A replacement of the active-site aspartic acid residue 293 in mouse cathepsin D affects its intracellular stability, processing and transport in HEK-293 cells

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The substitution of an active-site aspartic acid residue by asparagine in the lysosomal protease cathepsin D (CTSD) results in a loss of enzyme activity and severe cerebrocortical atrophy in a novel form of neuronal ceroid lipofuscinosis in sheep [Tyynelä, Sohar, Sleat, Gin, Donnelly, Baumann, Haltia and Lobel (2000) *EMBO J.* **19**, 2786–2792]. In the present study we have introduced the corresponding mutation by replacing aspartic acid residue 293 with asparagine (D293N) into the mouse CTSD cDNA to analyse its effect on synthesis, transport and stability in transfected HEK-293 cells. The complete inactivation of mutant D293N mouse CTSD was confirmed by a newly developed fluorimetric quantification system. Moreover, in the heterologous overexpression systems used, mutant D293N mouse

CTSD was apparently unstable and proteolytically modified during early steps of the secretory pathway, resulting in a loss of mass by about 1 kDa. In the affected sheep, the endogenous mutant enzyme was stable but also showed the shift in its molecular mass. In HEK-293 cells, the transport of the mutant D293N mouse CTSD to the lysosome was delayed and associated with a low secretion rate compared with wild-type CTSD. These data suggest that the mutation may result in a conformational change which affects stability, processing and transport of the enzyme.

Key words: fluorimetric substrate, lysosomal storage, neuronal ceroid lipofuscinosis, overexpression.

INTRODUCTION

Cathepsin D (CTSD; EC 3.4.23.5) is a lysosomal enzyme belonging to the pepsin family of aspartic proteases. Removal of the signal sequence in the human CTSD generates an inactive proCTSD with a molecular mass of 53 kDa [1,2]. During transport to the lysosome the enzyme is proteolytically processed. The cleavage of the N-terminal propeptide results in a single-chain active enzyme form of 43 kDa. In most species, this single-chain form undergoes further proteolytic processing yielding the mature form, composed of the light (14 kDa) and heavy (31 kDa) chains held together by disulphide bonds. These internal proteolytic cleavages are poorly understood but cysteine proteases appear to be involved in the processing [3].

CTSD is a glycoprotein with two N-linked oligosaccharides modified with 1–2 mannose 6-phosphate (M6P) residues that are required for binding to M6P-specific receptors in the Golgi apparatus [4]. Varying amounts of CTSD fail to bind to the receptors and become secreted [5–7]. Depending on the cell type, CTSD, as well as other lysosomal enzymes, may also be targeted to the lysosome in an M6P-independent manner [8–12].

CTSD has a bi-lobed structure, with an active site between two β -barrel domains [13,14]. Both of the domains contain one catalytically essential aspartic acid residue [Asp-97 and Asp-293 in mouse CTSD (mCTSD)], holding a water molecule which is involved in the nucleophilic attack during substrate hydrolysis [15]. During synthesis and maturation, the N-terminal propeptide is thought to be bent into the active-site cleft to assist

folding and prevent premature degradation [16,17]. CTSD has an acidic pH optimum, varying from pH 3.5 to 5.0 depending on assay conditions and the substrate used, and its activity is inhibited by pepstatin A [18]. CTSD has been reported to be involved in a number of biologically important processes related to cell proliferation and apoptosis [19–27].

There are two animal models described with a loss of CTSD activity. Disruption of the CTSD gene in mice [28] causes ileal atrophy and loss of lymphoid cells leading to death in the fourth week of life. Neuropathologically, the affected mice show neuronal storage with typical features of neuronal ceroid lipofuscinoses (NCL) [29], a group of diseases which comprise the most common reason for progressive encephalopathies of children [30]. A naturally existing Asp²⁹⁵ → Asn (D295N) mutation in the active site of CTSD causes a recessive form of NCL in newborn sheep [31]. The sheep are severely affected at birth and show an extreme brain atrophy of the cerebral cortex accompanied by marked accumulation of storage material in neurons, but no abnormalities of systemic tissues. Recently Glondou et al. [32] have shown that the corresponding mutation (D295N; with amino acid numbering of the translated pre-proCTSD) in human CTSD (hCTSD) abrogates its proteolytic activity but does not affect the non-enzymic properties of CTSD to stimulate proliferation of cells.

In the present study we have introduced the corresponding mutation found in the affected sheep into the mCTSD cDNA. The analysis of the overexpressed mutant D293N mCTSD in HEK-293 cells revealed that the mutation resulted not only in a

Abbreviations used: AMCA-Hb, 6-[(7-amino-4-methylcoumarin-3-acetyl)amino]hexoic acid coupled to haemoglobin; CHO, Chinese hamster ovary; CONCL, congenital ovine NCL; CTSD, cathepsin D; DMEM, Dulbecco's modified Eagle's medium; hCTSD, human CTSD; mCTSD, mouse CTSD; M6P, mannose 6-phosphate; NCL, neuronal ceroid lipofuscinosis; PDI, protein disulphide isomerase.

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complete inactivation of the enzyme but also affected the trafficking, processing and stability of mCTSD.

EXPERIMENTAL

Materials

[³⁵S]Methionine (> 1000 Ci/mmol) and Rainbow™ coloured protein molecular-mass markers were purchased from Amersham Biosciences (Freiburg, Germany). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, antibiotics (penicillin/streptomycin), Lipofectamine™ 2000 and *Taq* DNA polymerase came from Gibco Life Technologies (Karlsruhe, Germany). *Eco*RI and T4 DNA ligase came from New England Biolabs (Frankfurt, Germany). Protease inhibitor cocktail, M6P, cycloheximide and glycerol gelatin were from Sigma; Bradford reagent, Trans-Blot nitrocellulose membrane (0.2 µm) and pre-stained SDS/PAGE marker (Low Range) were obtained from Bio-Rad (Munich, Germany). Oligonucleotides were synthesized by MWG-Biotech (Munich, Germany) and pGEM-T Easy vector was purchased from Promega (Madison, WI, U.S.A.).

Antibodies

Polyclonal antibodies against CTSD purified from mouse liver and human placenta were raised in rabbits [33,34] and used for HEK-293 cell experiments. Commercial rabbit anti-hCTSD antiserum was obtained from Biodesign (Kennebunk, ME, U.S.A.) and used for Western blotting experiments of sheep fibroblasts in Finland. The monoclonal antibody H4 A3 developed by J. T. August against human (h) Lamp-1 was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, U.S.A., and the monoclonal antibody against protein disulphide isomerase (PDI) was purchased from Stress-Gen Biotechnologies Corp. (Victoria, Canada). Anti-mouse IgG Cy3, and anti-rabbit IgG-FITC conjugates were purchased from Sigma. Peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

DNA constructs

Total RNA purified from mouse liver was used to clone the mCTSD cDNA through reverse transcriptase PCR using oligo-d(T)₁₆ primer (Applied Biosystems, Foster City, CA, U.S.A.). PCR reactions were performed according to the manufacturer's protocol using *Taq* DNA polymerase with the primers (ctsd-for) 5'-CAG GGT GAG GAA CCA CAG AAC AGA ACA-3' and (ctsd-rev) 5'-GCT CTC TTC GGG CCG CCG CGA-3'. The PCR product was cloned into the pGEM-T Easy vector with T4 DNA ligase. Transformation of competent *Escherichia coli* DH5α cells was carried out by the heat-shock method. mCTSD cDNA was subcloned into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen, Groningen, The Netherlands) with *Eco*RI followed by transformation into DH5α cells, and positive colonies were confirmed by colony PCR with primers T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and ctsd-rev. Mutation of aspartic acid residue 293 to asparagine (D293N) was performed by PCR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.) and the primers (ctsd293-for) 5'-GAG GCT ATT GTG AAC ACA GGG ACA TC-3' and (ctsd293-rev) 5'-GAT GTC CCT GTG TTC ACA ATA GCC TC-3'. The nucleotide sequences of the plasmids were verified by sequencing.

Cell culture and transfections

HEK-293 cells were cultured in DMEM containing 10% fetal calf serum (PAA Laboratories, Linz, Austria) and 1% antibiotics (DMEM/FCS). Cells seeded on 35 mm plates at a density of 5×10^5 cells/well were transiently transfected after 24 h of cultivation using 4 µg of wild-type or mutant D293N mCTSD cDNA construct and 4 µl of Lipofectamine 2000 in 0.4 ml of Opti-MEM. For a control the cells were transfected with pcDNA3.1(+) vector alone. After 5 h of incubation at 37 °C transfection media were replaced with DMEM/FCS. Fibroblasts of the control and affected [congenital ovine NCL (CONCL)] sheep were cultivated in DMEM/FCS.

Western blotting analysis

Extracts obtained from vector-transfected, wild-type and mutant D293N mCTSD-expressing HEK-293 and Chinese hamster ovary (CHO) cells, as well as from control and CONCL sheep fibroblasts, were prepared in PBS/0.5% sodium deoxycholate/0.05% Triton X-100/protease inhibitors. After centrifugation, aliquots of the supernatants were separated by SDS/PAGE (12.5 or 15% gels), followed by electrotransfer for 90 min at 900 mA (Hofer Pharmacia Biotech, San Francisco, CA, U.S.A.) to nitrocellulose membrane. After blocking [5% milk powder in 10 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1% Tween (TBST)] the membrane was incubated with anti-mCTSD (1:300 in TBST) or anti-hCTSD (1:500 in TBST) antibody for 1 h. Immunoreactive bands were visualized after incubation with the goat anti-rabbit IgG coupled to horseradish peroxidase (1:3000) and ECL (Pierce, Rockford, IL, U.S.A.) and quantified by densitometric scanning (Hewlett-Packard Scan Jet 4c/T and the Advance Image Data Analyser program, Raytest, Straubenhardt, Germany).

Fluorimetric CTSD activity assay

CTSD activity was measured using 6-[(7-amino-4-methylcoumarin-3-acetyl)amino]hexoid acid coupled to haemoglobin (AMCA-Hb) as a substrate. For substrate preparation 5 mg of the reactive fluorophore AMCA succinimidylester, (AMCA-X; Molecular Probes Europe, Leiden, The Netherlands) was dissolved in 0.5 ml of DMSO and added gently to 10 ml of 0.15 M NaCl/0.075 M boric acid buffer (adjusted to pH 8.0 with NaOH) containing 50 mg of Hb. The mixture was incubated for 2 h at room temperature in the dark and extensively dialysed against 10 mM Tris/HCl (pH 7.3) at 4 °C with several buffer changes. Aliquots containing 10 mg/ml AMCA-Hb were stored at -20 °C.

Cell extracts and media (20 µl) were incubated with 20 µl of the substrate solution [0.1 µg/µl AMCA-Hb/0.4 µg/µl Hb/ acetic and formic acid (combined to a concentration of 25 mM and adjusted to pH 3.7 with NaOH)/50 µM leupeptin] for 5 h at 37 °C. The reaction was terminated by addition of 10 µl of 50% trichloroacetic acid. After incubation for 30 min on ice the samples were centrifuged and the supernatants were neutralized with an equal volume of 1 M Tris (pH 9.0). The fluorescence was measured in a fluorimeter vial using 455–465 nm emission and 340–380 nm excitation filters (VersaFluor; Bio-Rad).

Metabolic labellings and immunoprecipitation

After transfections (48 h) HEK-293 cells were incubated in methionine-free DMEM containing 0.1% BSA for 30 min followed by metabolic labelling with [³⁵S]methionine for the indicated time periods (20 min–17 h with 50–125 µCi/ml). After the pulse, the cells were either washed three times with ice-cold PBS

and harvested or chased for various time periods in DMEM/0.1% BSA/10 mM M6P to prevent the re-uptake of lysosomal enzymes. Immunoprecipitation of mCTSD was carried out as described previously [33] by means of Protein A–agarose followed by SDS/PAGE and fluorography.

Confocal immunofluorescence microscopy

After transfection (24 h) the cells were trypsinized and plated on to poly-L-lysine-coated 12 mm microscope glass coverslips. After a further 24 h of incubation the cells were fixed with 3% paraformaldehyde for 40 min at room temperature. Alternatively, 6 h prior to fixation, the cells were treated with 0.5 mM cycloheximide in the culture medium. After fixation, the cells were washed three times with PBS, incubated with 50 mM NH_4Cl for 10 min followed by two washes with PBS. The cells were permeabilized three times for 5 min with PBS/0.5% saponin/0.1% BSA (PSB). Cells were incubated with rabbit anti-mCTSD and mouse anti-hLamp-1 (1:100 dilution in PSB) or mouse anti-human-PDI (anti-hPDI; 2.5 $\mu\text{g}/\text{ml}$, diluted with PSB) at room temperature for 2 h. After three washes with PSB, the FITC-conjugated anti-rabbit and Cy3-conjugated anti-mouse IgG (1:200 dilution in PSB) were added, and incubated for 2 h in the dark. The coverslips were mounted on to object glasses with glycerol gelatin. The double fluorescence was viewed with a LSM 510 laser confocal microscope (Zeiss, Jena, Germany) set at excitation wavelengths 488 (FITC) and 552 nm (Cy3) and emission wavelengths 575 (FITC) and 570 nm (Cy3).

Other methods

Protein concentrations were determined using the Bradford protein assay with BSA as a standard. Immunoprecipitated mCTSD was treated with 10 m-units of peptide N-glycosidase F (Roche Molecular Biochemicals, Mannheim, Germany) for 20 h at 37 °C [35].

RESULTS

CTSD activity

To determine the activity of CTSD in many samples simultaneously and rapidly, a sensitive fluorimetric assay was developed using AMCA-Hb as a substrate. Initially the conditions were established with CTSD purified from human placenta [33]. The assay was adapted to HEK-293 cells transiently transfected with pcDNA3.1-CTSD, an expression vector containing the entire cDNA sequence of mCTSD. After transfection (48 h), cell extracts were assayed for CTSD activity and Western immunoreactivity. The optimum activity of CTSD was observed in cell extracts at pH 3.9 (Figure 1A), coinciding with that of the purified CTSD. The activity was measured in aliquots of cell extracts containing up to 50 μg of protein. In this range the enzyme activity increased in proportion to the protein concentration (results not shown) and the activity was 90–95% inhibited in the presence of 0.1 mM pepstatin A (Figure 1B). The expression of mCTSD was followed by Western blotting using a specific antibody. The blot revealed a protein band at 45 kDa which was not detectable in non-transfected or vector-transfected cells (Figure 1B, inset). In other experiments a doublet of 45 and 43 kDa was detected (Figure 1C, inset; Figure 2A). Equal loading of transfected cell samples was confirmed by examining parallel blots for endogenous CTSD background with antibodies against the hCTSD. In these blots, the intensities of the immunoreactive 33 and 31 kDa polypeptides, representing the mature forms of hCTSD [33], were similar in all cell extracts.

Figure 1 Expression and activity of mCTSD in overexpressing HEK-293 cells

(A) The pH dependency of CTSD activity was determined in extracts of mCTSD-overexpressing HEK-293 cells using AMCA-Hb as a substrate. The activity was expressed in arbitrary fluorescence units. The values are given as means $\pm$ S.D. from three different transfections carried out in triplicate. (B) CTSD activity in cell extracts of non-transfected (—), vector-transfected and wild-type mCTSD-expressing cells was measured in the presence (white bars) or absence (black bars) of 0.1 mM pepstatin A. (B, inset) Immunoblot of non-transfected, vector-transfected and wild-type mCTSD-expressing HEK-293 cells using an antibody (ab) against the mouse (m) and human (h) CTSD. The positions of molecular mass markers (in kDa) are indicated. (C) CTSD activity in cell extracts of wild-type and mutant D293N mCTSD-expressing HEK-293 cells was determined in relation to vector-transfected cells. The values are means $\pm$ S.D. from five independent transfection experiments carried out in triplicate. (C, inset) Western blot analyses of extracts from transfected cells were carried out using antibodies against the mouse and human CTSD.

Figure 2 Stability and processing of mCTSD

(A) After transfection (24 h) the HEK-293 cells were grown for a further 24 h in the presence or absence of pepstatin A and leupeptin (Peps/Leup.; 0.1 mM each). Cell extracts were prepared and the media collected. Aliquots of the cell extracts containing 20 µg of protein, and the media in the same proportion were run on SDS/PAGE gels (12.5% acrylamide) and analysed by mCTSD Western blotting. (B) Fibroblasts of the control and affected (CONCL) sheep were collected, and aliquots of the cell extracts containing 20 µg of protein were run on SDS/PAGE gels (15% acrylamide) and analysed by CTSD Western blotting.

Figure 3 Deglycosylation of wild-type and mutant D293N mCTSD

mCTSD was immunoprecipitated from wild-type- and mutant D293N-expressing HEK-293 cells labelled for 4 h with [³⁵S]methionine (0.1 mCi/ml of medium), and incubated in the presence or absence of 10 m-units of peptide N-glycosidase F (PNGase F). The samples were analysed by SDS/PAGE (12.5% acrylamide) and fluorography.

A missense mutation at the active site of CTSD resulted in inactivation of the enzyme and lead to severe neurodegeneration and early death in newborn sheep [31]. The corresponding mutation (D293N) was introduced into mCTSD cDNA by oligonucleotide-mediated mutagenesis. When the mutant D293N mCTSD was expressed in HEK-293 cells, the CTSD activity in cell extracts was similar to vector-transfected cells, whereas it was on average 8-fold increased in cell extracts transfected with wild-type mCTSD (Figure 1C). The CTSD content in cell extracts was determined by densitometric evaluation of Western blots, revealing about 3-fold more immunoreactive mCTSD in wild-type-transfected cells than in mutant-transfected cells (Figure 1C, inset).

Figure 4 Transport and secretion of wild-type and mutant D293N mCTSD

HEK-293 cells expressing the wild-type or mutant D293N mCTSD were labelled for 20 min with [³⁵S]methionine (0.1 mCi/ml of medium). Thereafter the cells were either harvested or chased for the indicated times in the presence of unlabelled methionine and 10 mM M6P. mCTSD was immunoprecipitated from the cell extracts and media. The samples were analysed by SDS/PAGE (12.5% acrylamide) and fluorography.

Stability and processing of the mutant D293N mCTSD

The steady-state concentration of mutant D293N mCTSD expressed in HEK-293 cells (Figure 2A) or in CHO cells (results not shown) was constantly lower than the wild-type mCTSD, presumably due to instability. In contrast, the endogenously expressed mutant enzyme was stable in sheep fibroblasts (Figure

Figure 5 Subcellular localization of the wild-type and mutant D293N mCTSD in transfected HEK-293 cells examined by confocal fluorescence microscopy

Upper panels: 42 h after transfection with the appropriate vectors the cells were grown for 6 h in the presence of 0.5 mM cycloheximide, and stained for mCTSD (green) and the lysosomal membrane marker hLamp-1 (red). Lower panels: alternatively, 48 h after transfection, the cells grown in the absence of cycloheximide were fixed and double-stained for mCTSD (green) and the endoplasmic reticulum marker hPDI (red). Yellow indicates co-localization of mCTSD with the marker. Differential interference contrast images are shown in upper left corner of each panel. Also, non-transfected cells are visible in the images. Scale bars, 10 μ m.

2B). Incubation of HEK-293 cells with a mixture of leupeptin and pepstatin A increased the stability of the wild-type and especially of the mutant D293N mCTSD, as shown by Western blotting (Figure 2A). Further, the mutant D293N mCTSD showed increased electrophoretic mobility compared with the wild-type enzyme (Figure 2A). The increased mobility of the mutant enzyme, corresponding to a shift of 1 kDa in the molecular mass, was observed not only in the overexpressed mutant D293N mCTSD but also in the fibroblasts of the affected sheep (Figure 2B).

To examine whether the differences in the electrophoretic mobility between the wild-type and mutant D293N mCTSD were due to altered glycosylation, CTSD immunoprecipitates from [35 S]methionine-labelled transfected HEK-293 cells were treated with peptide N-glycosidase F. After a 4 h pulse labelling, the mutant D293N mCTSD was represented predominantly by a

precursor 44 kDa form whereas the wild-type mCTSD was represented by 45 and 43 kDa precursor and processed polypeptides (Figure 3). After treatment with peptide N-glycosidase F, the apparent molecular masses of both wild-type and mutant D293N mCTSD were reduced by approx. 1.5–2 kDa. Thus the apparent mass of the 44 kDa mutant D293N mCTSD was reduced to approx. 42 kDa (Figure 3). The lack of a second mutant D293N mCTSD form corresponding to the 43 kDa wild-type mCTSD is presumably due to a decrease in stability (see below). The identity of an additional peptide N-glycosidase F-sensitive weak band at 48 kDa (wild-type mCTSD) and 47 kDa (mutant D293N mCTSD) is unclear. These findings demonstrate that both the mutant and wild-type labelled mCTSD forms are glycosylated, and that differences in glycosylation are not responsible for the higher electrophoretic mobility of the mutant D293N mCTSD.

Intracellular transport and secretion of the mutant D293N mCTSD

To examine whether the different steady-state concentrations of wild-type and mutant D293N mCTSD (seen in Figure 2A) were due to differences in the rates of synthesis or degradation, or in sorting efficacy, transfected HEK-293 cells were metabolically labelled with [³⁵S]methionine for 20 min and the radioactivity was chased up to 100 min. mCTSD was immunoprecipitated from cell extracts and media. To prevent the possible reuptake of secreted lysosomal enzymes, M6P was added to the chase medium. After a 20 min pulse, two bands of 45 and 43 kDa were precipitated from cells expressing the wild-type mCTSD. Also, in mutant D293N mCTSD expressing cells, two polypeptides with slightly increased mobilities of 44 and 42 kDa were precipitated (Figure 4). After a 20 min chase, both the wild-type and mutant enzymes were observed only in cell extracts. After a 40 min chase, about 70 % of the newly synthesized wild-type mCTSD was detected in the medium as a single band at 46 kDa, whereas no mutant enzyme was observed in the medium. After a longer chase (100 min) small amounts (approx. 10 % of the total synthesized) of the labelled 45 kDa mutant D293N mCTSD appeared in the medium. In cellular extracts of wild-type and mutant D293N mCTSD-expressing cells only the 45 and 44 kDa CTSD forms, respectively, were observed. The 42 kDa mutant D293N mCTSD precipitated after a 20 min labelling period disappeared within 100 min of chase, suggesting its rapid degradation. These results might also explain the absence of the 42 kDa mutant D293N mCTSD after a 4 h labelling period (Figure 3). Furthermore, the different intensities of newly synthesized wild-type and mutant D293N mCTSD precipitated from cell extracts after the 20 min labelling period, and for the mutant mCTSD in dependence of the chase time (Figure 4), are not related to unequal loading but rather to different rates of transport between the endoplasmic reticulum and the Golgi, and/or different folding kinetics. The latter affects the efficacy of the antibody binding (N. Muschol and T. Bräulke, unpublished work). These data indicate that early transport steps in the biosynthetic pathway and the sorting of the mutant D293N mCTSD are impaired.

Subcellular localization of the mutant D293N mCTSD

The intracellular distribution of mCTSD was studied in HEK-293 cells expressing the wild-type and mutant D293N mCTSD by confocal immunofluorescence microscopy. The cells were stained with mCTSD-specific antibodies and co-stained with markers of lysosomes (anti-hLamp-1) and endoplasmic reticulum (anti-hPDI). Then, 6 h after stopping protein synthesis with cycloheximide wild-type mCTSD was detected exclusively in lysosomes as shown by complete co-staining with hLamp-1 (Figure 5, upper left panel). As with the wild-type enzyme, the majority of the mutant D293N mCTSD was detected in lysosomes, although some staining in hLamp-1-negative organelles was also observed (Figure 5, upper right panel). In the absence of cycloheximide, wild-type mCTSD still co-localized with hLamp-1 (results not shown), and showed no overlap with PDI (Figure 5, lower left panel). By contrast, the majority of immunoreactive mutant D293N mCTSD was found in PDI-positive structures (Figure 5, lower right panel).

DISCUSSION

Initially, the D295N mutation in CTSD was observed in CONCL sheep, characterized by severe brain atrophy and neuronal death [31]. In the present study we have characterized the mutant

enzyme by introducing the corresponding mutation into mCTSD cDNA and overexpressing it in HEK-293 cells. The results presented here indicate that the substitution of the active-site aspartic acid residue 293 by asparagine in mCTSD affects the enzymic activity, stability, kinetics of transport, proteolytic processing and secretion of the enzyme.

The newly developed fluorimetric activity assay proved to be specific for CTSD because the hydrolysis of the AMCA-Hb substrate used was (i) more than 95 % inhibited by pepstatin A and (ii) not detectable in extracts from CTSD-deficient mouse fibroblasts (S. Partanen, unpublished work). Overexpression of the wild-type mCTSD resulted in increased CTSD activity compared with vector-transfected HEK-293 cells. Immunoblotting experiments using mCTSD-specific antibodies indicated the predominance of a 45 kDa form with a minor band of 43 kDa in the overexpressing HEK-293 cells. The proteolytically processed 30 kDa mature mCTSD, which was earlier shown to be present in small amounts in mouse splenocytes, hepatocytes or fibroblasts [11,36], was never observed in the overexpressing HEK-293 cells. The mutant D293N mCTSD was inactive in HEK-293 cells, as expected, thus being in agreement with the earlier observations on the cathepsin D-deficient sheep [31], and the mutant D295N human CTSD [32].

Loss of the enzymic activity was accompanied by changes in the stability, size, rate of transport and secretion of the mutant D293N mCTSD. Compared with the wild-type mCTSD, the mutant enzyme was constantly unstable in cells of both human (HEK-293) and hamster (CHO) origin. This was not the case in CONCL sheep, where the steady-state level of the endogenous mutant D295N CTSD was markedly higher than the amount of the wild-type enzyme in controls (Figure 2 and [31]). Furthermore, Glondou et al. [32] found that the mutation in the catalytic site does not impair the stability of mutant D295N hCTSD expressed in 3Y1-Ad12 rat cancer cells. These differences in the stability of the CTSD mutants may depend on the heterologous overexpression systems used (transient expression of mCTSD in HEK-293 cells compared with stable transfection of hCTSD in rat tumour cells), species-specific differences in the processing of CTSD or distinct cellular protease patterns in these systems (e.g. no endogenous CTSD activity in sheep cells; low expression level of endogenous CTSD in the rat cancer cell line [37]; high endogenous CTSD activity in HEK-293 cells). In the presence of pepstatin A and leupeptin, potent inhibitors of lysosomal aspartic and cysteine proteases, the amount of the mutant D293N mCTSD was increased, suggesting that the degradation may take place in lysosomes. Alternatively, the rapid degradation may occur in the endoplasmic reticulum, where the mutant seemed to be retained for longer than the wild-type enzyme, possibly due to improper folding. Thus the rapid preferential disappearance of the newly synthesized 42 kDa mutant D293N mCTSD form within 2 h (Figure 4) led to prominent expression of the 44 kDa mutant form, detectable after long-term labelling (Figure 3).

After short metabolic labelling periods clear alterations in the transport and secretion of the mutant D293N mCTSD were observed. While the rates of synthesis of the wild-type and mutant D293N mCTSD expressed in HEK-293 cells were roughly comparable, the kinetics of transport of the mutant D293N mCTSD appeared to be delayed during the first 2 h after synthesis (Figure 4). In addition, secretion of the mutant enzyme was slower and much less efficient (only approx. 10 % secreted) than that of the wild-type mCTSD. These findings are in a good agreement with the data of double immunofluorescence staining, which showed a clear retention of the mutant D293N mCTSD in the endoplasmic reticulum (Figure 5). However, within 6 h, the

majority of the mutant enzyme finally reached the Lamp-1-positive lysosomes.

Both the mutant D293N mCTSD overexpressed in HEK-293 cells (as well as in CHO cells; S. Partanen, unpublished work) and the mutant ovine CTSD in CONCL fibroblasts were found to be modified such that their molecular masses were reduced by 1–2 kDa (corresponding to 9–18 amino acid residues). Whereas the altered electrophoretic mobility of the mutant CTSD was not initially observed in brain extracts of CONCL sheep [31], re-evaluation of CTSD in CONCL sheep fibroblasts in an SDS gel system with a higher resolution confirmed the difference in electrophoretic mobility of the mutant enzyme (Figure 2B). Enzymic deglycosylation experiments showed that the difference in the electrophoretic mobility was not due to altered glycosylation (Figure 3), suggesting that proteolytic cleavage may be responsible for the reduced mass. The putative cleavage, presumably occurring in the endoplasmic reticulum, appears to be an early event, as it is detectable after a short pulse of 20 min in HEK-293 cells (Figure 4). During synthesis and maturation, the N-terminal propeptide of CTSD is believed to interact with the active-site residues, assist folding and prevent degradation [16,17]. Thus the substitution of the acidic Asp-293 in the active site by asparagine may affect the conformation of the precursor and allow proteolytic cleavage in one of the early biosynthetic compartments. It has not been determined whether the reduction in mass of the mutant CTSD is due to N- or C-terminal processing. Recently, it has been shown that mutant human pro-CTSD can be cleaved at alternative sites in the pro-sequence by as-yet-unidentified proteases [38]. Furthermore, in porcine kidney cells the heavy chain of CTSD undergoes a reduction in mass by approx. 1 kDa 4–8 h after synthesis, which was proposed to occur by C-terminal processing [39]. It may be pointed out that in ovine cells mutant D295N CTSD displays the reduction in size even in its mature 30 kDa chain. Because this chain represents the C-terminal fragment of the protein, its alteration in the mutant suggests strongly that the abnormal processing affects the C-terminus. Because the C-terminus of CTSD is part of a β -sheet [13] the deletion of 9–18 amino acid residues should have a drastic effect on protein structure.

In conclusion, we have shown that the D293N mutation affects the activity, stability, processing, transport and secretion of the mCTSD in HEK-293 cells, by a mechanism possibly involving a conformational change leading to slow or incorrect folding and, further, to altered susceptibility to proteolytic processing. It is not clear how these changes would affect non-enzymic functions of CTSD. Recently Glondou et al. [32] reported that the secreted form of CTSD mediates mitogenic activity that does not require catalytic activity. Thus, although the amount of the mutant CTSD is abnormally high in the CONCL sheep, a failure in its transport or secretion in brain cells may affect its mitogenic functions, contributing to the development of NCL disease in newborn sheep. However, an understanding of the causes of the differential processing of the mutant enzyme, and the role of CTSD in neurons, is needed to clarify the pathogenetic processes triggered by the mutant CTSD.

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