Mutations in classical late infantile neuronal ceroid lipofuscinosis disrupt transport of tripeptidyl-peptidase I to lysosomes

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Classical late infantile neuronal ceroid lipofuscinosis is an autosomal recessive disease caused by mutations in the CLN2 gene resulting in functional defects of the gene product tripeptidyl-peptidase I. This disease is associated with a progressive neurodegenerative course beginning at the age of two years with developmental stagnation, finally leading to a complete loss of motor function, vision and speech by the age of 10 years. We analyzed the functional consequences of the mutations R127Q, R208X, N286S, I287N, T353P and Q422H, which were previously identified in patients with late infantile ceroid lipofuscinosis, with regard to enzymatic activity, stability, post-translational processing and intracellular localization of tripeptidyl-peptidase I. We could not detect any translational product for the mutant R208X. We found that four missense mutations, N286S, I287N, T353P and Q422H, which are located in conserved protein regions of tripeptidyl-peptidase I, decreased the enzymatic activity dramatically, blocked processing to mature size peptidase and led to protein retention in the endoplasmatic reticulum and rapid degradation in non-lysosomal compartments. We conclude that these amino-acid substitutions induce major misfolding of the precursor peptidase and hence prevent post-translational processing and lysosomal targeting of tripeptidyl-peptidase I. In contrast, the amino-acid substitution R127Q within a non-conserved protein region did not significantly affect enzymatic activity, stability, processing and lysosomal targeting of tripeptidyl-peptidase I. Thus, our functional analyses of CLN2 mutations reveal novel insight into the molecular defect underlying dysfunction of tripeptidyl-peptidase I.

INTRODUCTION

The neuronal ceroid lipofuscinoses (NCL) are neurodegenerative diseases of childhood and adolescence that share similar clinical features such as developmental regression, visual loss, movement disorder, epilepsy and the name-giving presence of autofluorescent lysosomal storage material. At present, seven different genes have been identified for distinct forms of NCL in humans and other mammals. Deficiencies of three lysosomal enzymes are associated with NCL forms having an early onset of disease (1): CLN1—infantile NCL (2), CLN2—classical late infantile NCL (3), and cathepsinD—ovine congenital NCL (4). Mutations in genes of transmembrane proteins are linked to later onset of symptoms (5): CLN3—juvenile NCL (6), CLN5—Finnish variant late infantile NCL (7), CLN6—variant late infantile NCL (8,9) and CLN8—Northern epilepsy (10).

Mutations in the CLN2 gene are associated with classical late infantile NCL (MIM no. 204500), a fatal autosomal recessive disorder with onset at the age of two years, rapidly progressive neurodegeneration and complete disability by the age of 10 years. The CLN2 gene encodes a lysosomal peptatin insensitive tripeptidyl-peptidase I (TPP-I, EC 3.4.14.9) and is synthesized as an inactive 67 kDa precursor that is autocatalytically converted to an active 46 kDa serine protease at acidic pH (11). We recently established a phenotype–genotype correlation for patients with late infantile NCL and defined...
mutations in the CLN2 gene (12). Our studies revealed a uniform severe clinical disease progression for those patients carrying premature stop mutations in the CLN2 gene. However, several patients with missense mutations showed a significantly milder phenotype. The measured residual TPP-I activity in fibroblasts were equal or below 5% for all patients and thus could not explain divergent clinical phenotypes.

To elucidate further the molecular mechanism of TPP-I inactivation conferred by specific missense mutations in the CLN2 gene, we expressed various mutant TPP-I proteins in mammalian cells and studied maturation and trafficking of TPP-I mutants, which has not been investigated up to now.

RESULTS
Expression of TPP-I mutants

The functional consequences of several CLN2 mutations on the biosynthesis of TPP-I was studied by transient and stable expression of mutant cDNA in HEK and CHO cells. The cDNA of the complete CLN2 coding sequence was cloned into the expression vector pcDNA3. Mutations were generated by site-directed mutagenesis and short fragments comprising the mutations of interest were introduced in the wild-type CLN2 cDNA. The identity of the subcloned fragments was confirmed by sequencing. In order to control efficacy of transient transfection in the HEK and CHO cells, wild-type and mutant TPP-I was co-expressed with β-galactosidase. Cells were harvested and the cell lysates were assayed for TPP-I activity as well as β-galactosidase activity 48 h after transfection. All transfected cells had similar β-galactosidase activity, suggesting comparable transfection efficiency. Western blotting of TPP-I mutants expressed in the HEK cells revealed the presence of a 67 kDa as well as a 46 kDa signal for wild-type TPP-I and mutant R127Q (Fig. 1A). The TPP-I proteins carrying mutation N286S, I287Q, T353P or Q422H showed a signal with slightly higher mobility (65–66 kDa) and among these N286S exhibited the highest electrophoretic mobility. None of these four mutants revealed a lower signal at ~46 kDa. No TPP-I specific signals were detected for mutant R208X or vector-transfected cells. Mutant R208X, N286S, I287N, T353P and Q422H had low activities that did not differ from vector-transfected cells (Table 1). Mutant R127Q showed 60–70% activity of wild-type transfected cells. These findings suggest that only wild-type TPP-I and mutant R127Q were processed to the active 46 kDa TPP-I form.

Metabolic labeling of TPP-I mutants

The measured activities of wild-type and mutant TPP-I in the HEK and CHO cells indicated differences in processing of various mutants. To this end, we transiently expressed wild-type and mutant TPP-I in CHO cells and metabolically labeled cells with radioactive methionine for 30 min. After exchanging the medium with non-radioactive one, cells were harvested after chase times of 1/2, 4 and 20 h. For wild-type TPP-I and mutant R127Q, the 67–66 kDa TPP-I precursor protein and the mature 46 kDa TPP-I protein were immunoprecipitated from homogenates of transfected cells by anti-TPP-I antibodies coupled to Sepharose beads. The intensity of the upper 66 kDa signal decreased with longer chase times (Fig. 1B). After 4 h of chase, ~70% of wild-type TPP-I and ~85% of mutant R127Q were detected at 46 kDa; after 20 h of chase, both wild-type and mutant R127Q showed exclusively a signal at 46 kDa. All other mutants, namely N286S, I287N, T353P and Q422H were reduced significantly in intensity after 4 h of chase and were undetectable after 20 h; they never displayed a signal at 46 kDa. Stability of these TPP-I mutants was not increased by the addition of either leupeptin or pepstatin A, which are inhibitors of serine and thiol proteases or of acidic proteases, respectively, to the medium during the pulse chase experiment (data not shown). The data thus indicate that the intracellular half-life of mutant N286S, N287I, T353P or Q422H is significantly shortened. Further, it suggests that lysosomal proteases are not involved in their accelerated degradation.

![Figure 1](image)

Expression of wild-type and mutant TPP-I in HEK and CHO cells. (A) Immunoblotting of wild-type and mutant TPP-I. Lysates (30 μg protein per lane) from HEK cells transiently transfected with either pcDNA3 vector alone or constructs containing TPP-I wild-type or TPP-I mutant R127Q, N286S, I287N, T353P, Q422H and R208X were subjected to SDS–PAGE, transferred to nitrocellulose membrane, and stained with chicken anti-TPP-I antibodies as described. The molecular weight of the unprocessed TPP-I precursor (67 kDa) and of the mature TPP-I protein (46 kDa) are indicated. (B) Metabolic labeling of wild-type and mutant TPP-I. Vector-transfected CHO cells and cells transfected with TPP-I wild-type or TPP-I mutant R127Q, N286S, I287N, T353P, Q422H, and R208X were metabolically labeled with [35S]Met for 30 min and chased for 30 min, 4 h and 20 h in DMEM supplemented with 10% (v/v) fetal calf serum. The TPP-I was immunoprecipitated from cell lysate using chicken anti-TPP-I antibodies coupled to Sepharose beads and resolved on SDS–PAGE.
Intracellular targeting of TPP-I mutants

Since the mutant TPP-I proteins were not processed to the mature form, we assumed that intracellular trafficking might be altered. Using immunolabeling, we determined the intracellular localization of TPP-I mutants expressed in the CHO cells. The cells were co-transfected with the CD4ER variant that is retained in the ER (13). To reveal co-localization, the cells were double-stained with anti-TPP-I antibody and either in combination with anti-Lamp-2 antibody, with anti-CD4 antibody or with anti-PDI antibody. Prior to the beginning of the immunolabeling procedure, half of the coverslips from one transfection well were separated and treated with 100 μg/ml cycloheximide (CHX) to block protein translation. The CHX-treated and untreated cells were stained in parallel and the effect of the CHX-treatment on TPP-I localization and TPP-I stability was examined. We observed co-localization of anti-TPP-I immunostaining with the lysosomal marker Lamp-2 in the wild-type and the R127Q mutant (Fig. 2). The CHX-treatment had no effect on the intracellular localization of wild-type and R127Q mutant and only caused minor degradation (Table 1). In contrast, mutant N286S, I287N, T353P and Q422H immunoreactivities co-localized mainly with the ER marker and other non-lysosomal compartments (Fig. 2). CHX-treatment for 5 h resulted in major degradation of the mutants: Q422H > N286S > I287N > T353P (Table 1). These findings were confirmed by Percoll density-gradient fractionation of homogenates derived from the CHO cells stably expressing wild-type or mutant TPP-I. Wild-type TPP-I and R127Q mutant activities and immunoblot intensities were mainly recovered from lysosomal fractions (Fig. 3). In contrast, mutant N286S and Q422H showed neither activity nor immunoblot reactivity in lysosomal fractions; only low immunoreactivity was detected in the ER fractions. Altogether, these results demonstrate severely altered intracellular trafficking for most mutant TPP-I proteins and suggest ER retention and rapid degradation.

Molecular genetic analysis of the mutation c.380G>A (R127Q) in exon 4

Metabolic labeling and intracellular localization of mutant R127Q demonstrated comparable processing and lysosomal targeting with respect to wild-type TPP-I. The enzymatic activity of mutant R127Q amounted to 60–70% of wild-type TPP-I and thus was inconsistent with the low TPP-I activity of the patient fibroblasts, which was about 5% of normal (12). To reveal the molecular mechanism of TPP-I deficiency caused by the mutation c.380 G>A, we analyzed the cDNA of the patient. Amplification of a correctly spliced 500 bp DNA fragment comprising exon 4–7 with the primer pair F1 and R gave significantly smaller quantity of product from patient cDNA than from equivalent template of control cDNA (Fig. 4A and B). The patient’s PCR fragments were subcloned and individual clones were sequenced. The majority of clones carried the maternal null mutation R208X; only two out of 24 clones carried the paternal allele indicating that mutation c.380 G>A may cause incorrect splicing. To reveal possible splicing errors, a second primer F2 was designed that included the paternal allele (Fig. 4B). Since primer F2 spanned the splice junction of exon 4 and intron 4, the primer pair F2 and R would not amplify the correctly spliced cDNA sequence but rather any uncorrectly spliced cDNA. PCR amplification of the patients’ cDNA with primer pair F2 and R displayed an aberrant 690 bp splice product that contained the complete intron 4 sequence (Fig. 4A). Since intron 4 has a stop codon in frame at the very beginning, the resulting translation product would be a truncated protein without enzymatic activity (Fig. 4C).

DISCUSSION

In the present study, we investigated the molecular pathology underlying TPP-I dysfunction in late infantile NCL. We studied activity, post-translational processing, intracellular trafficking and stability of mutant TPP-I proteins in heterologous expression systems.

TPP-I exhibits significant sequence similarity with bacterial pepstatin-insensitive carboxypeptidases (14) and has been suggested to belong to the sedolisin family of serine-carboxyl peptidases (15). Among mammals, TPP-I is highly conserved considering published sequences of human, macaque, mouse, rat, cow and dog TPP-I. The amino-acid sequence similarity between human and mouse TPP-I is 88.6% for the N-terminal domain, 81.9% for the mid-domain and 86.8% for the C-terminal domain.
Figure 2. Immunohistochemical localization of TPP-I mutants expressed in CHO cells. The CHO cells were seeded on glass coverslips and cotransfected with CD4ER and with either TPP-I wild-type or TPP-I mutants. Forty hours after transfection, half of the coverslips with cells from one transfection well were separated and treated with 100 μg/ml cycloheximide (CHX) for 5 h. Fixed cells were co-stained with chicken anti-TPP-I antibodies and secondary Alexa Fluor 488 conjugates (displayed in green) and either mouse anti-Lamp-2 antibody (lysosomal marker, indicated by LYSO) or mouse anti-CD4 antibody and secondary Alexa Fluor 546 conjugates (displayed in red, indicated by ER). Alternatively, the marking of ER was achieved by anti-PDI antibody staining. Nuclei were stained with TOTO-3 iodide (displayed in blue). Each image is labeled with the TPP-I mutant type (left upper corner) and the type of antibody staining (right upper corner). CHX is marked (left lower corner) when cells were treated with cycloheximide. (A) Upper row: mutant R127Q (green) co-localizes with the lysosomal marker (red); in contrast, there is no co-localization of mutant R127Q with the ER (overlay only). Lower row: mutant Q422H (green) co-localizes with the ER marker (red), whereas there is only minor co-localization of mutant Q422H with the lysosomes (overlay only). (B) Upper row: mutants N286S and I287N do not co-localize with lysosomes but with the ER (overlays only). Lower row: mutant T353P does not co-localize with lysosomes but with the ER (overlays only), wild-type TPP-I co-localizes with lysosomes and mutant R208X (truncated protein) is not detected with anti-TPP-I antibody (overlays only).
cleavage product (residues 1–195) and 93.5% for the C-terminal part (residues 196–557). Further, TPP-I from more distantly related species as the pufferfish fugu shares 52.8% sequence similarity at the N-terminus (residues 31–195) and 77.6% sequence similarity at the C-terminus (residues 196–557).

Figure 3. Subcellular fractionation of wild-type and mutant TPP-I was performed on cell homogenates by Percoll density-gradient centrifugation. Higher fraction numbers possess lower density. (A) The profiles of lysosomal β-hexosaminidase and NADPH:cytochrome c reductase activities reveal lysosomal fractions indicated by lysosomes and ER fractions indicated by ER. (B) Most of the activity of wild-type and mutant R127Q TPP-I are recovered in the lysosomal fractions. No significant activities are detectable for mutant N286S and Q422H. (C) Immunoblot reactivity of wild-type and mutant R127Q show a preferential lysosomal localization. Immune reactivity of mutant N286S and Q422H are only recovered from ER fractions and are undetectable in lysosomal fractions. wt, wild-type; β-Hex, β-hexosaminidase; cyt c red, NADPH:cytochrome c reductase.

Figure 4. Analysis of the CLN2 mutation c.380G>A (R127Q) in exon 4. Patient (P) cDNA and control (C) cDNA were used to amplify specific CLN2 fragments. (A) Amplification of a correctly spliced cDNA fragment comprising exon 4–7 with the primer pair F1 and R gave 56% less product (500 bp, triple asterisk) than the equivalent amplification with control cDNA as template. The quality of the cDNA was controlled by amplification of a GAPDH fragment that resulted in equal product yield. A second forward primer F2 included the paternal allele and spanned the splice junction of exon 4 and intron 4. Therefore primer pair F2 and R can only amplify incorrectly spliced cDNA and not the correctly spliced cDNA. Amplification with the primer pair F2 and R displayed a 690 bp band for the patient that was absent in control cDNA. Sequencing of the 690 bp fragment (double asterisk) disclosed an aberrant splice product containing the complete intron 4 sequence. The upper 950 bp band (asterisk) is the amplification product of contaminating genomic DNA. (B) Schematic representation of the CLN2 gene (exon 4–7) and of the amplification procedures. The location of primers F1, R and F2 are indicated by arrows. ‘Y’ marks the position of the paternal mutation c.380A>G, ‘X’ the position of the maternal null allele (R208X). Primers F1 and R are located in exon 4 and exon 7, respectively. Primer pair F1 and R will preferentially amplify the correctly spliced cDNA sequence (triple asterisk). Since primer F2 spans the splice junction of exon 4 and intron 4, the amplification with primer pair F2 and R will not detect the correctly spliced sequence but will rather identify the incorrectly spliced transcripts (double asterisk) or non-spliced genomic DNA (asterisk). (C) Extract from the sequencing of the aberrant splice product (s.a.) displaying the c.380G>A mutation (arrows) and the premature stop codon (underlined) that is located in reading frame at the very beginning of intron 4.
195–557) with human TPP-I. Alignment of human, mouse and fugu TPP-I reveals structurally conserved sequence elements that are preferentially located in the C-terminal region corresponding to the mature TPP-I protein (Fig. 5).

Among the six CLN2 mutations we expressed in the HEK and CHO cells, four are located in conserved sequence regions (N286S, I287N, T353P and Q422H), one contained an amino-acid substitution at a non-conserved position (R127Q) and one causes premature stop of translation (R208X). We could not detect any protein in cells expressing the R208X mutant. For all other mutants, TPP-I proteins were detected by western blotting, immunoprecipitation and immunofluorescence analysis. Our experiments revealed similar properties for the mutants N286S, I287N, T353P and Q422H that affected evolutionarily conserved amino-acid residues. First, the enzymatic activities of these mutants were comparable to those of vector-transfected or R208X-transfected cells. Second, the intracellular half-life of these mutants was considerably reduced as estimated by metabolic labeling or treatment with CHX. Third, the majority of the mutant proteins was retained in the ER and was not sorted to lysosomes, suggesting that these four mutations may cause general folding defects and hence rapid degradation in non-lysosomal proteasomal compartments. Further, western blotting and immunoprecipitation from metabolically labeled cell lysates of TPP-1 mutants N286S, I287N, T353P and Q422H demonstrated a defect in post-translational processing to the mature 46 kDa TPP-I protein. In addition, the unprocessed precursor protein (66 kDa) of these mutants exhibited a slightly lower molecular weight than the wild-type enzyme. In particular, the N286S mutant exhibited the highest electrophoretic mobility. In case of mutant N286S, this could be explained by the lack of one of five potential glycosylation sites. Deglycosylation of mutant N286S with either N-glycosidase F or endoglucosaminidase H resulted in the same increase in electrophoretic mobility (16), indicating that the precursor protein carries largely high-mannose oligosaccharides, which are the predominant N-linked carbohydrate residues in the ER. Similarly, the TPP-I mutant N286Q has recently been shown to be predominantly retained in the ER (17). Incomplete ER-type glycosylation might also explain the increased electrophoretic mobility of mutant I287N, T353P and Q422H. The data give further evidence that these mutants are misfolded, degraded by the cellular quality control system and hence not transported to the lysosomal compartment. In contrast, mutant R127Q undergoes post-translational maturation and lysosomal targeting in a manner that is not distinguishable from wild-type TPP-I. The high enzymatic activity and the regular metabolic processing of mutant R127Q are in good agreement with its amino-acid substitution in a non-conserved position (Fig. 5).

However, molecular genetic analyses of the underlying mutation c.380G>A disclosed that <10% of the transcripts correspond to the correctly spliced R127Q allele. Most of the transcripts are incorrectly spliced and result in a truncated translational product. Hence, the alteration c.380G>A represents largely a splice site mutation. The slow disease progression of the patient, who was compound heterozygous for the c.380G>A allele and a null mutation (12), can be explained by the minor biosynthesis of the full length R127Q mutant.

![Figure 5. Alignment of human, mouse and the partial pufferfish (fugu) TPP-I sequence.](image-url)
Together, our functional analyses of the molecular defects in selected CLN2 gene mutations of patients with late infantile NCL show that either truncation of CLN2-protein or a defect in post-translational processing and lysosomal misrouting are the relevant pathomechanisms of TPP-I deficiency. Considering that the vast majority of patient mutations lies within highly conserved TPP-I regions, we expect that the majority of missense mutants will cause misfolding and hence premature TPP-I degradation. Our data are in agreement with recent findings in TPP-I mutant G389E or R447H. When expressed in CHO cells, these TPP-I mutants showed altered maturation resulting in the absence of mature precursor protein, whereas the mature protein was not detectable on western blotting (18). Presently, the only reported missense mutation that shows a processed 46 kDa CLN2-protein is the mutant S475L, which affects the active centre of TPP-I (19). Processing of mutant S475L proenzyme requires the assistance of an unidentified AEBSF-sensitive protease (20). Additional insight into the structure of TPP-I will help to understand the mechanism of TPP-I activity as well its malfunction in late infantile NCL.

MATERIALS AND METHODS

Materials

Cell culture medium (DMEM, Opti-MEM), G418, Lipofectamine 2000 and Taq DNA polymerase were purchased from Invitrogen (Groningen, The Netherlands). Oligonucleotides were synthesised by MWG-Biotech (Munich, Germany); primer F1 has the sequence 5'-TGACTTGCTGCTGAGCATCC-3', primer F2 5'-GCTGGCTGAGCATCCAGTGAG-3' and primer R 5'-TGTGGCACCAGCCTCATCAG-3'. [35S]methionine and molecular weight protein markers were obtained from Amersham Biosciences (Freiburg, Germany). Restriction enzymes were supplied by New England BioLabs (Frankfurt, Germany). Mouse anti-CD4 antibody was acquired from Zymed Laboratory Inc. (San Francisco, CA, USA), anti-Lamp-2 antibody from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Biotin SP-conjugated donkey anti-chicken antibody was purchased from Jackson ImmunoResearch Laboratory Inc. (West Grove, PA, USA), anti-PDI antibody from Stressgen Biotechnologies (Victoria, Canada). Alexa Fluor 488 goat anti-rabbit antibody, streptavidin Alexa Fluor 488 conjugate, Alexa Fluor 546 goat anti-mouse antibody and TOTO-3 dye were obtained from Molecular Probes (Leiden, The Netherlands). The BigDye terminator kit was used for sequencing according to the recommendation of the manufacturer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). All other reagents were purchased from Sigma-Aldrich (Munich, Germany).

Expression of TPP-I wild-type and TPP-I mutants in the HEK and CHO cells

The CLN2 cDNA was received from the IMAGE consortium (RZPD, Berlin, Germany, clone id IMAGp998C171716) and was cloned into the expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands). Site-directed mutagenesis was performed using a QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the supplier’s protocol. DNA fragments of between 300 and 700 bp containing the introduced mutations were obtained from the mutant pcDNA3–CLN2 plasmid by double digestions with the appropriate restriction enzymes and were subcloned into the parental pcDNA3–CLN2 plasmid. All final constructs were verified by semiautomated sequencing.

The HEK and CHO cells were seeded on 35 mm plates at a density of 2 × 10³ cells/well. After 24 h culturing, the cells were transfected with 2 μg of plasmid pcDNA 3.1–CLN2, 0.5 μg of plasmid pSV–β-galactosidase (Promega, Madison, WI, USA) and 5 μl of Lipofectamin 2000 in 0.5 ml Opti-MEM. Medium was replaced with DMEM/FCS after 6 h of incubation. Forty eight hours after transfection, TPP-I and β-galactosidase activities were assayed in cellular homogenates using the substrate Ala-Ala-Phe-[7-amido-4-methylcoumarin] as described by Vines and Warburton (21) or the substrate o-nitrophenyl-β-D-galactopyranoside (Promega, Madison, WI, USA).

For stable transfection of wild-type and mutant TPP-I in CHO cells, selection with G418 was started 48 h after transfection. For immunofluorescence labeling, cells were seeded on glas coverslips and cells were co-transfected with the plasmid pcDNA3.1–CD4ER, where CD4ER stands for a mutant CD4 that contains the C-terminal ER retention signal KKTN (13).

Anti-TPP-I antibodies

Rabbit polyclonal antiserum and chicken polyclonal antiserum against human TPP-I were generated with recombinant TPP-I expressed in CHO cells. Stably transfected CHO cells were selected for clones with high TPP-I expression. Recombinant TPP-I was purified from the culture medium by anion-exchange chromatography (22). A measured amount (200 μg) of purified recombinant TPP-I was used to immunize one animal. Antiserum was passed through protein G Sepharose or protein L Sepharose columns. The specificity of the antibodies was demonstrated by western blotting in the presence or absence of competing recombinant TPP-I in the incubation buffer. Staining of blotted TPP-I was performed with secondary antibody anti-rabbit antibody or rabbit anti-chicken antibody conjugated to peroxidase using the SuperSignal chemiluminescent detection kit (Pierce Biotechnology, Rockford, IL, USA). For immunoprecipitation, 2 mg of purified anti-TPP-I antibodies were coupled to 800 μl of NHS-Sepharose beads (Amersham Biosciences).

Metabolic labeling and immunoprecipitation of TPP-I mutants

Forty eight hours after transfection with either wild-type or mutant TPP-I cDNA, the CHO cells were washed twice with methionine-free DMEM (Invitrogen), incubated at 37°C for 1 h in the same medium and then incubated again for 30 min in the same medium supplemented with 100 μCi/ml [35S]Met (Amersham Biosciences). The radioactive medium was removed; cells were washed twice with Hank’s balanced salt solution and chased in DMEM/FCS with or
without proteinase inhibitor pepstatin A and leupeptin (10 μg/ml each). At 30 min, 4 h and 20 h, cells were washed three times with cold PBS and then lysed in immunoprecipitation buffer containing 150 mM NaCl, 10 mM Na2HPO4/K2HPO4 (pH 7.4), 0.5% Triton X-100, 0.3% sodium deoxycholate, 0.2% SDS, 1% BSA and proteinase inhibitor mix (Sigma-Aldrich, Munich, Germany). The lysates were centrifuged at 13 000g for 10 min and the supernatants were incubated for 4 h with 1/10 volume of preimmune serum coupled to Sephardose beads. The suspension was centrifuged at 200 g for 1 min and the supernatant was incubated overnight with 20 μl anti-TPP-I antibody coupled to Sephardose beads. The suspension was centrifuged again and the beads were washed three times with immunoprecipitation buffer. The proteins bound to the anti-TPP-I antibody beads were eluted by boiling in sample buffer containing 60 mM Tris (pH 6.8), 25% glycerol, 2% SDS, 14 mM 2-mercaptoethanol, 0.1% bromphenol-blue. The denatured proteins were subjected to SDS–PAGE according to Laemmli (23). The gels were fixed in an aqueous solution of 10% acetic acid and 50% methanol, soaked for 30 min in AmplifyTM solution (Amersham Biosciences), vacuum-dried at 60 °C and analyzed by either autoradiography and scanning densitometry or by quantitative fluorometry.

**Immunofluorescent microscopy**

Forty hours after transfection, half of the coverslips with cells from one transfection well were separated and treated with 100 μg/ml CHX for 5 h. The transfected CHO cells were washed twice with PBS, and fixed with 3% paraformaldehyde in PBS for 20 min at 37 °C. The cells were washed again four times with PBS and then permeabilized and blocked with 2 mg/ml saponin in PBS containing 10% horse serum and 0.3% BSA for 1 h at RT. Subsequently, the permeabilized cells were incubated with primary antibodies—anti-TPP-I antibody anti-Lamp-2 antibody, anti-CD4 antibody or anti-PDI antibody—for 2 h at dilution of 1:200 in carrier solution containing 0.5 mg/ml saponin plus 1% horse serum in PBS. After three times washing with PBS, the cells were incubated with the appropriate secondary antibodies conjugated with Alexa Fluor 488 or 546 (1:2000 in carrier solution) for 1 h in the dark. The cells were either co-stained with anti-TPP-I antibody and anti-Lamp-2 antibody, with anti-TPP-I antibody and anti-CD4 antibody or with anti-TPP-I antibody and anti-PDI antibody. After three washes with PBS, nuclei were stained for 5 min with TOTO-3 iodide (1:8000 in PBS) and the cells were again washed three times with PBS. Finally, coverslips were mounted on object glasses with Vectashield (Vector Laboratories, Burlingame, CA, USA). The triple fluorescence was viewed with a Leica NTSC laser confocal microscope (Leica, Bensheim, Germany) in sequential scan mode.

**Percoll density-gradient fractionation**

Confluent CHO cells stably expressing wild-type or mutant TPP-I were washed three times with cold PBS and once with fractionation buffer containing 250 mM sucrose, 20 mM HEPES, 1 mM EDTA supplemented with protease inhibitor cocktail (Sigma-Aldrich). The cells were scrapped from tissue culture flasks in 5 ml fractionation buffer, cell pellets were obtained at 600g for 10 min and the pellets resuspended in fractionation buffer and placed on ice for 10 min. Cell homogenization was carried out at 4 °C using a Dounce homogenizer (10 passages). The resulting homogenate was adjusted to 5 ml with fractionation buffer and 3 ml of an 80% Percoll solution was added. The gradient was performed by centrifugation for 35 min at 100 000g at 4 °C. Fractions of 400 μl were collected from the bottom of the tubes with a needle mounted to a syringe.

The β-hexosaminidase activity as lysosomal marker and NADPH:cytochrome c reductase activity as ER marker were determined for each collected fraction. The lysosomal β-hexosaminidase activity was measured in 200 mM sodium acetate, 0.1% Triton-X 100, pH 4.5 with 1 mM of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide substrate. Fluorescence was measured with 340 nm excitation and 450 nm emission. NADPH:cytochrome c reductase activity was measured in 50 mM phosphate, 0.1 mM EDTA, pH 7.7 with 1 mg/ml NADPH and 25 mg/ml cytochrome c as substrates. Absorbance was measured at 550 nm.

TPP-I activity was measured according to Vines and Warburton (21). Total activity of TPP-I wild-type was defined as 100% and the activities of TPP-I mutants were related to it.

The TPP-I protein of each fraction was assessed by dot-blot analysis with anti-TPP-I antibody and chemiluminescent detection. Signal intensities were measured by a digital CCD imaging system, assigned arbitrary units and converted to the percentage of total wild-type TPP-I intensity (100%).

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