Lysosomal degradation of cholecystokinin-(29–33)-amide in mouse brain is dependent on tripeptidyl peptidase-I: implications for the degradation and storage of peptides in classical late-infantile neuronal ceroid lipofuscinosis

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Tripeptidyl peptidase-I (TPP-I) is a lysosomal exopeptidase which removes tripeptides from the N-terminus of small peptides. Mutations in the TPP-I gene result in a lethal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis (CLN2). This disease is characterized by the accumulation of proteinaceous and autofluorescent material within the lysosomes of neurons, which undergo massive cell death during the course of the disease. The absence of TPP-I may result in the lysosomal accumulation of small peptides and proteins, which eventually compromises lysosomal functions critical to the survival of neurons. To investigate the metabolism of small peptides, we have studied the degradation of cholecystokinin-(29–33)-amide (GWMDF-NH₂; cholecystokinin C-terminal pentapeptide) by lysosomal fractions isolated from mouse brain and several other tissues. GWMDF-NH₂ is cleaved at only one peptide bond by brain lysosomes, to produce GWM and DF-NH₂. Inhibitor studies demonstrate that this reaction is catalysed by TPP-I. In contrast, lysosomal fractions from other mouse tissues additionally cleave a second peptide bond to produce GW and MDF-NH₂.

Inhibitor studies indicate that this reaction is catalysed by dipeptidyl peptidase-I (DPP-I; cathepsin C). Inhibitors of TPP-I are sufficient to completely block the degradation of GWMDF-NH₂ by brain, but inhibitors of both TPP-I and DPP-I are required to completely inhibit the degradation of GWMDF-NH₂ by other mouse tissues. Enzyme assays confirm the low activity of DPP-I in brain. An unrelated neuropeptide, neuromedin B, is degraded by a pathway that is partially dependent on TPP-I. These results indicate that TPP-I is required for the partial or complete digestion of certain neuropeptides by brain lysosomes. In the absence of TPP-I, neuropeptides or their degradation products will accumulate in brain lysosomes and may contribute to the pathogenesis of CLN2. Other tissues are spared because they express another peptidase, DPP-I, which has extensive activity on peptides and can compensate for the loss of TPP-I.

Key words: Batten disease, dipeptidyl peptidase-I, neuromedin B, neuropeptide.

INTRODUCTION

Mutations in the lysosomal enzyme tripeptidyl peptidase-I (TPP-I; EC 3.4.14.9) result in the inherited neurodegenerative disease classical late-infantile neuronal ceroid lipofuscinosis [CLN2, the late-infantile form of Batten disease; OMIM (Online Mendelian Inheritance In Man) 204500] [1,2]. TPP-I commonly acts as an exopeptidase that removes tripeptides from the N-terminus of peptides [3]. The identity of the natural substrates for TPP-I remains unclear, although the enzyme appears to act predominantly on peptides smaller than 5 kDa [4]. TPP-I has also been reported to cleave tripeptides from the N-terminus of mitochondrial ATP synthase subunit *c* (molecular mass 7500 kDa), initiating its further degradation by other lysosomal proteases. ATP synthase subunit *c* is observed to accumulate in several cell types in patients with CLN2 [5,6]. Although TPP-I has a ubiquitous distribution, the pathological effects of mutations in TPP-I are manifested only in neurons. Children with this disease are symptom-free until the age of about 3 years. They then suffer from progressive neurodegeneration with associated seizures, increasing spasticity, dementia and blindness. Death occurs before the age of 10 years [7]. Autopsy reveals a dramatic loss of neuronal cells, and to a lesser extent of other brain cell types. Brain cells that remain contain autofluorescent ceroid lipopigment and other proteinaceous material.

In addition to TPP-I, most cells contain a second TPP, TPP-II (EC 3.4.14.10). Both TPP-I and TPP-II belong to the subtilisin class of serine proteinases and show some similarities in the nature of the synthetic substrates that they hydrolyse [8]. TPP-II exists in both soluble (cytoplasmic) and membrane-bound forms. In rat brain membranes, approx. 30% of TPP-II activity occurs as a glycosylphosphatidylinositol-anchored enzyme, probably located at the cell surface [9]. This form of TPP-II is responsible for the degradation of the C-terminal octapeptide (CCK-8) of cholecystokinin (CCK) at neutral pH, and consequently plays a role in regulating the extracellular concentration of CCK [9]. CCK is a neuropeptide that has several biological effects, including the regulation of appetite and anxiety. Certain neuronal cells have CCK receptors (CCKR-A and CCKR-B). On binding CCK, the CCK-receptor complex is internalized into the endosomal/lysosomal system, where CCK is degraded and a proportion of the receptor is recycled to the cell surface [10].

The end-point for the lysosomal degradation of peptides is not entirely clear. Activities of lysosomal aminopeptidases and dipeptidases are low compared with corresponding activities in the cytoplasm and, based on the presence of dipeptide and tripeptide transporters in the lysosomal membrane, it has been suggested that dipeptides and tripeptides, in addition to amino acids, constitute an end-point to the lysosomal degradation of proteins/peptides [11,12]. Lysosomal enzymes which release

Abbreviations used: Boc, butoxycarbonyl; CCK, cholecystokinin; CCK-5, cholecystokinin C-terminal pentapeptide [cholecystokinin-(29–33)-amide; GWMDF-NH₂]; CLN2, classical late-infantile neuronal ceroid lipofuscinosis (ceroid lipofuscinosis, neuronal 2); DPP, dipeptidyl peptidase; E64, *trans*-epoxysuccinyl-L-leucylamide(4-guanidino)butane; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; -NHMeC, 7-(4-methyl)coumarinylamide; TFA, trifluoroacetic acid; TPP, tripeptidyl peptidase.

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Figure 1 Degradation of CCK-5 by a mouse brain lysosomal extract and by purified TPP-I

CCK-5 was incubated with a mouse brain lysosomal extract for (A) 1 h, (B) 2 h or (C) 4 h. In (D) CCK-5 was incubated with purified TPP-I for 2 h. The digest was chromatographed on a C18 reverse-phase HPLC column. Peak (1) is DF-NH₂, peak (2) is GWM and peak (3) is the undegraded CCK-5 (GWMDF-NH₂). OD, absorbance.

di- and tri-peptides might play an essential role in the terminal phase of peptide breakdown. An uncharacterized, acidic TPP has been implicated in the endosomal degradation of glucagon [13]. Given the specificity of TPP-II, a role for TPP-I in the degradation of internalized CCK, under the acidic conditions prevailing in the endosomal/lysosomal system, can be envisaged. We now explore the role of TPP-I in the degradation of the CCK C-terminal pentapeptide [CCK-5; cholecystokinin-(29–33)-amide] GWMDF-NH₂ (Gly-Trp-Met-Asp-Phe-NH₂) by a mouse brain lysosomal fraction at acid pH. GWMDF-NH₂, a substrate for TPP-II, is the shortest CCK-derived peptide to retain some biological activity.

EXPERIMENTAL

Preparation of a mouse brain lysosomal fraction

Mouse brains (or other tissues) were minced and resuspended in 0.25 M sucrose/10 mM Tris/HCl (pH 7.4)/5 mM EDTA and then homogenized using 25 strokes of a motor-driven Dounce homogenizer rotating at 720 rev./min. The homogenate was digested with DNase I (250 µg/ml for 30 min) and centrifuged for 10000 *g* · min to remove nuclei and intact cells. The supernatant was centrifuged for 30000 *g* · min to remove a high proportion of the mitochondria and plasma membrane vesicles. The supernatant was finally centrifuged for 300000 *g* · min to prepare an enriched lysosomal fraction [14]. The pellet was resuspended in the homogenization buffer, layered on top of

4 ml of 21.85% Percoll in the same buffer and centrifuged for 1125000 *g* · min in a Sorvall SV-80 vertical rotor. The band collecting at a density of 1.035–1.045 g/ml was collected and stored at –85 °C. The overall enrichment of lysosomes was estimated as 11-fold using TPP-I as a marker and 14-fold using β-D-galactosidase as a marker. Lysosomal fractions were sonicated for 30 s before use in digestion experiments.

Digestion of CCK peptides

Peptides (100 µg) were digested with purified lysosomal fractions for various time periods at 37 °C in the presence of 100 mM sodium acetate, pH 4.0, 20 mM NaCl and 5 mM EDTA. Lysosomal fractions were normalized for protein content and were used at a protein content of 5 µg/ml. Incubation of CCK-5 with purified dipeptidyl peptidase-I (DPP-I) (25 µ-units) or TPP-I (100 µ-units) was carried out for 15 min or 2 h in the standard digestion buffer. Inclusion of 4 mM cysteine did not alter the pattern of digestion products. Reactions were stopped by the addition of trifluoroacetic acid (TFA) to 0.1% and analysed by reverse-phase HPLC using a Dynamax C18-300 column (4.6 mm × 250 mm) and a solvent system of 0% acetonitrile/0.1% TFA for 10 min, a 0–37.5% acetonitrile/0.1% TFA gradient for 60 min and a 37.5–60% acetonitrile/0.1% TFA gradient for 15 min. The flow rate was 0.5 ml/min. The extent of peptide degradation was estimated by measuring the peak areas of CCK-5 and its digestion products. CCK peptides were purchased from Research Plus Inc. (South Plainfield, NJ, U.S.A.). Amino acid analysis of peaks collected from the HPLC column was carried

Figure 2 pH-activity profile for the degradation of CCK-5 and Ala-Ala-Phe-NHMec by a mouse brain lysosomal extract

The degradation of CCK-5 (Δ , $\square$, $\circ$) and Ala-Ala-Phe-NHMec ($\blacktriangle$, $\blacksquare$, $\bullet$) by a mouse brain lysosomal extract was determined by measuring the peak height of the major degradation product (GWM) or fluorimetrically (release of amino-methylcoumarin) respectively. The buffers used were sodium citrate (Δ , $\blacktriangle$), sodium acetate ($\square$, $\blacksquare$) and sodium phosphate ($\circ$, $\bullet$).

out as described previously [15]. In some experiments, the degradation of neuromedin B (50 μ g) by mouse brain lysosomes was investigated as described above.

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS

A Kratos Axima-CFR MALDI-TOF mass spectrometer was used to obtain data. Peptide samples were collected from the HPLC column, concentrated using a centrifugal evaporator and mixed with an equal volume of colloidal cobalt (10 mg/ml in methanol/ethylene glycol, 100:1, v/v). CCK-(4-8)-NH₂, CCK-(7-8)-NH₂ and CCK-(4-6)-OH were used as calibration standards. Spectra were averaged over 100 laser scans.

Enzymes and enzyme assays

Assays for TPP-I and β -D-galactosidase were performed as described previously [2,3]. Ala-Ala-Phe-NHMec [where -NHMec is 7-(4-methyl)coumarylamide] was used as a synthetic substrate for measuring TPP-I activity. TPP-I was purified from rat spleen as described previously [3]. DPP-I was assayed as described by Dolenc et al. [16], except that Gly-Phe-NHMec was used as substrate. Recombinant rat DPP-I was a gift from Dr C. Lauritzen (UNIZYME Laboratories, Horsholm, Denmark) [17]. DPP-II was assayed using Lys-Ala-NHMec as substrate [18]. All assays were performed in the absence and presence of specific inhibitors (Ala-Ala-Phe-CH₂Cl for TPP-I, Gly-Phe-CHN₂ for DPP-I and Lys-Ala-CH₂Cl for DPP-II). Final substrate concentrations were 100 μ M and inhibitor concentrations were 5 μ M. Sodium acetate buffers (100 mM) were used at pH 4.0 (TPP-I), pH 4.5 (DPP-I) or pH 5.0 (DPP-II). DPP-I assays additionally contained 20 mM NaCl and 4 mM cysteine. The pH optima were determined using a liver lysosomal fraction. Enzyme substrates and inhibitors were purchased from Sigma or Bachem. Gly-Phe-CHN₂ (Gly-Phe-diazomethyl ketone) and Lys-Ala-CH₂Cl were obtained from Enzyme System Products (Livermore, CA, U.S.A.). Protein was estimated using the Coomassie Plus Assay Reagent (Pierce).

Tissue culture

Two normal human skin fibroblast cell lines were obtained from The Montreal Children's Hospital (Montreal, Quebec, Canada): MCH65 (from a 3.5-year-old female) and MCH75 (from an 8-year-old male). The CLN2 fibroblasts used were WG0305 (from a 7-year-old female) and WG0308 (from a 5.5-year-old male) (Montreal Children's Hospital) and GM09404 (from a female; Coriell Cell Repository, Camden, NJ, U.S.A.). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cells were used 3-6 passages after receipt (passages 12-15). After washing twice with PBS, cells were harvested by scraping into PBS. The cells were collected by centrifugation (5000 g $\cdot$ min), resuspended in water and homogenized with a Polytron homogenizer. Immediately before use, the homogenates were sonicated for 30 s.

RESULTS

Degradation of CCK-5 by mouse brain lysosomes

Incubation of CCK-5 (GWMDNF-NH₂) with a purified mouse brain lysosomal fraction at pH 4.0 resulted in the degradation of CCK-5 and the appearance of two degradation products eluting from a C18 reverse-phase HPLC column at 17 and 45 min (Figures 1A-1C). These were identified as DF-NH₂ (Asp-Phe-NH₂) and GWM (Gly-Trp-Met) respectively by comparison with the elution positions of standards, MALDI-TOF MS and amino acid analysis. These were the only degradation products observed even after prolonged digestion (48 h) or after digestion in the presence of 4 mM cysteine. This pattern of degradation products was identical with that obtained when GWMDNF-NH₂ was digested with purified TPP-I (Figure 1D). Neither Boc-GWMDNF-NH₂ (where Boc- is butoxycarbonyl) nor GWMDNF-OH was hydrolysed by the lysosomal extract or by purified TPP-I (results not shown). The production of GWM was linear with time until about 50% of the substrate had been degraded (Figures 1A-1C). The GWM produced appeared to be stable and did not decrease on prolonged incubation (48 h), suggesting that GWM is resistant to degradation by brain lysosomal peptidases. A pH-activity profile for the degradation of CCK-5 by mouse brain lysosomes demonstrated maximum activity at pH 4 (Figure 2). The pH-activity profile for the degradation of CCK-5 was identical with that for the hydrolysis of Ala-Ala-Phe-NHMec, the synthetic substrate commonly used for assaying TPP-I activity.

Effects of inhibitors on the degradation of CCK-5 by mouse brain lysosomes

Incubation of CCK-5 with a mouse brain lysosomal fraction in the presence of Ala-Ala-Phe-CH₂Cl inhibited the degradation of CCK-5 by approx. 90% (Figures 3A and 3B). Ala-Ala-Phe-CH₂Cl appears to be a specific inhibitor of TPP-I at acidic pH [3,4,16]. The degradation of CCK-5 was not inhibited by PMSF, EDTA, E64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane] or pepstatin. Butabindide, a compound designed as a specific inhibitor of TPP-II [9], also inhibited CCK-5 degradation at pH 4.0 (Figure 3C). However, concentrations of butabindide above 100 μ M were required for inhibition, compared with 1 μ M for the inhibition of TPP-II [9]. The *K*_i for the inhibition of TPP-I by butabindide was 10 μ M, whereas that for the inhibition of TPP-II is reported to be 7 nM [9]. An excess of the non-degradable substrate analogue GWMDNF-OH also inhibited the degradation of CCK-5 (Figure 3D).

Figure 3 Effects of inhibitors on the degradation of CCK-5 by a mouse brain lysosomal extract

CCK-5 was incubated with a mouse brain lysosomal extract for 4 h in the absence of inhibitors (**A**) or in the presence of 5 μ M Ala-Ala-Phe-CH₂Cl (**B**), 50 μ M butabindide (**C**) or 250 μ g/ml GWMDf-OH (**D**), and the digest was chromatographed on a C18 reverse-phase HPLC column. Peak (1) is DF-NH₂, peak (2) is GWM, peak (3) is undegraded CCK-5 (GWMDF-NH₂) and peak (4) is GWMDF-OH. OD, absorbance.

Degradation of CCK-5 by lysosomal fractions from other tissues

CCK-5 was also incubated with purified lysosomal fractions obtained from other mouse tissues (gall bladder, pancreas, liver, kidney and heart). Two of these tissues (gall bladder and pancreas) are also responsive to CCK *in vivo*. In addition to the two degradation products observed with brain lysosomes, two additional degradation products were obtained when CCK-5 was incubated with lysosomal fractions isolated from all the other tissues. These two products eluted at 32 and 38 min and were identified as GW (Gly-Trp) and MDF-NH₂ (Met-Asp-Phe-NH₂) respectively (Figures 4A, 4C and 4D). Inclusion of Ala-Ala-Phe-CH₂Cl in the incubation mixture containing CCK-5 and gall bladder lysosomes abolished the production of GWM and DF-NH₂, but did not block the production of GW or MDF-NH₂ (Figure 4B). The production of GW and MDF-NH₂ was inhibited by E64, but not by PMSF, EDTA or pepstatin. This pattern of degradation products and its inhibition by a cysteine protease inhibitor suggests the activity of a DPP, probably DPP-I (EC 3.4.14.1). Incubation of CCK-5 with purified recombinant DPP-I produced two degradation products, which were identified as GW and MDF-NH₂ (Figure 5C). When CCK-5 was incubated with the gall bladder lysosome extract in the presence of Gly-Phe-CHN₂, an inhibitor of DPP-I [19], the production of GW and MDF-NH₂ was prevented, whereas the production of DF-NH₂ and GWM was unaffected (Figures 5A and 5B). When the incubation of CCK-5 with the gall bladder lysosomal extract was performed in the presence of Lys-Ala-CH₂Cl (an inhibitor of DPP-II) or PMSF, the production of the four major degradation products was not inhibited, but the amount of MDF-NH₂ was

substantially increased (Figure 5D). There was no evidence for the involvement of aminopeptidase or carboxypeptidase activity in the degradation of CCK-5 by any of the tissues examined.

The effects of the inhibition of TPP-I and DPP-I on the kinetics of CCK-5 degradation by lysosomal fractions from mouse brain, gall bladder, heart and pancreas were investigated further (Figure 6). Over 12 h, Ala-Ala-Phe-CH₂Cl reduced the degradation of CCK-5 by mouse brain lysosomes by about 97%. Over the same time period, Ala-Ala-Phe-CH₂Cl reduced the degradation of CCK-5 by heart, gall bladder and pancreas lysosomal fractions by approx. 63%, 42% and 26% respectively. Gly-Phe-CHN₂ did not inhibit the degradation of CCK-5 by a brain lysosomal fraction, but inhibited its degradation by heart, gall bladder and pancreas lysosomal fractions by approx. 31%, 58% and 64% respectively. A combination of Ala-Ala-Phe-CH₂Cl and Gly-Phe-CHN₂ inhibited CCK-5 degradation by at least 90% in all four tissues. Lys-Ala-CH₂Cl did not inhibit the degradation of CCK-5 by lysosomal fractions from any of the tissues.

Degradation of CCK-5 by normal and CLN2 fibroblasts

Homogenates prepared from normal human skin fibroblasts or from fibroblasts isolated from CLN2 patients were incubated with CCK-5. CCK-5 was readily hydrolysed to GW, MDF-NH₂, GWM and DF-NH₂ by normal fibroblasts, but was degraded to a lesser extent by CLN2 fibroblasts (Figures 7A and 7B). The TPP-I-catalysed reaction was clearly absent from CLN2 fibroblasts. Identical results were obtained with two normal and three

Figure 4 Degradation of CCK-5 by lysosomal fractions from mouse gall bladder, pancreas and heart

CCK-5 was incubated with lysosomal fractions purified from mouse gall bladder (**A**), gall bladder in the presence of Ala-Ala-Phe-CH₂Cl (**B**), pancreas (**C**) or heart (**D**) for 4 h and the digest was chromatographed on a C18 reverse-phase HPLC column. Peak (1) is DF-NH₂, peak (2) is GW, peak (3) is MDF-NH₂, peak (4) is GWM and peak (5) is undegraded CCK-5. OD, absorbance.

CLN2 fibroblast cell lines. Addition of Ala-Ala-Phe-CH₂Cl to the incubation mixture prevented the degradation of CCK-5 by normal fibroblasts to GWM and DF-NH₂ (results not shown). Although GWM and DF-NH₂ were the major degradation products in all fibroblast cell lines, the ratio of GW to GWM produced (i.e. DPP-I/TPP-I) varied by about 3-fold.

TPP-I, DPP-I and DPP-II activities in tissues

The specific activities of TPP-I, DPP-I and DPP-II in mouse tissues are shown in Table 1. TPP-I activities varied 9-fold, with pancreas having the lowest and kidney the highest specific activity. Brain, heart and gall bladder had similar activities. DPP-I activities varied 21-fold, with brain having the lowest and gall bladder the highest specific activity. Substantial DPP-II activity was found in all tissues except heart. Of the four tissues examined in detail for their degradation of CCK-5, the DPP-I/TPP-I ratio was in the order brain < heart < gall bladder < pancreas.

Degradation of neuromedin B by mouse brain lysosomes

The ability of a mouse brain lysosomal fraction to degrade a neuropeptide, neuromedin B (GNLWATGHFM-NH₂), unrelated to CCK was investigated (Figure 8). The digestion of neuromedin B into several degradation products was also sensitive to inhibition by Ala-Ala-Phe-CH₂Cl. Although inhibition was not complete, degradation of neuromedin B decreased from approx. 95% of the sample to 35% in the presence of Ala-Ala-Phe-CH₂Cl. The production of the major degradation product,

eluting at 49 min, was partially inhibited, whereas the production of several minor degradation products, eluting between 31 and 37 min, was completely inhibited.

DISCUSSION

One of the puzzling features of CLN2 is that, despite the ubiquitous distribution of TPP-I, the deleterious effects of mutations in the TPP-I gene are manifested only in neuronal cells, resulting in brain damage, whereas most other tissues are spared pathological damage [7]. Mutations in lysosomal peptidases and proteinases are comparatively rare. Apart from that in CLN2, the only presently known mutations to cause human disease occur in the DPP-I gene, resulting in the Papillon-Lefevre syndrome [20]; in the cathepsin A gene, resulting in galactosialidosis [21]; and in the cathepsin K gene, which causes pycnodystosis [22]. Mutations in cathepsin D cause a neurodegenerative disease in sheep [23]. Interestingly, cathepsin D-deficient mice, unlike cathepsin B- or cathepsin L-deficient animals, develop symptoms similar to those of the neuronal ceroid lipofuscinoses, including accumulation of ATP synthase subunit *c* [24,25]. The overlapping specificities of lysosomal proteases may preclude the development of pathological consequences by providing alternative degradative pathways that are able to compensate for the missing activity. The experiments that we describe in the present paper provide a partial explanation for the specific storage of peptides in brain tissue and evidence for the presence of an alternative degradative pathway in other tissues which may compensate for the loss of TPP-I activity.

Figure 5 Effects of inhibition of DPP-I and DPP-II on the degradation of CCK-5 by a lysosomal fraction from mouse gall bladder

CCK-5 was digested with a lysosomal fraction from mouse gall bladder for 5 h in the absence (A) or presence of (B) 5 μ M Gly-Phe-CHN₂ or (D) 5 μ M Lys-Ala-CH₂Cl. The degradation of CCK-5 by purified DPP-I is also shown (C). Digests were fractionated on a C18 reverse-phase HPLC column. Peak (1) is DF-NH₂, peak (2) is GW, peak (3) is MDF-NH₂, peak (4) is GWM and peak (5) is undegraded CCK-5. OD, absorbance.

Clearly, as proteins are degraded to small peptides, the number of peptide bonds susceptible to cleavage by any particular protease decreases. In the case of CCK-5, only one peptide bond is susceptible to cleavage by lysosomal peptidases in brain. Several experiments demonstrate that this reaction is catalysed by TPP-I. The reaction produces the N-terminal tripeptide (GWM), and this is prevented by a specific inhibitor of TPP-I, Ala-Ala-Phe-CH₂Cl. Purified TPP-I produces the same degradation products as brain lysosomes. TPP-I requires that the N-terminus of its substrate is not blocked [3]. Boc-CCK-5 is resistant to hydrolysis by brain lysosomes and purified TPP-I. TPP-I hydrolyses peptides with a blocked C-terminus more readily than the corresponding non-blocked peptide [26]. GWMDF-OH is not hydrolysed by brain lysosomes or purified TPP-I, and acts as an inhibitor of the hydrolysis of GWMDF-NH₂. The degradation of CCK-5 by brain lysosomes is also inhibited by butabindide, a specific inhibitor of TPP-II [9], although the K_i is 1000-fold higher for the inhibition of TPP-I than for that of TPP-II. At high concentrations (> 100 μ M), butabindide is reported to inhibit other serine proteases [9]. The pH optima for the hydrolysis of CCK-5 and Ala-Ala-Phe-NHMec, a synthetic substrate for TPP-I, by brain lysosomes are identical. Furthermore, normal human fibroblasts mostly degrade CCK-5 to GWM and DF-NH₂, whereas in fibroblasts from CLN2 patients, which lack TPP-I, the production of these degradation products is inhibited.

All the other mouse tissues examined (heart, gall bladder, pancreas, kidney and liver) contain a second pathway for the lysosomal degradation of CCK-5. This pathway involves the degradation of CCK-5 by DPP-I into GW and MDF-NH₂. Again, several experiments support this conclusion. Incubation of CCK-5 with purified recombinant DPP-I produces the same degradation products. Gly-Phe-CHN₂, a specific inhibitor of DPP-I, inhibits the production of GW and MDF-NH₂ in all tissues. DPP-I is a cysteine peptidase [27], and the production of the two reaction products is inhibited by E64, but not by inhibitors of other classes of peptidase. Lys-Ala-CH₂Cl, a specific inhibitor of DPP-II, does not block the production of GW and MDF-NH₂, but actually increases the amount of MDF-NH₂ detected. DPP-II is a serine peptidase [28], and an inhibitor (PMSF) of this class of peptidase also increases the amount of MDF-NH₂ detected. These results suggest that, in all tissues except brain, CCK-5 is also degraded by DPP-I to GW and MDF-NH₂, and that MDF-NH₂ is degraded further to MD and F-NH₂ by DPP-II. DPP-II has greatest activity towards tripeptides and activity decreases rapidly with increasing peptide length, whereas DPP-I acts on proteins and peptides of any length [29,30]. DPP-II is expressed in all tissues, including brain [18]. However, DPP-I is expressed moderately to abundantly in all mouse and human tissues except brain, where neither DPP-I protein nor mRNA transcripts could be detected [31,32]. The low expression of DPP-I in brain relative to the activity in other

Figure 6 Comparison of the extent of the inhibition of CCK-5 degradation in mouse tissues by inhibitors of TPP-I and DPP-I

CCK-5 was digested for 12 h with lysosomal fractions from mouse brain (A), heart (B), gall bladder (C) and pancreas (D) in the absence or presence of 5 μ M Ala-Ala-Phe-CH₂Cl (AAF-CMK), 5 μ M Gly-Phe-CHN₂ (GF-DMK) or a combination of the two inhibitors. The amount of lysosomal fraction was adjusted to give 50% degradation of CCK-5. Digests were fractionated on a C18 reverse-phase HPLC column and the peak area of the undegraded CCK-5 determined.

Figure 7 Degradation of CCK-5 by normal fibroblasts and fibroblasts derived from CLN2 patients

CCK-5 was digested by homogenates prepared from normal human skin fibroblasts (A) or CLN2 fibroblasts (B). Digests were fractionated on a C18 reverse-phase HPLC column. Peak (1) is DF-NH₂, peak (2) is GW, peak (3) is MDF-NH₂, peak (4) is GWM and peak (5) is undegraded CCK-5. OD, absorbance.

Table 1 Specific activities of TPP-I, DPP-I and DPP-II in mouse tissues

Mouse tissues were homogenized in water with a Polytron homogenizer. Triton X-100 was added to 0.1% (v/v) and the homogenate was centrifuged for 60 000 $g \cdot \min$. The supernatant was assayed for TPP-I, DPP-I and DPP-II activities as described in the Experimental section. Results are means $\pm$ S.E.M. ($n = 3$).

Tissue	$10^{-2} \times$ Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)			
	TPP-I	DPP-I	DPP-II	DPP-I / TPP-I
Brain	2.16 ± 0.17	0.31 ± 0.02	3.36 ± 0.02	0.14
Heart	2.40 ± 0.14	0.71 ± 0.03	0.66 ± 0.03	0.30
Gall bladder	2.01 ± 0.09	6.45 ± 0.21	16.51 ± 0.08	3.20
Pancreas	0.75 ± 0.08	3.53 ± 0.29	12.40 ± 0.65	4.71
Kidney	6.92 ± 0.30	4.19 ± 0.17	11.23 ± 0.33	0.61
Liver	5.38 ± 0.04	5.00 ± 0.02	10.53 ± 0.04	0.93

tissues was confirmed in the present enzyme assays. Several studies, using Western and Northern blotting techniques, have demonstrated the expression of TPP-I in the major tissues of both rodents and humans [33–35].

Experiments with specific peptidase inhibitors enable us to estimate the relative contributions of the two pathways to the degradation of CCK-5 in each tissue. In brain, DPP-I plays little role in the degradation of CCK-5, as the digestion is completely inhibited by a TPP-I inhibitor. Pancreas shows the greatest involvement of DPP-I in CCK-5 degradation, with Gly-Phe-CHN₂ inhibiting degradation by 64%. In support of these results, the ratio of DPP-I to TPP-I activity is lowest in brain and highest in pancreas, with a 30-fold difference between the two tissues. Other tissues show intermediate activity ratios. Use of both synthetic substrates and CCK-5 to measure the DPP-I/TPP-I ratio in the lysosomal fractions demonstrated that the tissues are ranked in the order brain < heart < gall bladder < pancreas, supporting the suggested role for a combination of the two activities in the degradation of CCK-5. However, the ratio of DPP-I to TPP-I in brain appears to be approx. 7-fold higher when using the synthetic substrates than when using CCK-5. Several factors might account for this, such as the relative K_m values of the two enzymes for CCK-5, or the presence of inhibitory or competing peptides in the lysosomal preparations. The ability of a combination of inhibitors of TPP-I and DPP-I to almost completely inhibit the lysosomal degradation of CCK-5 in all tissues emphasizes the important, and in this case exclusive, role that these two enzymes play in the lysosomal degradation of small peptides. The combination of the three peptidases produces amino acids, dipeptides and tripeptides as the final degradation products, confirming the necessity for a dipeptide/tripeptide transporter in the lysosomal membrane [12].

The degradation of neuromedin B, a neuropeptide unrelated to CCK, by mouse brain lysosomes was also dependent on TPP-I. The release of the N-terminal tripeptides (Gly-Asn-Leu) from neuromedin B by TPP-I has recently been demonstrated [33]. However, inhibition was incomplete, suggesting a role for other peptidases in the degradation of neuromedin B. As neuromedin B has nine peptide bonds and CCK-5 has only four, the involvement of other peptidases in neuromedin B degradation is more likely. The sequence of peptide bond cleavage during the degradation of neuromedin B is presently under investigation.

A feature of CLN2 that is common (but to a lesser extent) to other forms of neuronal ceroid lipofuscinoses is the intra-lysosomal storage of the highly hydrophobic mitochondrial ATP synthase subunit *c* [5,7]. Studies in sheep models suggest that this protein is the major component of the insoluble matter that

Figure 8 Degradation of neuromedin B by a mouse brain lysosomal extract

Neuromedin B was digested with a mouse brain lysosomal extract for 4 h in the absence (A) or presence (B) of Ala-Ala-Phe-CH₂Cl and the digest was chromatographed on a C18 reverse-phase HPLC column. Peak (1) is undigested neuromedin B. OD, absorbance.

accumulates within the lysosomes [36]. However, the pathological consequences of the storage of subunit *c* are unknown. Incubation of homogenates of fibroblasts from CLN2 patients, which have accumulated ATP synthase subunit *c*, with purified TPP-I results in the digestion of the N-terminal region of subunit *c*, followed rapidly by the degradation of the remainder of the protein [6]. This suggests that TPP-I initiates the degradation of subunit *c* by other proteases by removing the N-terminal region, although it does not rule out the possibility that TPP-I activates another protease which subsequently degrades subunit *c*. Interestingly, DPP-I is required for the activation of other proteases, especially those of cytotoxic lymphocytes, including granzymes A and B and mast cell chymase [37,38]. Indeed, many of the pathological effects of DPP-I deficiency are postulated to derive from loss of activation of other proteases, rather than from a general disturbance of protein breakdown [20]. The specific activity of DPP-I in some normal and CLN2 fibroblast cell lines is similar to the low activity found in brain (F. Bernardini and M. J. Warburton, unpublished work), but it is not known if DPP-I can substitute for TPP-I in the initiation of the degradation of subunit *c*. An examination of the amino acid sequence of human mitochondrial ATP synthase subunit *c* (accession number NP_005167) suggests that TPP-I should release a maximum of 12 tripeptides and DPP-I a maximum of 19 dipeptides from the N-terminus of subunit *c* before both reactions are stopped by proline-40, as neither enzyme hydrolyses peptide bonds involving a proline residue [26,30]. On this basis, DPP-I might be able to substitute for TPP-I in initiating the degradation of subunit *c*. The relative deficiency of DPP-I activity in brain may consequently be a contributory factor to the excessive accumulation of subunit *c* and other peptides in this tissue in patients with CLN2.

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