Classical late infantile neuronal ceroid lipofuscinosis fibroblasts are deficient in lysosomal tripeptidyl peptidase I

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Abstract Tripeptidyl peptidase I (TPP-I) is a lysosomal enzyme that cleaves tripeptides from the N-terminus of polypeptides. A comparison of TPP-I amino acid sequences with sequences derived from an EST database suggested that TPP-I is identical to a pepstatin-insensitive carboxyl proteinase of unknown specificity which is mutated in classical late infantile neuronal ceroid lipofuscinosis (LINCL), a lysosomal storage disease. Both TPP-I and the carboxyl proteinase have an M_r of about 46 kDa and are, or are predicted to be, resistant to inhibitors of the four major classes of proteinases. Fibroblasts from LINCL patients have less than 5% of the normal TPP-I activity. The activities of other lysosomal enzymes, including proteinases, are in the normal range. LINCL fibroblasts are also defective at degrading short polypeptides and this defect can be induced in normal fibroblasts by treatment with a specific inhibitor or TPP-I. These results suggest that the cell damage, especially neuronal, observed in LINCL results from the defective degradation and consequent lysosomal storage of small peptides.

Key words: Tripeptidyl peptidase I; Late infantile neuronal ceroid lipofuscinosis; Carboxyl proteinase; Peptide degradation

1. Introduction

Tripeptidyl peptidase I (TPP-I) is a lysosomal enzyme that removes tripeptides from the amino-terminus of peptides [1–4]. It is structurally and genetically unrelated to tripeptidyl peptidase II, a cytoplasmic enzyme with a similar action which is a member of the subtilisin class of serine proteases [5]. TPP-I is unusual in that it is not inhibited by any of the classical inhibitors of serine, cysteine, aspartate or metalloproteases [4]. TPP-I is potently inhibited by chloromethylketone analogues of tripeptidyl substrates but the kinetics of inhibition are not consistent with the enzyme being a serine or cysteine proteinase [4]. The enzyme has an M_r of 46 kDa and has a ubiquitous distribution in mammalian tissues. TPP-I acts on small peptides in vitro, e.g. angiotensins and glucagon, which are completely degraded to tripeptides, but appears to be inactive on proteins, e.g. casein [4]. There is also evidence for a tripeptidyl peptidase activity in hepatic endosomes which contributes to the degradation of glucagon [6]. TPP-I has previously been reported to degrade synthetic collagen-like polymers producing Gly-Pro-X triplets which can be further degraded by dipeptidyl peptidases and dipeptidase [2]. Inhibitors of TPP-I inhibit bone resorption (where type I collagen is the major protein being degraded) in an in vitro assay suggesting that TPP-I contributes to protein turnover in some physiological situations [3,4].

Recently, the N-terminal sequence and two internal sequences of rat spleen TPP-I have been reported [4]. Searching of these sequences against an expressed sequence tag (EST) database identified human and mouse ESTs which contained the homologues of all three rat sequences. Sequencing of these ESTs demonstrated that they contained full length clones and that the sequence of the human clone is identical to that of a recently identified lysosomal pepstatin-insensitive carboxyl proteinase [7]. This enzyme shows homology to a family of bacterial pepstatin-insensitive carboxyl proteinases [8,9] but it is the only known eukaryotic member of this family and its function is unknown.

Mutations in this pepstatin-insensitive carboxyl proteinase have been identified in classical late infantile neuronal ceroid lipofuscinosis, a lysosomal storage disease (LINCL, CLN2) [7]. The neuronal lipofuscinoses are a heterogeneous group of diseases associated with progressive neurodegeneration, blindness and musculo-skeletal defects in infants and young children [10]. In classical LINCL, death occurs around the age of 10 when there is a dramatic decrease in the number of neuronal cells. Other brain cell types are diminished in number and contain cytoplasmic (lysosomal) vacuoles filled with fluorescent lipopigment. Similar inclusions are observed in many different cell types and in most other tissues although cell viability is not as dramatically reduced [11]. We now show that fibroblasts which have been demonstrated to have mutations in the pepstatin-insensitive carboxyl proteinase gene are deficient in TPP-I activity.

2. Materials and methods

2.1. Sequencing of EST clones

Amino-terminal and internal sequences of rat spleen TPP-I [4] were searched against an EST database (dbEST). A single human EST clone (EST T7210) containing the human homologues of both internal sequences was identified. The sequence of this EST was used to search dbEST and clones overlapping both 5′ and 3′ ends were identified. Further iterative dbEST searching of these and subsequent overlapping EST clones allowed a 1500 bp contiguous sequence to be built up. This composite sequence was used to perform a BLAST search of

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Abbreviations: AAF-CMK, Ala-Ala-Phe-chloromethylketone; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EST, expressed sequence tag; LINCL, late infantile neuronal ceroid lipofuscinosis; NHMec, 7-(4-methyl)coumarylamide; PMSF, phenylmethylsulphonyl fluoride; TFA, trifluoroacetic acid; TPP-I, tripeptidyl peptidase I

The mouse nucleotide sequence reported in this paper has been submitted to the EBI Data Bank/GenBank with accession number AJ011912.

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a non-redundant nucleotide database. The largest human and mouse EST clones identified were AA287343 and AA119072 respectively and these were sequenced in their entirety using −28M13 rev2 ET and oligo-dT(20). Internal sequencing primers were designed using the Human Genome Mapping Project (HGMP) Staden sequence processing package primer design routine, from sequences obtained. ESTs were obtained from HGMP, Hinxton, Cambridge, UK. Sequencing was performed with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit using an automated sequencer (PE Applied Biosystems, model 377 XL). Alignment of sequences was performed using PILEUP and calculations of homologies using GAP (EGCG package at HGMP, Hinxton, Cambridge).

2.2. Cell culture

Two normal human skin fibroblast cell lines (H1 and H2, males aged 5 and 18 years) were obtained from The Heart Science Centre, Harefield Hospital and two from The Montreal Children's Hospital (MCH65 and MCH75, female 3.5 years and male 8 years). The LINCL fibroblasts used were WG0305 and WG0308 (female 7 years and male 5.5 years, Montreal Children's Hospital) and GM09404 (female, Coriell Cell Repository, Camden, NJ). Batten and Tay-Sachs disease fibroblasts (WG0277 and WG0312, male 5.5 years and female 5 years respectively) were also obtained from The Repository for Mutant Human Cell Strains, The Montreal Children's Hospital, Montreal, Quebec, Canada. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum. Cells were used for enzyme assays three passages after receipt (passages 12–15).

2.3. Enzyme assays

Cells were washed with phosphate buffered saline, harvested by scraping and homogenised in 50 mM sodium acetate pH 4.0/0.1% Triton X-100 in a Dounce homogeniser. Fluorimetric assays for TPP-I, cathepsins B and L, aminopeptidase and β-galactosidase, using Ala-Ala-Phe-NHMec, CBZ-Phe-Arg-NHMec, Phe-NHMec and methylumbelliferyl-β-D-galactopyranoside as substrates, were carried out as described previously [3,12,13]. Proteinase assays were performed using FITC-casein or FITC-gelatin as substrates [14]. The fluorescamine procedure was used to determine protein concentrations [15]. A Hitachi F-2000 fluorimeter was used for fluorescence measurements.

To investigate the degradation of peptides by normal and LINCL fibroblasts, cells were homogenised in 100 mM sodium acetate pH 4.0, frozen and thawed three times and treated with protease inhibitors (1 μM E-64/1 mM PMSF/5 μg/ml pepstatin and 2.5 mM EDTA or additionally with 1 μM AAF-CMK) and incubated with 500 μg of peptide for 16 h. The reaction was stopped by adding trifluoroacetic acid (TFA) to 0.1% and analysed by reversed phase HPLC using a Dynamax C18-300 column (4.6×250 mm) and a 0–40% acetonitrile/0.1% TFA gradient over 60 min. Peaks were collected and analysed by amino acid analysis [16].

3. Results and discussion

Four lines of evidence suggest that TPP-I is the enzyme which is mutated in classical LINCL. Firstly, the partial se-

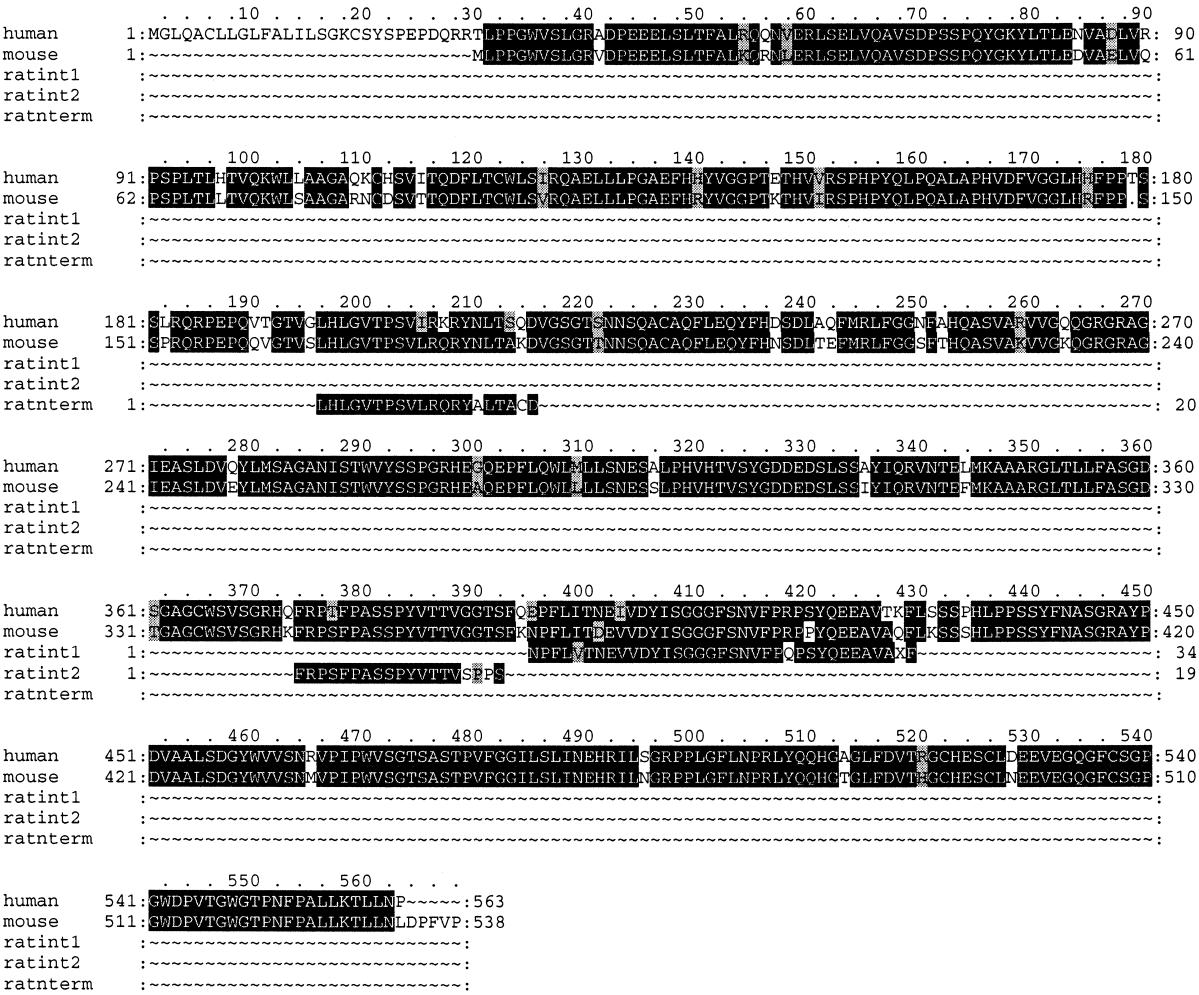

Fig. 1. Alignment of human and mouse TPP-I deduced amino acid sequences and the N-terminal (ratnterm) and two internal (ratint1 and ratint2) sequences of rat TPP-I obtained by direct sequencing. Heavy shading denotes identical amino acids and light shading similar amino acids.

quences of rat TPP-I are highly homologous to the deduced sequence of the protein mutated in LINCL. Secondly, fibroblasts from LINCL patients are deficient in TPP-I activity. Thirdly, there is specific loss of acidic TPP activity whereas alkaline TPP activity remains normal. Fourthly, LINCL fibroblasts are defective in their ability to degrade small peptides and this deficiency can be induced in normal cells by a specific inhibitor of TPP-I.

3.1. Comparison of TPP-I and pepstatin-insensitive carboxyl proteinase sequences

The amino acid sequences of the N-terminus and two internal peptides of rat spleen TPP-I were searched against dbEST and a single EST clone containing potential human homologues of both internal sequences was obtained. The sequence of this EST was used to search dbEST and several clones overlapping both 5′ and 3′ ends were obtained and partially sequenced. The largest of these (AA287343, 2.7 kb) was sequenced in its entirety and found to contain the full coding sequence for a putative pepstatin-insensitive carboxyl proteinase which is mutated in the lysosomal storage disease, LINCL [7]. Similar searches identified a mouse EST (AA119072) which contained the full coding sequence of a mouse homologue (Fig. 1). Alignment of the deduced amino acid sequences indicated that they were 89% identical and 91% similar. The overall identity between the rat and human amino acid sequences was 83% and between the rat and mouse sequences, the identity was 89%. The mouse EST contained an open reading frame of 1617 nucleotides with an ATG translation initiation codon 84 nucleotides 3′ of the human initiation codon. This would result in the loss of 29 amino acids at the N-terminus. The nucleotide sequence indicates an insertion of two nucleotides into a putative ATG start codon located in the same position as the start of the human open reading frame to give ATGTG. Correcting for this insertion and the resulting frame shift would give a sequence encoding a full length amino acid sequence homologous to that of the human sequence. In two of the LINCL cell lines used in this study, a single base substitution converts Cys^{365} to either Tyr (WG0305) or Arg (WG0308) [7]. The sequence and structural organisation of the gene (CLN2) have now been determined [17].

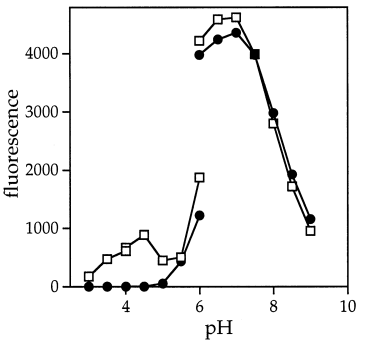

Fig. 2. pH optima of TPP activity in normal and LINCL fibroblasts. Homogenates of normal (MCH65) (□) and LINCL (WG0308) (●) fibroblasts were incubated with Ala-Ala-Phe-NHMec over the pH range 3–9 and the liberated aminomethylcoumarin measured fluorimetrically. The buffers used were 0.1 M sodium citrate (pH 3–4), 0.1 M sodium acetate (pH 4–6) and 0.1 M sodium phosphate (pH 6–9) containing 0.5 M NaCl.

Both TPP-I and the protein that is mutated in LINCL have an M_r of 46 kDa [4]. Homology searching revealed that the LINCL protein is related to a family of bacterial pepstatin-insensitive carboxyl proteinases [7–9]. These enzymes differ from other carboxyl (aspartate) proteinases in having an aspartate and a glutamate rather than two aspartates in the active site. The LINCL protease is the only known eukaryotic member of this family of proteolytic enzymes. The inability of serine, cysteine, aspartate and metalloproteinase inhibitors to inhibit TPP-I is now easily explained.

Table 1
Enzyme activities in normal and mutant fibroblasts

Cell line	Description	Specific activity (units/mg protein)$\times 10^{-2}$						Relative specific activity
		TPP-I	AAF-CMK-sensitive TPP-I	protease inhibitor-resistant TPP-I	amino-peptidase	cathepsin B/L	β-galactosidase	pepstatin-insensitive acid proteinase
H1	normal	1.34 ± 0.08 (103)	1.11 ± 0.04 (105)	1.36 ± 0.12 (101)	0.27 ± 0.04 (113)	18.4 ± 1.2 (106)	3.17 ± 0.27 (126)	92 ± 8
H2	normal	1.42 ± 0.05 (109)	1.17 ± 0.09 (110)	1.40 ± 0.10 (104)	0.23 ± 0.04 (96)	14.7 ± 0.8 (85)	3.33 ± 0.22 (133)	97 ± 12
MCH 65	normal	1.30 ± 0.07 (100)	1.06 ± 0.02 (100)	1.34 ± 0.07 (100)	0.24 ± 0.02 (100)	17.3 ± 1.1 (100)	2.51 ± 0.18 (100)	100 ± 9
MCH 75	normal	1.28 ± 0.03 (98)	1.01 ± 0.05 (95)	1.41 ± 0.10 (105)	0.27 ± 0.05 (113)	20.1 ± 1.3 (116)	2.87 ± 0.19 (114)	145 ± 14
WG 0277	Batten disease	1.56 ± 0.07 (120)	1.35 ± 0.09 (128)	1.58 ± 0.13 (118)	0.20 ± 0.02 (83)	15.4 ± 0.6 (89)	3.02 ± 0.22 (120)	127 ± 11
WG 0312	Tay-Sachs	1.36 ± 0.06 (105)	1.20 ± 0.11 (113)	1.39 ± 0.12 (104)	0.16 ± 0.02 (67)	20.1 ± 0.9 (116)	3.42 ± 0.20 (136)	122 ± 13
WG 0305	LINCL	0.07 ± 0.01 (5)	0.06 ± 0.01 (6)	0.06 ± 0.01 (5)	0.30 ± 0.04 (125)	19.5 ± 1.1 (113)	3.79 ± 0.14 (151)	110 ± 10
WG 0308	LINCL	0.03 ± 0.01 (2)	0.03 ± 0.01 (3)	0.03 ± 0.01 (2)	0.27 ± 0.04 (113)	18.2 ± 1.3 (105)	3.42 ± 0.17 (136)	120 ± 11
GM 0904	LINCL	0.04 ± 0.01 (3)	0.04 ± 0.01 (4)	0.04 ± 0.01 (3)	0.30 ± 0.03 (125)	18.8 ± 0.7 (109)	3.28 ± 0.16 (131)	86 ± 9

Results are the means ± S.E.M. of five determinations (TPP-I assays) or three determinations (other activities). Numbers in parentheses are the values normalised to MCH = 100. Casein was used as the substrate for the acid proteinase assays. The relative specific activity for the acid proteinase measurements was obtained by dividing the fluorimeter reading by the protein content and normalising the readings to MCH 65 = 100.

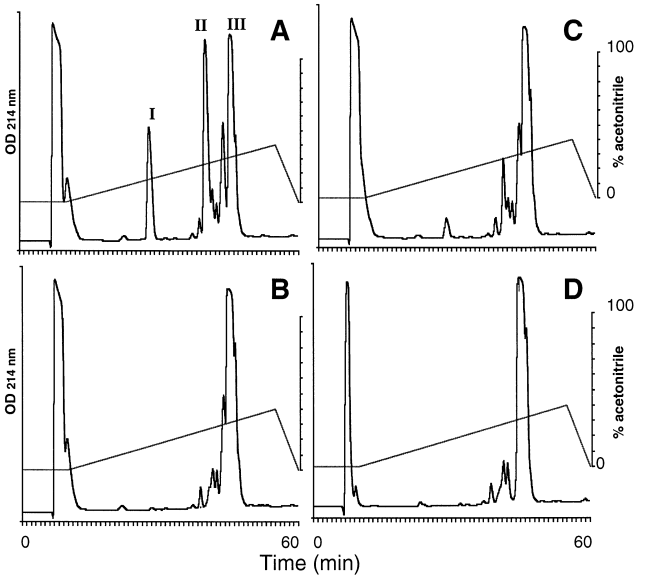

Fig. 3. Degradation of telopeptide-2 by normal and LINCL fibroblasts. Homogenates of normal (MCH65) and LINCL (WG0308) fibroblasts were incubated with 500 μg of telopeptide-2 for 16 h at 37°C at pH 4 (100 mM sodium acetate) after the addition of 1 mM PMSF, 1 μM E-64, 5 μg/ml pepstatin and 2.5 mM EDTA. Reactions were stopped by adding TFA to 0.1% and analysed by reversed phase HPLC using a Dynamax C18-300 column (4.6×250 mm) and a 0–40% acetonitrile/0.1% TFA gradient over 60 min. Peaks were collected and analysed by amino acid analysis. A: MCH65 digest, peak I is DEKGVGLGPG-NH_2, peak II is AQY-DEKGGLGPG-NH_2 and peak III NFAAQYDEKGVGLGPG-NH_2 (undigested telopeptide-2). B: WG0308 digest, note the absence of peaks I and II. C: MCH65 digest treated with AAF-CMK prior to the digestion, peaks I and II are greatly reduced. D: Telopeptide-2 added to the MCH65 reaction mixture at the end of the 16 h digestion.

3.2. Lysosomal enzyme activities in LINCL fibroblasts

Homogenates prepared from four normal and three LINCL fibroblast cell lines were assayed for several lysosomal enzyme activities (Table 1). As further controls, homogenates prepared from Tay-Sachs disease (an unrelated lysosomal storage disease) and Batten disease (juvenile neuronal ceroid lipofuscinosis) fibroblasts were also assayed. There is a dramatic reduction in TPP-I activity in the three LINCL fibroblast cell lines. Activities ranged from about 2 to 6% of those of normal, Tay-Sachs and Batten disease fibroblasts. A similar result was obtained when the assays were carried out in the presence of protease inhibitors. A more precise determination of TPP-I activity, i.e. TPP-I activity sensitive to inhibition by AAF-CMK, gave an identical result. The TPP-I substrate (Ala-Ala-Phe-NHMec) may also be a substrate for aminopeptidases. However, aminopeptidase activity was similar in all the cell lines. Two other lysosomal enzyme activities in LINCL fibroblasts, cathepsin B/L and β-galactosidase, were also similar to those of normal cells. Homogenates of brain tissue from LINCL patients have been reported to be deficient in an acidic pepstatin-insensitive proteinase active on haemoglobin [7]. Using casein as a substrate, we were unable to detect any differences in pepstatin-insensitive protease activity between normal and LINCL fibroblasts. No activity against gelatin was observed in normal or LINCL fibroblasts. This is in agreement with our previous results which suggest that TPP-I has little activity on proteins although the size limit of its activity is unknown [4]. However, peptidases are able to synergise with proteinases in the degradation of proteins [18] and the extent of this synergy may be tissue dependent. If TPP-I acts synergistically with other proteolytic enzymes, the greatest effect might occur in brain as most of the tissue damage in LINCL patients is observed in this tissue.

Patients with Batten disease have similar symptoms to those with LINCL but with a later onset. The nature of the material stored within the lysosomes is also similar in the two diseases. The function of the gene involved in Batten disease is unknown [19]. TPP-I activities are normal in Batten disease fibroblasts and it therefore seems unlikely that the product of the Batten disease gene is involved in the transport or processing of TPP-I.

3.3. pH optima of TPP-I activity in normal and LINCL fibroblasts

There were two distinct pH optima for TPP activity in normal fibroblasts at pHs 4.5 and 7 (Fig. 2). The activity at pH 7 was about five times the activity at pH 4.5. The acidic TPP activity was totally absent from LINCL fibroblasts whereas the activity at pH 7 was similar to that in normal cells. The acidic TPP activity was resistant to inhibition by inhibitors of serine (PMSF), cysteine (E-64), aspartate (pepstatin) and metalloproteinases (EDTA) but was inhibited by AAF-CMK suggesting that this activity is TPP-I. The TPP activity at pH 7 was partially inhibited by all the inhibitors suggesting that it results from the action of several peptidases. This experiment provides further evidence for the specific loss of an acidic TPP activity in LINCL fibroblasts.

3.4. Degradation of peptides by normal and LINCL fibroblasts

TPP-I is capable of degrading short peptides to tripeptides [4]. To investigate if the degradation of peptides is impaired in LINCL cells, homogenates from normal and LINCL fibroblasts were incubated with a model, test peptide (NFAAQY-DEKGVGLGPGN-H_2, the N-terminal telopeptide of the α2 chain of type I collagen), the reaction products separated by reversed phase HPLC and characterised by amino acid analysis (Fig. 3). Two major reaction products were observed when the telopeptide was incubated with a normal cell homogenate (Fig. 3A). The peptide eluting after 40 min was identified as AQYDEKGVGLGPG-NH_2, the telopeptide with the three N-terminal amino acids removed, and the peptide eluting after 28 min as DEKGVGLGPG-NH_2, the telopeptide with the six N-terminal amino acids removed. It is likely that the telopeptide has been subjected to the sequential removal of N-terminal tripeptides. Neither of the two degradation products was observed when the telopeptide was incubated with extracts or LINCL fibroblasts (Fig. 3B). These incubations were carried out in the presence of PMSF, E-64, EDTA and pepstatin. When the normal fibroblast extract was additionally treated with AAF-CMK, a specific inhibitor of TPP-I in these cells, before incubation with the telopeptide, the appearance of both degradation products was greatly reduced (Fig. 3C). Thus, inhibition of TPP-I in normal fibroblasts and mutations in the LINCL gene produce a similar defect in the degradation of short peptides. This result is consistent with TPP-I being the enzyme which is mutated in LINCL.

About 60% of the material stored within lysosomes in LINCL is believed to be protein. An unusual feature of the

storage material in LINCL is that a major component is subunit c of mitochondrial ATP synthase [20,21]. Subunit c is a small (M_r 7600 kDa), very hydrophobic protein. Its accumulation in LINCL cells has been clearly shown to result from a reduced rate of degradation rather than increased synthesis and normal fibroblasts contain a proteolytic activity which can degrade the subunit c stored within the lysosomes of LINCL fibroblasts [22]. It will be interesting to determine if TPP-I has any activity on subunit c. However, subunit c accumulation is also observed in Batten disease and certain unrelated lysosomal storage diseases suggesting that it may be a non-specific response to the lysosomal storage of other material [23]. Our results support the suggestion of Sleat et al. [7] that LINCL results from defects in a proteolytic enzyme involved in the degradation of peptides especially those acting on neurones. Recently, TPP-II has been identified as the enzyme which inactivates the neuropeptide cholecystokinin possibly regulating signalling pathways associated with this peptide [24]. Elucidating the role of TPP-I in the metabolism of neurone-specific peptides might enhance our understanding of the disease process in LINCL.

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