measured concentrations of the dipeptides γ-Glu-Cys and Cys-Gly suggest that their reliable assessment necessitates immediate SBD-F derivatization.

We gratefully acknowledge financial support from Fresenius-Kabi, Bad Homburg, Germany. We thank S. Cvek and M. Wolter for excellent technical assistance, are indebted to Prof. Dr. H-C. Bode, Robert Bosch Hospital, Stuttgart, for providing us with the human mucosal biopsy specimens.

References


Enzyme-based Diagnosis of Classical Late Infantile Neuronal Ceroid Lipofuscinosis: Comparison of Tripeptidyl Peptidase I and Pepstatin-insensitive Protease Assays, Istvan Sohar, Li Lin, and Peter Lobel* (Center for Advanced Biotechnology and Medicine, Department of Pharmacology, Robert Wood Johnson Medical School-University of Medicine and Dentistry of New Jersey, 679 Hoes Lane, Piscataway, NJ 08854; * author for correspondence: fax 732-235-5289, e-mail lobel@cabm.rutgers.edu)

The neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative diseases that include infantile NCL (INCL; OMIM 256730), where the defective gene is CLN1, classical late infantile NCL (LINCL; OMIM 204500, where the defective gene is CLN2), two variant late infantile NCLs (OMIM 256731, where the defective gene is CLN5; and OMIM 601780, where the defective gene is CLN6), juvenile NCL (JNCL; OMIM 204200; defective gene, CLN3), a juvenile onset epilepsy with progressive mental retardation (EPMD; OMIM 600143; defective gene, CLN8), and adult NCL (Kufs disease; OMIM 204300; defective gene, CLN4) [reviewed in Ref. (1)]. Patients experience a progressive and profound loss of neurons in the central nervous system that generally is associated with increasingly severe epileptic seizures, visual failure, ataxia, mental deterioration, and early death. At the cellular level, the NCLs are characterized by accumulation of autofluorescent storage material in lysosome-like organelles in neurons and other cell types. Different forms of NCLs have traditionally been classified on the basis of age of onset of symptoms and cellular morphology and, more recently, by molecular criteria. Molecular analysis has revealed that different mutations in a given NCL gene can produce distinct clinical presentations [e.g., CLN2 deficiencies can lead to late infantile as well as juvenile onset disease (2), CLN1 deficiencies to infantile as well as late infantile and juvenile onset disease (3), and CLN3 deficiencies to juvenile as well as delayed onset disease (4, 5)]. Because of the existence of multiple NCL disease genes that can have overlapping clinical manifestations, simple clinical assays for NCL classification are useful before proceeding to DNA-based analysis.

Enzyme-based assays are now available for diagnosis of CLN2 deficiencies. The CLN2 gene encodes a lysosomal protein (6) that has sequence similarity to bacterial pepstatin-insensitive endoproteinasises (7). We developed a diagnostic assay (termed “pepinase”, for pepstatin-insensitive protease) based on the ability of specimens from healthy individuals but not patients with LINCL to degrade hemoglobin into trichloroacetic acid-soluble products in the presence of aspartyl and cysteinyl protease inhibitors (8). However, this assay required a reagent that is not commercially available [fluorescein isothiocyanate (FITC)-labeled hemoglobin] and required multiple labor-intensive steps. Another specialized assay for pepstatin-insensitive protease activity that entails monitoring cleavage of a chromophore-labeled peptide after HPLC has also been described (9). The recent realization that the CLN2 protein is identical to the lysosomal enzyme tripeptidyl peptidase I (TPP-I) (10–12) should simplify detection of CLN2 deficiencies because this assay is simpler and uses a commercially available substrate. However, to date the TPP-I assay has been applied only to cultured fibroblasts, with the TPP-I activity of LINCL fibroblasts being ∼5% of healthy controls (10). When the same cells were analyzed for “pepstatin-insensitive acid proteinase activity” using a FITC-casein substrate, the activity of control and LINCL fibroblasts were indistinguishable. Given this apparent discrepancy and the fact that we previously validated the pepinase assay on a large number of nonpathological and genetically verified disease specimens (8), in this study
we performed the TPP-I and pepinase assays in parallel on a variety of nonpathological and disease specimens as well as on recombinant CLN2 protein. We find that both assays detect function of the same underlying gene product.

Blood specimens were obtained using an internal review board-approved informed consent protocol. Cleared homogenates from different specimens were prepared and analyzed for pepinase activity and protein content as described previously (8).

TPP-I activity was assayed in 96-well format plates using the following modification of the method described by Vines and Warburton (12): Samples (10 μL) were transferred to individual wells of a clear polystyrene 96-well plate (Nalge Nunc International), and the reactions were initiated by adding 40 μL of substrate solution to each well. The substrate solution consisted of 250 μmol/L Ala-Ala-Phe 7-amido-4-methylcoumarin (cat. no. A3401; Sigma; diluted freshly from a 25 mmol/L stock solution in dimethyl sulfoxide stored at −20 °C) in 0.15 mol/L NaCl-1 g/L Triton X-100-0.1 mol/L sodium acetate, adjusted to pH 4.0 at 20 °C. For kinetic assays, plates were centrifuged briefly to dispel bubbles and placed in a 37 °C thermostated sample chamber of a PerSeptive Biosystems CytoFluor 4000 TC plate reader (PE Biosystems). The plates were read from the bottom using 360/20 nm excitation and 460/25 nm emission filters. Plates were read at 1-min intervals for 30 min and mixed for 5 s before each reading. For endpoint assays, after the addition of substrate solution, the plates were sealed with a polyester film (SealPlate; EXCEL Scientific), covered with aluminum foil, and incubated for 1 h at 37 °C with continuous shaking on a miniorbital shaker. The reactions were terminated by the addition of 100 μL of 0.1 mol/L monochloroacetic acid-0.13 mol/L NaOH-0.1 mol/L acetic acid, pH 4.3. The plates were shaken and, if there were any bubbles in the wells, centrifuged without covers for 1 min at room temperature.

The fluorescence of each well was measured at ambient temperature. For both kinetic and endpoint assays, 8–12 wells were used for each determination, consisting of four different sample concentrations (undiluted and diluted two-, four-, and eightfold into the 0.15 mol/L NaCl-1 g/L Triton X-100 homogenization buffer) assayed in duplicate or triplicate. Data were corrected for background using a minimum of eight wells per plate that contained 0.15 mol/L NaCl-1 g/L Triton X-100 instead of sample. Specific activity was calculated using at least two different dilutions that fell within the linear range of the assay or by linear regression of the different dilutions. Product formation was converted from fluorescent units to nanomoles using 7-amino-4-methylcoumarin calibrators.

For analysis of recombinant CLN2 protein, CHO cells were transfected with the CLN2 cDNA (6) subcloned into the pSFFVneo expression vector (13) using standard molecular biology techniques. Cells were cultured in DMEM-F12 (Sigma) containing 100 mL/L fetal bovine serum. After selection with G418, drug-resistant colonies were picked, expanded, and plated into 3.5-cm diameter plates. After the cells formed confluent monolayers, the complete media were removed and replaced with 2 mL of serum-free media. After 48 h, the conditioned media were collected and centrifuged at 2000 g for 10 min. To activate the inactive recombinant CLN2 proenzyme secreted by the cells, 10-μL samples (undiluted or diluted two-, four-, or eightfold in phosphate-buffered saline) were mixed with 20 μL of 150 mmol/L NaCl-1 g/L Triton X-100-50 mmol/L formic acid/sodium formate, pH 3.5, and incubated for 30 min at 37 °C. Pepinase and TPP-I assays were then conducted as described above except that 20 μL of substrate solution containing twice the usual concentration of substrate was used to initiate the reactions.

We evaluated both kinetic and endpoint assays to measure TPP-I activity. Representative data for the kinetic analysis of TPP-I activity measured with different amounts of buffy coat leukocytes from a single subject are plotted in the inset in Fig. 1A. After a lag of ∼10 min while the samples reach thermal equilibrium, product formation was linear with respect to time for the protein concentrations typically used in the assay. After correcting for the slight decrease in fluorescence of the reagent blank, the linear portion of the curve was used to calculate a reaction rate for each well. The increase in activity was relatively linear with respect to protein content for both the kinetic and endpoint assays (Fig. 1A): for the kinetic assay, slope (mean ± SE) = 195 ± 2; y-intercept = 18 ± 4; r² = 0.998; for the endpoint assay, slope = 226 ± 5; y-intercept = 29 ± 8; r² = 0.995). Note that in both assays, the y-intercepts were not zero, suggesting that the reagent blanks underestimate the true background. Although this affected the specific activity calculation for specimens with low activity, the effect was relatively minor at the higher protein concentrations, with the two lowest dilutions giving specific activities that approach those calculated using linear regression. In general, the specific activities determined using the kinetic assay were 10–20% lower than those determined using the endpoint assay. This may be attributable to photobleaching during the kinetic assay, which could decrease detection of the fluorescent reaction product.

We then compared the pepinase and TPP-I activities of a variety of cellular or tissue homogenates specimens, including human fibroblasts (Fig. 1B), leukocytes, lymphoblasts, and brain (data not shown). In all cases, the two activities were tightly correlated (for fibroblasts, slope = 0.449 ± 0.014; y-intercept = −8.95 ± 11.92; r² = 0.979; n = 23; for leukocytes, slope = 0.441 ± 0.042; y-intercept = 21.66 ± 18.18; r² = 0.882; n = 17; for lymphoblasts, slope = 0.578 ± 0.014; y-intercept = 1.15 ± 1.04; r² = 0.996; n = 9; for brain, slope = 0.440 ± 0.15; y-intercept = 30.76 ± 16.38; r² = 0.984; n = 16). The two activities were also tightly correlated in conditioned media from different CHO cell lines that overexpress recombinant CLN2 protein (Fig. 1C; slope = 0.125 ± 0.004; y-intercept = 12.11 ± 16.40; r² = 0.990; n = 10). The slopes of the plots indicate that the ratio of pepinase to TPP-I activity is approximately threefold higher in the condi-
tioned media than in the cellular homogenates. This may be attributable to the presence of proteins in the cellular extracts that are more efficient substrates for the CLN2 protein compared with the FITC-hemoglobin. Regardless, the important conclusion is that the TPP-I and pepinase assays both measure the activity of the same protein.

The analytic sensitivity of the TPP-I assay is superior to that of the pepinase assay. In terms of mole-equivalents of liberated fluorophore, the activity measured in the TPP-I assay is >100-fold higher than that in the pepinase assay (note the differences in units on the x- and y-axes in Fig. 1). The total signal-to-noise (reagent blank) ratio of each assay depends on incubation time and amount of input sample. For the pepinase assay on buffy coat leukocyte preparations, a 4-h incubation using 25 μL of leukocyte homogenate (equivalent to 125 μL of whole blood) typically yielded a signal-to-noise ratio of 4 and a CV of 3% (within-run variability using triplicate determinations). In comparison, for the TPP-I endpoint assay, a 1-h incubation using 10 μL of leukocyte homogenate (equivalent to 50 μL of blood) typically yielded a signal-to-noise ratio of 6 and a CV of 2%.

In terms of clinical applicability, we previously validated the pepinase assay on specimens that were verified to have mutations in the CLN2 gene by DNA sequence analysis (2, 8). Using a threshold value of 15% of control for leukocytes and 5% of control for other specimens [see below and Ref. (8)], all 51 specimens analyzed (representing leukocytes, cultured cells, or brain autopsy specimens from 46 different LINCL families) could easily be discriminated from carriers and controls. Thus, the sensitivity of the assay appears to be 100% [true positives/(true positives + false negatives)]. Two additional NCL cases had no detectable pepinase activity, but no CLN2 mutations were found (2). Whereas these may represent false positives, it is quite possible that these are true positives and that the pepinase-deficient specimens harbor rare CLN2 mutations that have not yet been detected. There were 14 cases where pathophysiological findings suggested LINCL but the specimens had detectable pepinase activity and there were no mutations found. In addition, all 220 controls, other neurological disease specimens, and carriers tested had clearly detectable pepinase activity [Ref. (8) and unpublished observations]. Thus, the specificity of the assay was 99.2–100% [true negatives/(true negatives + false positives)] = 234 of 236 or 234 of 234, depending on whether the two cases with no detectable pepinase activity represent false or true positives]. Taken together, these results indicate that the pepinase assay is well suited for LINCL screening.

We have analyzed fewer cases using the TPP-I assay. However, all 10 cases with confirmed CLN2 mutations had negligible activity (<5% of control). All 80 samples from healthy controls, LINCL carriers, and patients with other neurological diseases had robust activity. In cases where both the TPP-I and pepinase assays were conducted in parallel, both assays could discriminate LINCL individuals from normals, but a lower threshold could be used when analyzing leukocytes with the TPP-I assay (Table 1). For example, analysis of the data in Table 1 reveals that the highest LINCL leukocyte specimen had 2% or 13% of the mean control activity when measured by either the TPP-I or pepinase assay, respectively. This can

Fig. 1. Characterization of the TPP-I assay and comparison with the pepinase assay.
(A), concentration dependence of the TPP-I kinetic and endpoint assays on buffy coat leukocytes. Each symbol represents background-corrected data for each replicate of triplicate determinations. (Inset), time dependence of the TPP-I kinetic assay on buffy coat leukocytes. (B), comparison of TPP-I and pepinase assays in fibroblasts. □, control; ◯, other neurological disease; ○, LINCL. (C), comparison of TPP-I and pepinase assay in conditioned media. ○, samples of different clones transfected with a CLN2 expression construct; □, untransfected controls.
be explained by our previous finding that leukocytes contain variable amounts of a neutral serine protease that has some residual pepinase activity at pH 3.5 [typically ~5% of the CLN2-derived activity, but sometimes more, depending on the granulocyte content of the specimens (8)]. Although there also exists a neutral tripeptidyl peptidase, experiments investigating the pH dependence of tripeptidyl peptidase activity in control and LINCL leukocytes, lymphoblasts, fibroblasts, and brain indicated that it did not contribute to the CLN2-derived activity at pH 4.0 (data not shown).

In conclusion, we find that the TPP-I and pepinase assays both measure function of the CLN2 gene product. Given that the pepinase assay is more laborious and has no obvious advantages over the TPP-I assay, the TPP-I assay appears more suitable for routine enzyme-based diagnosis of CLN2 deficiencies.

This work was supported by Grants DK45992 and NS37918 from the NIH. We thank families from the Batten Disease Support and Research Association and the NCL research alliance and their healthcare providers for participating in this study. Some specimens were obtained from the collections "Cell Lines and DNA Bank from Patients Affected by Genetic Disorders” (Instituto G. Gaslini, Genova, Italy), supported by TELETHON project C. 20; the Brain and Tissue Bank for Developmental Disorders, supported by the National Institute of Child Health and Development contract HD-8-3248; the McGill University Repository for Mutant Human Cell Strains (Toronto, Canada); and the Batten Disease Cell Bank at Indiana University School of Medicine.

### Table 1. Enzyme activity of leukocytes and fibroblasts.∗

<table>
<thead>
<tr>
<th></th>
<th>TPP-I, nmol · h⁻¹ · mg⁻¹</th>
<th>Pepinase, pmol · h⁻¹ · mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LINCL (n = 2)</td>
<td>1 ± 4 (&lt;0:4)</td>
<td>7 ± 71 (&lt;0:57)</td>
</tr>
<tr>
<td>Carrier (n = 6)</td>
<td>192 ± 52 (124:285)</td>
<td>407 ± 168 (280:733)</td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td>232 ± 86 (147:431)</td>
<td>452 ± 176 (290:794)</td>
</tr>
<tr>
<td><strong>Fibroblasts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LINCL (n = 3)</td>
<td>0.2 ± 0.5 (&lt;0:0.7)</td>
<td>11 ± 5 (7:17)</td>
</tr>
<tr>
<td>Other disease</td>
<td>362 ± 184 (188:883)</td>
<td>827 ± 357 (429:1747)</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>283 ± 244 (56:721)</td>
<td>649 ± 605 (88:1736)</td>
</tr>
</tbody>
</table>

*a Data are expressed as mean ± SD (minimum:maximum).

*b Note that the calculated activity of some LINCL specimens is below zero.

This occurs because in the absence of enzyme activity, the measured fluorescence fluctuates around background. The negative values have been included in the mean and SD calculations.

### References


11. Rawlings ND, Barrett AJ. Tripeptidyl-peptidase I is apparently the CLN2 protein absent in classical late-infantile neuronal ceroid lipofuscinosis fibroblasts are deficient in lysosomal tripeptidyl peptide I. Biochim Biophys Acta 1999;1429:496–500.
