC_{50} 6–8 nM), indicating that, at high concentrations, there was considerable uptake through MPR-independent mech-

Figure 6 Stability of recombinant CLN2p in LINCL fibroblasts

Cells were incubated with 31 nM CLN2p for 24 h and then cultured in the absence of CLN2p for an additional 1, 3 or 7 days before assaying cell lysates for TPP-I activity. Data were fitted to a one-phase exponential decay model. Results are means $\pm$ S.E.M. for quadruplicate determinations.

Figure 7 Subunit c in normal and LINCL fibroblasts

Confluent fibroblasts were cultured in RPMI 1640/10% FBS, containing 30 nM CLN2p where indicated, for 7 days. Cells were solubilized in lithium dodecyl sulphate sample buffer and proteins separated by SDS/PAGE on precast NuPAGE 10% Bis-Tris gel using Mes running buffer (Invitrogen). Western blotting was conducted using rabbit antibodies against subunit c [4] and enhanced chemiluminescence (Renaissance; New England Nuclear).

Figure 8 Uptake of recombinant CLN2p by rat cerebellar granule neurons

Cerebellar granule neurons were cultured in 48-well plates for one day in medium containing the indicated concentrations of CLN2p in the presence (closed symbols) or absence (open symbols) of 10 mM Man-6-P. Cells were lysed as described in the Experimental section and then assayed for TPP-I activity using the kinetic assay (see Figure 3) after a 30 min preincubation at 37 °C in pH 3.5 buffer to activate proenzyme (circles) or at 0 °C in pH 7.5 buffer to prevent proenzyme activation (triangles). The dotted line represents the endogenous TPP-I activity level. Results are means $\pm$ S.E.M. for triplicate determinations. Data were fitted to a sigmoidal dose-response model using Prism 3.0. The inset shows the difference between the activity associated with the cells incubated in the absence and presence of Man-6-P, with the circles representing pre-activated samples and the triangles representing non-pre-activated samples.

anisms. Nonetheless, even when MPR-independent mechanisms predominated, $\approx 80\%$ of the endocytosed enzyme was converted into the active form, suggesting proper lysosomal targeting of the recombinant CLN2p.

DISCUSSION

In the present paper we have described a production system for recombinant human CLN2p and demonstrated that this protein can be delivered to lysosomes of CLN2p-deficient LINCL fibroblasts and correct the metabolic defect. Similar CHO-based production systems have been used to produce large quantities of other lysosomal enzymes for protein characterization and enzyme-replacement studies [15–18]. Consistent with our findings, overexpression of a given recombinant lysosomal enzyme typically results in its disproportionate secretion [15,16]. The effect on intracellular targeting of endogenous lysosomal enzymes is variable, with either no noticeable changes [15,16] or relatively minor effects ([16]; the present study). This is somewhat surprising, given that the overexpressed protein would be expected to compete with other lysosomal enzymes for intracellular targeting by MPRs. For α -galactosidase A, the lack of competition was attributed to the enzyme forming large aggregates in the *trans*-Golgi network that were unable to bind to MPRs and were constitutively secreted. If this occurs for CLN2p, the properties of the secreted protein (see below) indicate that any aggregation is readily reversible. The decrease in intracellular levels of several lysosomal enzymes found after high-level expression of CLN2p may be due to some mistargeting as a result of competition for MPRs, and differential effect on the secretion of individual lysosomal enzymes may reflect their relative affinities for the different MPRs [19]. Alternatively, it is possible that the high level of active lysosomal CLN2p affects the stability of other lysosomal enzymes.

The properties of the CLN2p precursor differ in a number of aspects from that of the mature protein. First, the precursor is enzymically inactive, but, upon acidification, is autocatalytically processed to the mature, active form (the present study; see also [10]). Secondly, the mature enzyme is rapidly inactivated when incubated at 37 °C at neutral pH [20,21]. In contrast, the proenzyme is stable at neutral pH (Figure 4) and can subsequently be converted into the active form (Figure 8 and results not shown). Finally, the quaternary structure and physical properties of the proenzyme and mature enzyme appear to be quite different. For instance, published procedures for purification of mature CLN2p/TPP-I from mammalian tissues utilize detergent to maintain the protein in solution [21–26], and gel-filtration analysis indicates that the mature protein forms aggregates of 250–700 kDa [23,25]. In contrast we show here that the proenzyme behaves as a soluble monomer.

Recombinant CLN2p produced from CHO cells has a number of properties that appear useful for enzyme-replacement therapy in LINCL. First, as the proenzyme is inactive and stable in an extracellular environment until delivery to the lysosome, and the mature form is unstable with little activity at neutral pH, concerns about unwanted proteolysis of extracellular structures by TPP-I activity after enzyme administration should be minimized. Secondly, the protein is efficiently delivered to the lysosome by Man-6-P-mediated endocytosis (fibroblasts and neurons) and possibly by other endocytic mechanisms (neurons). Thirdly, the endocytosed enzyme restores the deficient TPP-I activity of LINCL fibroblasts. Fourthly, the internalized CLN2p can reverse a biochemical marker of the disease, i.e. storage of subunit c. Finally, the internalized active protein has a long half-life within the lysosome, which will be important in considering dosing regimes.

Administration of glucocerebrosidase has proved remarkably effective in treating many patients with Gaucher's disease [27], and there are ongoing clinical trials for a number of other lysosomal-storage disorders [28–31]. However, to date, treatment is restricted to visceral symptoms, as peripherally administered enzyme does not penetrate the blood/brain barrier. While a wide variety of cell types exhibit lysosomal storage in LINCL, the most striking manifestations of the disease occur in the central nervous system, and thus brain delivery is essential for any therapy. Innovative bioengineering and possibly protein engineering approaches will be required to deliver protein across the blood/brain barrier. Our results showed that large quantities of recombinant CLN2 protein can be readily obtained from our CHO-cell system. This will facilitate enzyme-replacement studies in animal models and development of novel delivery methods for treatment of LINCL.

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