Human tripeptidyl-peptidase I (TPP I, CLN2 protein) is a lysosomal serine protease that removes tripeptides from the free N termini of small polypeptides and also shows a minor endoprotease activity. Due to various naturally occurring mutations, an inherited deficiency of TPP I activity causes a fatal lysosomal storage disorder, classic late infantile neuronal ceroid lipofuscinosis (CLN2). In the present study, we analyzed biosynthesis, glycosylation, transport, and proteolytic processing of this enzyme in stably transfected Chinese hamster ovary cells as well as maturation of the endocyted proenzyme in CLN2 lymphoblasts, fibroblasts, and N2a cells. Human TPP I was initially identified as a single precursor polypeptide of ~68 kDa, which, within a few hours, was converted to the mature enzyme of ~48 kDa. Compounds affecting the pH of intracellular compartments, those interfering with the intracellular vesicular transport as well as inhibition of the fusion between late endosomes and lysosomes by temperature block or 3-methyladenine, hampered the conversion of TPP I proenzyme into the mature form, suggesting that this process takes place in lysosomal compartments. Digestion of immunoprecipitated TPP I proenzyme with both N-glycosidase F and endoglycosidase H as well as treatment of the cells with tunicamycin reduced the molecular mass of TPP I proenzyme by ~10 kDa, which indicates that all five potential N-glycosylation sites in TPP I are utilized. Mature TPP I was found to be partially resistant to endo H treatment; thus, some of its N-linked oligosaccharides are of the complex/hybrid type. Analysis of the effect of various classes of protease inhibitors and mutation of the active site Ser275 on human TPP I maturation in cultured cells demonstrated that although TPP Izymogen is capable of autoactivation in vitro, a serine protease that is sensitive to AEBSF participates in processing of the proenzyme to the mature, active form in vivo.

Degradation of polypeptides requires the collective action of various endo- and exopeptidases, finally releasing free amino acids and dipeptides reused in the cell cytoplasm according to the metabolic needs of the cell. Two tripeptidyl peptidases identified to date in mammalian cells sequentially cleave tripeptides from the N termini of oligopeptides: tripeptidyl peptidase I (TPP I,1 CLN2 protein) and tripeptidyl peptidase II (TPP II) (for a recent review, see Ref. 1). TPP II is a cysteine enzyme that belongs to the subtilisin subclass of serine peptidases (2). TPP I (EC 3.4.14.9) is a lysosomal exopeptidase with an acidic pH optimum (3, 4) and a minor endoprotease activity (5). Naturally occurring mutations in TPP I are associated with a fatal lysosomal storage disorder, the classical late infantile form of neuronal ceroid lipofuscinosis (CLN2, Jansky-Bielschowsky disease) (6, 7). This autosomal recessive disorder starts at the age of 2–4 years with poorly controllable seizures and dementia, followed by visual loss and cerebellar and pyramidal and extrapyramidal signs, leading to death in the second decade of life. Rare, atypical cases with later onset of the disease and more protracted course also have been documented (for a recent review, see Ref. 8). Curvilinear profiles, lysosomal inclusions typical for CLN2, have been demonstrated ultrastructurally in amniotic fluid cells from around 16 weeks’ gestation and in fetal skin and lymphoblasts from around 20 weeks’ gestation (9), which correlates well with the early expression and developmental regulation of TPP I (10–12).

TPP I in humans is encoded by a gene mapped to chromosome 11p15 (13). The deduced amino acid sequence of TPP I consists of 563 amino acid residues and includes a 19-amino acid signal sequence and a 176-amino acid prepropeptide removed during the maturation process to yield a mature enzyme of 368 amino acid residues (6, 14, 15). By SDS-PAGE, the mature enzyme, which was purified from human osteoclastomas (3), rat spleen (4, 5) and kidney (16), and bovine and human brain (15, 17), has an apparent molecular mass of 46–48 kDa, whereas the proenzyme has a mass of 66 kDa (15, 18). However, by non-denaturing PAGE and gel filtration, the molecular mass of the rat TPP I was calculated to be 280 kDa and 290 kDa in the absence and presence of β-mercaptoethanol, respectively, which suggests that the enzyme is composed of six identical subunits (16).

Natural substrates of TPP I are unknown; however, it ap-
pears that this peptidase is involved in degradation of small unstructured polypeptides with unsubstituted N terminus and uncharged amino acid in the P1 position (4, 19). In vitro, TPP I cleaved peptide hormones such as angiotensin II, glucagon (4), substance P (17), angiotensin III, and neuromedin B (16) as well as synthetic amyloid-β peptide 1–42 and 1–28 (17) and most probably collagen (4) and subunit c of mitochondrial ATP synthase (17, 18), a proteolipid that accumulates in all types of neuronal ceroid lipofuscinoses except for the infantile form in Lp. The activity of TPP I can be inhibited efficiently by the tripeptide analogue of the substrate Ala-Ala-Phe-chloromethylketone (AAF-CMK) (3, 4, 17, 21). Recent data have demonstrated that TPP I is a serine protease inhibitable by 3,4-dichloroisocoumarin and diisopropyl fluorophosphate, with Ser475 representing the active site nucleophile and Asp360 and Asp517 being involved in catalytic activity (15).

The deduced amino acid sequence of TPP I zymogen has five potential N-glycosylation sites at amino acid positions 210, 222, 286, 313, and 443. Like many other lysosomal hydrolases, TPP I proenzyme is able to autoactivate in the acidic pH in vitro (15, 22). However, the role of glycosylation for the biology of TPP I and the process of maturation of TPP I zymogen in vivo have not yet been examined. Here we addressed these issues by analyzing the biosynthesis, glycosylation, and processing of hTPP I overexpressed in Chinese hamster ovary (CHO) cells. Our data suggest that maturation of TPP I takes place in lysosomal compartments and that glycosylation enables intracellular transport and maturation of TPP I. Furthermore, we show that a serine protease that is sensitive to 4-(2-aminomethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) participates in processing of the proenzyme to the mature, active form in vivo.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium components were from Invitrogen. pSmethionine/cysteine (1000 Ci/mmol; Tran32P-label) was purchased from ICN. Protease inhibitor mixture (Complete), AEBSF (Pe- fable SC Plus), E64, endoglycosidase H (endo H), and N-glycosidase F (PNGase F) and FuGENE 6 transfection reagent were from Roche (Indianapolis, IN). Polyclonal antibodies against calreticulin were from Affinity Bioreagents. Human brain cDNA library was from Clontech. pcDNA3.1Hygro vector was from Invitrogen, and pET22b vector was from Novagen. Secondary antibodies conjugated to Alexa Fluor 488 were from Molecular Probes, Inc. (Eugene, OR), and secondary antibodies conjugated to Cy3 were from Jackson ImmunoResearch. Vectorshield mounting medium was from Vector. All other chemicals were from Sigma.

TPP I Cloning and Cell Transfection—Open reading frame encoding full-length TPP I was amplified by polymerase chain reaction from human brain cDNA library (Marathon Ready) by using the primers forward (cgctgacataagggatcaagctcg) (F) and reverse (ccgacctg- cgggttgaag) (R) and subcloned into the KpnI/NcoI site of pcDNA3.1Hygro. CHO cells were grown in F-12 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere with 5% CO2. One day before transfection, the cells were seeded on 35-mm culture dishes. Cells were transfected by using FuGENE 6 transfection reagent, according to the manufacturer’s recommendations. Stably transfected cells were selected by using hygromycin B (200 μg/ml). Hygromycin-resistant individual colonies were picked up, expanded into cell lines, and maintained in selective medium.

To obtain a high level of secretion of TPP I proenzyme for in vitro studies, CHO cells deficient in asparagine- and methionine- rhodanese (DHFRmm) (ATCC CRL-9096) were transfected with plasmid encoding the dihydrofolate reductase (DHFR) and TPP I. The EcoRI/SphI fragment encompassing mouse dhfr cDNA cassette was isolated from plasmid pSV2-dhfr (ATCC), blunt-ended, and ligated in the same direction as the cytomegalovirus promoter into Xpl-digested plasmid pDNA3.1Hygro to make plasmid pcDNA-DHFR. cDNA encoding full-length hTPP I was PCR-amplified from the cDNA library, as above, by using primers F and R, restricted with KpnI and NotI, and introduced into corresponding restriction sites of pcDNA-DHFR expression vector to make pcDNA-DHFR-TPP I. The structural integrity of the insert was verified by dyeoxygen-mediated sequencing of the entire insert. Purified and linearized plasmid was transfected into CHO-DHFR™ cells grown in Isewo’s modified Dulbecco’s minimal essential medium, supplemented with 0.1 mM hypoxanthine and 0.016 mM thymidine and 10% FCS by using FuGENE 6 transfection reagent, as above. Two days after transfection, cells were subcultured and incubated in selection medium (ribonucleotide-free), supplemented with 10% FCS and hygromycin B (200 μg/ml). Single colonies were picked up by use of cloning rings, expanded, and tested for TPP I by using Western blotting and enzymatic assay. The highest expressors were then used for selection and amplification with methotrexate, as described (23). That optimization of cell culture conditions allowed us to obtain around 59 μg of recombinant TPP I per 1 ml of serum-free medium (OPTI-MEM I) conditioned for 4 days.

Antibodies—The cDNA sequence encoding the mature TPP I enzyme (amino acids 196–563) was expressed in E. coli in pET72b vector, where it was found mostly in inclusion bodies (not shown). Recombinant TPP I was purified from inclusion bodies by means of gel filtration and ion exchange chromatography in the presence of 8 M urea. Purified protein was devoid of any enzymatic activity toward TPP I substrate Ala-Ala-Phe-aminomethylcoumarin (AAP-AMC), unstable in aqueous solutions without detergents, and easily precipitated. Purified TPP I was used to raise both monoclonal and polyclonal antibodies in mice and rabbits, respectively. Monoclonal antibodies B4 and 2E12 were described by us previously (11). Antiserum RAS307 raised in rabbits against TPP I was affinity-purified on recombinant TPP I immobilized on CNBr-activated Sepharose (Amersham Biosciences). RAS307 does not recognize hamster TPP I (results not shown).

Cell Cultures—Primary skin fibroblasts from CLN2 subjects and controls as well as Epstein-Barr-transformed CLN2 lymphoblasts were from the Cell and Tissue Culture Repository at the Institute for Basic Research. Mouse neuroblastoma cells (N2a) (ATCC CCL-121) were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37 °C in a humidified atmosphere with 5% CO2 either in Dulbecco’s modified Eagle’s medium (fibroblasts, N2a) or RPMI 1640 medium (lymphoblasts), supplemented with 10% FCS, 2 mM glutamine, and antibiotics. For uptake experiments, cells were transfected to se- rum-free medium (OPTI-MEM I).

SDS-PAGE and Western Blotting—Cells were lysed in a buffer containing 50 mM Tris, pH 7.4, 1% Triton X-100, and protease inhibitor mixture (lysis buffer). The protein content was measured by using a BCA method and bovine serum albumin as a standard. Cell lysates were solubilized in sample buffer, and 2–40 μg of protein per lane was loaded onto 10% Tris/Tris buffer-sodium dodecyl sulfate-polyacrylamide gels. Mem- branes were subsequently blocked with 5% nonfat dry milk in phos- phate-buffered saline (PBS) with 0.05% Tween 20 (PBST), incubated overnight with primary antibodies, washed extensively in PBST buffer, incubated with peroxidase-conjugated secondary antibodies diluted 1:5000, and developed using the ECL kit.

In Vivo Labeling and Immunoprecipitation—Subconfluent (70–90% confluency) cell cultures (35- or 60-mm dishes) were stained in methi- onine- and cysteine-free medium for 1 h and then labeled with 100–250 μCi/ml of Tran32P-label. After a pulse, the cells and media were either harvested or subjected to chase in full medium, at the periods indicated. Afterward, the cells were lysed in lysis buffer, frozen, thawed, and disrupted with a probe homogenizer to remove nuclei. Lysates were adjusted to 250 mM NaCl, 0.5% Triton X-100, 500 mM NaCl, 0.5% RIPA buffer (1× RIPA buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Immune complexes were collected by using affinity-purified RAS307 IgG bound to Protein A-Sepharose, washed four times with RIPA buffer and once with 50 mM Tris, pH 7.4, boiled for 5 min in a 10% Tris-Triton X-100 buffer, and separated on 10% Tris-Triton X-100 buffer, and proteins were visualized by autoradiography.

Deglycosylation Experiments—hTPP I was in vivo labeled and immuno- precipitated, as described above. For PNGase F digestion, after four washes with RIPA buffer, beads were suspended in 0.5% SDS, 1% β-mercaptoethanol and boiled for 5 min, and the solution was adjusted to 250 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris, pH 7.5, and 0.6 units of PNGase F and incubated overnight at 37 °C. For endo H digestion, immunoprecipitated TPP I was denatured as above and digested in 50 mM sodium acetate, pH 6.5, overnight with 15 milliunits of endo H, followed by SDS-PAGE and autoradiography, as described above. Cold

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Fig. 1. hTPP I in CHO cells. A, subcellular vesicular structures visualized in CHO cells by pAb RAS307 anti-hTPP I (a, green), colocalize with structures labeled by mAb to LAMP I (b, red) on a composite image (c, yellow) generated by a laser-scanning confocal microscope. Original magnification is ×1000. B, immunoblot analysis of hTPP I in CHO cell lysates (lane 1) and culture medium (lane 2). Lane 1, 2 μg of protein per lane; lane 2, 10 μl of serum-free medium conditioned for 48 h. Blot developed with mAb 8C4. The arrow indicates the proenzyme. C, lysates of CHO cells and cell secretions were either mock-digested or treated with PNGase F, as indicated, and immunoblotted with mAb 8C4. Ten μg of protein of cell lysates are shown per lane; 10 μl of serum-free medium conditioned for 48 h were loaded per lane. The arrow indicates deglycosylated proenzyme.

hTPP I in cell lysates and cell secretions was denatured and digested with PNGase F, as above.

TPP I Activity Measurement—Cultured cells were lysed in 0.1% Triton X-100, 20 mM ammonium formate, pH 3.5, and protease inhibitor mixture. Protein concentration was determined by using BCA assay and bovine serum albumin as a standard. The reaction mixture contained 10 μg of cell lysate protein or 10 μl of clarified cell culture medium and 0.25 mM substrate (AAP-AMC), 0.1% Triton X-100 in a total volume of 100 μl of 0.1 M sodium acetate, pH 3.5. The reaction was carried at 37 °C for 30 min and was terminated by the addition of 50 μl of 10% SDS. These reaction conditions allowed for efficient activation of the immature TPP I present in the sample (if any) as well as reliable measurement of specific activity toward the reporter substrate. Liberated 7-amino-4-methylcoumarin was measured fluorometrically on CytoFluor (Applied Biosystems) (excitation 360 nm, emission 460 nm) after alkalizing the solution by adding 50 μl of 0.1 M Tris-HCl, pH 9.0.

Immunofluorescence Microscopy—For double immunostaining, CHO cells grown in LabTek chamber slides were fixed with methanol for 20 min at 4 °C. Nonspecific binding sites were blocked with 10% FCS in PBS for 1 h. After incubation with primary antibodies, either mAb 8C4 against TPP I and pAb to calreticulin or pAb RAS307 and mAb to LAMP I in 10% FCS in PBS overnight at 4 °C, cells were washed in PBS and incubated for 1 h at room temperature with species-specific secondary antibodies conjugated with fluorescent dyes: Alexa Fluor 488 (for TPP I and RAS307, green fluorescence) and Cy3 (for LAMP I and calreticulin; red fluorescence). The cover slips were mounted with Vectashield and viewed with a Nikon Eclipse E600 laser-scanning confocal microscope. Omission of the primary antibodies was used as a control of the method.

RESULTS

Biosynthesis of hTPP I—hTPP I expressed in CHO cells under the control of the cytomegalovirus promoter was present in the lysosomes, as revealed by double immunostaining and laser-scanning confocal microscopy (Fig. 1A), thus similar to endogenous enzyme in human cells (5, 11). On immunoblots of cell lysates, the majority of hTPP I appeared as a 48-kDa species representing a mature form of the enzyme and as a minor species with a mass of ~68 kDa, corresponding to the proenzyme (Fig. 1B, lane 1). hTPP I proenzyme also was found on immunoblots of conditioned media (Fig. 1B, lane 2), but its mass was about 2 kDa higher than that of its cellular counterpart. However, after PNGase F treatment (endoglycosidase cleaving off all asparagine-linked oligosaccharides), the SDS-PAGE mobility of intracellular and secreted proenzyme was identical (Fig. 1C). Thus, the observed difference in mass was caused by dissimilar oligosaccharide structures on secreted and intracellular proenzyme of hTPP I, a finding that was reported for other overexpressed mature acid hydrolases (24, 25) or their proforms (26). CHO cells also secreted very small amounts of mature enzyme, which could be visualized only after prolonged exposure of autoradiograms and immunoblots following immunoprecipitation (see below).

To analyze the biogenesis of hTPP I, CHO cells were pulse-labeled with [35S]methionine/cysteine and harvested after various periods. hTPP I was immunoprecipitated using RAS307 (affinity-purified rabbit anti-hTPP I antibody), separated by SDS-PAGE, electrotransferred, and autoradiographed. Autoradiographic images of the respective blots showed that hTPP I is synthesized as a precursor protein with an apparent molecular mass of ~68 kDa (Fig. 2A). Time resolution of this process by pulse/chase experiments revealed that the precursor disappeared slowly, whereas a species of ~50 kDa corresponding to the newly synthesized mature enzyme increased in intensity. At 8 h of chase, a band with mass of ~50 kDa started to thicken and form a distinct doublet, which at 24 h of chase appeared as...
two well separated bands with masses of 50 and 48 kDa. The estimated half-life ($t_{1/2}$) of the proenzyme is ~2.3 h, and the estimated $t_{1/2}$ of the mature form is ~20 h.

Given that under steady-state conditions, mature hTPP I appears as a single band with a mass of ~48 kDa (see Fig. 1B), posttranslational modification giving rise to two species of the processed hTPP I observed on autoradiograms could result either from oligosaccharide trimming or additional proteolytic cleavage of a small fragment of maturing enzyme. Because conversion of the 50-kDa to the 48-kDa species started late during the biosynthesis of hTPP I (after 8 h of chase), we reasoned that most likely it takes place in the lysosome. hTPP I proenzyme, like many other acid hydrolases labeled with mannose 6-phosphate (Man-6-P) recognition marker, is internalized by cells from extracellular milieu mostly through mannose 6-phosphate receptor (MPR)–mediated endocytosis (22).

Thus, to more closely characterize the nature and subcellular localization of this late event of hTPP I maturation, we analyzed intracellular processing of the proenzyme endocytosed by CLN2 lymphoblasts. These cells are devoid of endogenous hTPP I as a result of disease-associated mutation (27). As shown in Fig. 2B, hTPP I proenzyme added to the culture medium was taken up by CLN2 lymphoblasts and converted first to a 50-kDa species and then, between 8 and 24 h after administration, to a 48-kDa species. We observed similar results for mouse neuroblastoma cells (N2a) (not shown). Because under normal conditions endocytosed ligands need ~30 min to reach lysosomes (28), these data provide further evidence that conversion of the 50-kDa to the 48-kDa species does in fact occur in lysosomes. This experiment also revealed that the final trimming of maturing enzyme is not restricted to overexpressing cells but represents part of the normal processing of the enzyme.

To examine whether the 2-kDa trimming of maturing hTPP I results from the action of lysosomal glycosidases, CLN2 lymphoblasts were maintained for 8 and 24 h in media supplemented with hTPP I proenzyme and then lysed and subjected to PNGase F treatment. Longer running of the gel allowed better separation of the proteins in the range of 50 kDa and visualization of two species of maturing hTPP I with masses of ~50 and ~48 kDa (Fig. 2C). The higher band was distinctly stronger after 8 h of cell exposure to the proenzyme, whereas the lower band was much more prominent 24 h after proenzyme administration. Upon PNGase F treatment, the apparent molecular mass of deglycosylated mature hTPP I analyzed at both time points was the same (Fig. 2C). Thus, we conclude that the late step of maturation of hTPP I includes carbohydrate trimming in the lysosome. Trimming of oligosaccharide residues on acid hydrolases in the lysosomes was also documented for glucocerebrosidase (29) and $\alpha$-galactosidase A (25).

**Intracellular Transport and Maturation of hTPP I**

Most acid hydrolases are synthesized as preproenzymes in the rough endoplasmic reticulum (ER), where the signal peptide is cleaved co-translationally and the precursors undergo asparagine-linked glycosylation and carbohydrate processing, which continues in the Golgi apparatus (for a review, see Ref. 30). In the ER-Golgi intermediate and in the cis-Golgi compartments, lysosomal hydrolases acquire a Man-6-P marker. In the trans-Golgi network, the “uncovering” enzyme removes the covering GlcNAC residues (31), which allows for the specific, high affinity binding of the Man-6-P label of acid hydrolases to one of the two MPRs and their further vesicular transport to the endolysosomal system (for a review, see Ref. 32). In the trans-Golgi network, some acid hydrolases enter the constitutive secretory pathway and are secreted. Following dissociation from MPRs in late endosomes, which is pH-dependent and proceeds at pH below 5.5, acid hydrolases are converted to their mature forms either in prelysosomes or lysosomes. Studies in cells devoid of MPRs or deficient in the enzyme involved in the first step of generation of the Man-6-P marker (UDP-N-acetylglucosamine: lysosomal enzyme phosphotransferase) evidenced the existence of other, MPR-independent and cell type-specific pathway(s) for delivery of acid hydrolases to lysosomes (33).

To investigate intracellular transport and the site of maturation of hTPP I, first we examined the effect of brefeldin A (BFA), monensin, and bafilomycin A1 (bafA) on the processing and secretion of the enzyme. All of these compounds alter intracellular vesicular transport of lysosomal enzymes; however, their major site(s) action and molecular mechanisms differ. BFA produces disassembly and redistribution of the Golgi complex into the ER, most probably due to the inhibition of some of the proteins that activate ADP-ribosylation factors (for a recent review, see Ref. 34). Monensin is a carboxylic proton ionophore that neutralizes the intracellular acidic organelles. However, the major effect of monensin appears to be associated with the inhibition of the function of the trans cisternae of the Golgi apparatus, often near the point of exit of secretory vesicles or at low monensin concentration or short exposure time in the midregion of the stacked cisternae (for a review, see Ref. 35). bafA is a specific inhibitor of vacuolar ATPase, a major proton pump that generates H$^+$ gradients across the membranes of intracellular acidic compartments (36). Thus, like weak bases, monensin and bafA disturb normal trafficking of lysosomal enzymes via a pH-dependent MPR-mediated pathway by alkalizing intracellular acidic compartments. As a consequence, normal sorting, secretion, proteolytic processing, and endocytosis of lysosomal enzymes are altered, and protein degradation within lysosomes is inhibited (37–40). At least some of the effects exerted by bafA can be attributed to inhibition of fusion between late endosomes and lysosomes (41). It has not yet been documented whether monensin produces a similar effect. However, monensin prevents the formation of complex oligosaccharides on lysosomal enzymes, which can result either from bypassing of the trans-Golgi compartment by lysosomal enzymes or from inhibition of the oligosaccharide-processing enzymes in this compartment (42). Thus, the biological effects of bafA and monensin partially overlap, despite their molecular mechanisms being different.

CHO cells were pulsed for 1 h and chased for 3 h in the presence of BFA, bafA, and monensin, and hTPP I from both cell lysates and media was immunoprecipitated and analyzed by autoradiography and Western blotting. Treatment by all compounds tested caused a significant inhibition of hTPP I maturation, with increased cellular levels of hTPP I proenzyme (Fig. 3A, upper panel). When the maturation rate was expressed as a ratio of the precursor to the mature form by scanning the pixel density of the autoradiograms, the following values were obtained: for control cells, 2; for BFA-treated cells, 8; for bafA-treated cells, 6, and for monensin-treated cells, 5. These findings indicate that BFA was the most potent inhibitor of hTPP I maturation. However, neither of the compounds tested completely inhibited maturation of hTPP I. We believe that this was due to incomplete block of the MPR-mediated lysosomal pathway by tested compounds under the experimental conditions used. Consistent with our data, monensin incompletely blocked the segregation, transport to lysosomes, and maturation of another lysosomal proteinase, cathepsin D, in human skin fibroblasts, hepatoma cells, and monocye cell line (38). However, we cannot exclude the possibility that CHO cells are able to utilize an alternative pathway of acid hydrolase delivery to lysosomes that is less sensitive to the drugs applied. Higher doses of monensin and bafA and longer incubation time
led to significant degradation of hTPP I (not shown), which indicates that like TPP I in vitro (17), hTPP I is unstable under alkalinizing conditions also in vivo.

All tested compounds also affected secretion of hTPP I proenzyme into the medium (Fig. 3A, lower panel). BFA completely inhibited secretion of hTPP I proenzyme. In contrast, monensin and bafA increased secretion of hTPP I proenzyme. Increased secretion or even induction of secretion of the proenzymes of acid hydrolases is a well documented effect of alkalinizing compounds including monensin (37, 38) and bafA (39), which divert enzymes normally destined to lysosomes into the secretory pathway. The level of hTPP I proenzyme in cell secretions was slightly increased by the presence of Man-6-P in the culture media. Thus, inhibition of MPR-mediated endocytosis could not significantly contribute to increased levels of hTPP I proenzyme in the medium after treatment with both these compounds. Of note, the apparent molecular mass of hTPP I proenzyme secreted to the culture media by monensin-treated cells was slightly lower than in controls. This most likely reflects altered terminal glycosylation of hTPP I in monensin-treated cells, an effect elicited by monensin on numerous other glycoproteins (for a review, see Ref. 35).

Analysis of autoradiograms also showed very small amounts of mature enzyme with a mass of ~48 kDa in cell secretions (Fig. 3A, lower panel), which were slightly increased after bafA treatment. The presence of trace amounts of partially processed species of hTPP I with a mass of ~50 kDa in conditioned media most likely results from limited proteolysis of the proenzyme in the extracellular milieu.

To see whether the compounds studied also affected the steady-state pool of hTPP I, the enzyme was immunoprecipitated from CHO cells and culture media and visualized on immunoblots by using mAbs to hTPP I. All compounds tested increased the cellular level of hTPP I proenzyme, most prominently BFA (Fig. 3B). In conditioned media, hTPP I proenzyme was absent after BFA treatment, whereas its level was increased after monensin administration. Of interest, in four independent experiments, both bafA and monensin also increased the secretion of fully processed mature enzyme with a mass of ~48 kDa; however, the effect of bafA was much more prominent. This finding, together with our data indicating that processing of hTPP I leading to the formation of a ~48-kDa species takes place in lysosomes, implies that bafA and, to a lesser extent, also monensin induce secretion of mature hTPP I from lysosomes to the culture media. The similar effect of monensin and bafA was previously reported also for other acid hydrolases (37, 43).

Our data showing that maturation of hTPP I was hampered by inhibition of the intracellular transport system or alkalinization of the acidic compartments suggested that in vivo, this enzyme is processed to the mature form in the post-Golgi compartments, most likely endosomes or lysosomes. In an attempt to more closely characterize the intracellular site of hTPP I maturation, we analyzed processing of endocytosed proenzyme after temperature block and 3-methyladenine (3MA) treatment. It is well documented that at temperatures around 20 °C, the receptor-ligand complexes are endocytosed, but they accumulate in the prelysosomal compartments (28). 3MA, like bafA, blocks the transport from late endosomes to lysosomes but does not affect the pH of these compartments (44). In the presence of 3MA, the normal fusion between late endosomes and lysosomes was inhibited in vitro by 50–80% (45).

To analyze the effect of temperature block and 3MA on maturation of hTPP I, we used N2a cells. Under standard conditions, these cells internalize hTPP I by using MPR-mediated endocytosis more efficiently than CLN2 fibroblasts and lymphoblasts (not shown). Because endocytosis proceeds more slowly at 20 °C than at 37 °C (28), we expected that any differences in processing of hTPP I at 20 °C could be visualized more easily in N2a cells than in diseased cells. Cells were incubated for 4 h at 20 °C and 37 °C and then lysed and analyzed by Western blotting. Immunoblots of lysates of cells maintained at 37 °C showed that the majority of internalized proenzyme underwent maturation, whereas the maturation of internalized proenzyme was completely inhibited in cells maintained at 20 °C (Fig. 4A). To confirm that the proenzyme was indeed internalized and not associated with the cell membrane after temperature block, cells maintained for 4 h at 20 °C were extensively washed and incubated for an additional 3 h either at 20 or 37 °C in fresh medium without hTPP I. As shown in Fig. 4B, maturation of the proenzyme was almost completely inhibited in cells maintained even for 7 h at 20 °C, whereas the mature form predominated in cells incubated for 4 h at 20 °C followed by 3 h at 37 °C. Strong inhibition of maturation of endocytosed hTPP I proenzyme was also observed in N2a cells treated with 3MA (Fig. 4C). Thus, impairment of fusion between late endosomes and lysosomes by both temperature block and 3MA inhibited processing of hTPP I proenzyme into the mature form, which strongly suggests that maturation of hTPP I in vivo takes place in lysosomal compartments.

Glycosylation of hTPP I—To characterize glycosylation of hTPP I, we performed in vivo labeling and in vitro deglycosylation experiments. Cells were labeled in vivo with [35S]methionine/cysteine, and hTPP I was immunoprecipitated and in vitro digested with endo H, which removes only the high mannose type of asparagine-linked oligosaccharides, and with...
PNGase F, cleaving off all asparagine-linked oligosaccharides. Immediately after a pulse of \[^{[35S]}\text{methionine/cysteine}\], hTPP I proenzyme was prone to digestion by both endo H and PNGase F, which reduced the mass of the precursor to ~58 kDa (Fig. 5A). This indicates that the proenzyme contains only high mannose type, unmodified oligosaccharides and that this modification accounts for ~10 kDa of the proenzyme total mass. After a chase of 3 h, mature enzyme was found to be partially resistant to endo H digestion, demonstrating that some of its oligosaccharides had been converted to the complex or hybrid type. However, the proenzyme visualized on autoradiograms was still sensitive to endo H, which suggests that it represents a portion of the immature enzyme not yet delivered to the trans-Golgi compartments, where glycoconjugates of the complex type are formed. Consistent with this, hTPP I proenzyme secreted to the culture medium was partially resistant to endo H digestion (not shown).

When the cells were pulse-chased in the presence of tunicamycin, an inhibitor of N-type protein glycosylation, the cellular hTPP I appeared as a ~58-kDa species (Fig. 5B), further confirming that N-type glycoconjugates add 10 kDa to the TPP I mass. Moreover, upon tunicamycin treatment, maturation of the proenzyme was completely inhibited, whereas its secretion was only slightly reduced, which suggests that hTPP I is targeted to lysosomes but not to the extracellular milieu via the glycoconjugate-dependent (MPR) pathway. Of interest, upon tunicamycin treatment, the level of unglycosylated hTPP I precursor migrating at ~58 kDa was distinctly higher than the level of hTPP I proenzyme in untreated cells. By analyzing subcellular distribution of hTPP I after 24 h of tunicamycin treatment by using confocal microscopy, we observed that a portion of hTPP I is present in large vesicular structures associated with the ER compartments, colocalizing with calreticulin, an ER-resident protein involved in protein folding (Fig. 5C). These findings suggest that tunicamycin produces retention of a substantial portion of hTPP I in the ER compartments.

**Proteolytic Processing of hTPP I**—It was demonstrated that autoactivation of TPP I in vitro is a pH-dependent process, being the most efficient in a narrow pH range of 2.5–4 (15).

**Fig. 4.** The effect of temperature block and 3MA on maturation of hTPP I endocytosed by N2a cells. A, cells were maintained in serum-free medium supplemented with 5 μg/ml of hTPP I proenzyme for 4 h at 20 °C (lane 1) or 37 °C (lane 2), lysed, and analyzed by Western blotting with mAb SC4. B, cells were maintained in serum-free medium supplemented with 5 μg/ml of hTPP I proenzyme for 4 h at 4 °C, extensively washed, and incubated for an additional 3 h either at 4 °C (lane 1) or 37 °C (lane 2). Lysates of cells were analyzed on immunoblots. **C**, cells were maintained in serum-free medium supplemented with 5 μg/ml of hTPP I proenzyme for indicated periods in the presence of either vehicle only (Me2SO) or 5 mM 3MA, as indicated, and then lysed and analyzed by Western blotting with mAb SC4. Forty μg of protein are shown per lane.

**Fig. 5.** Glycosylation of hTPP I. **A**, cells were pulse-labeled for 1 h with \[^{[35S]}\text{methionine/cysteine}\] and chased for 0 and 3 h, as indicated. hTPP I was immunoprecipitated, denatured, and digested with endo H or PNGase F or mock-digested, electrophoresed, and analyzed by autoradiography. The asterisks indicate the mature form. **B**, hTPP I was immunoprecipitated from cell lysates and media of untreated cells and cells treated with tunicamycin (5 μg/ml) after 1 h of pulse with \[^{[35S]}\text{methionine/cysteine}\] and 3 h of chase, as indicated, and analyzed by autoradiography. **C**, untreated CHO cells (a–c) and cells exposed for 24 h to tunicamycin (2 μg/ml) (d–f) were double-labeled with pAb RAS307 to hTPP I (a and d) followed by secondary antibodies conjugated to Alexa Fluor 488 (green fluorescence) and calreticulin (b and e) visualized with secondary antibodies conjugated to Cy3 (red fluorescence). Merged images (c and f) were generated by laser-scanning confocal microscopy. Original magnification is ×1000.

However, most of the studies indicate that the lysosomal pH value is around 4.3–5 (46–48). Thus, we hypothesized that because the lysosomal milieu does not provide a favorable environment for spontaneous processing of TPP I zymogen in vivo, another protease could be involved in this process.

To investigate the enzymatic activity capable of in vivo processing of hTPP I, CHO cells overexpressing hTPP I were in vivo labeled and chased in the presence of inhibitors of all major classes of proteases, and TPP I was immunoprecipitated and analyzed by autoradiography. Neither E64 (an inhibitor of cysteine proteases), pepstatin A (an inhibitor of aspartic proteases), leupeptin (an inhibitor of serine and cysteine proteases), nor phosphoramidon (an inhibitor of metalloproteases) significantly affected the intracellular processing of hTPP I (Fig. 6, A and B). However, AEBSF, a specific, potent, and irreversible inhibitor of serine proteases, applied at 0.4 mM, almost totally inhibited the proteolytic maturation of hTPP I (Fig. 6A, lane 3). In some experiments, the amount of immature enzyme also was moderately reduced (up to 30%) upon AEBSF treatment in comparison with untreated cells, whereas the
mature hTPP I was invariably absent. Based on morphological criteria, under the experimental conditions used, AEBSF did not produce cell toxicity.

Because AEBSF may cause nonspecific covalent modification of proteins, in separate experimental approaches, cells were labeled and chased for 3.5 h in a cold medium to allow the proenzyme to mature, and then the inhibitors were applied for an additional 20 h of cold chase. Neither AEBSF nor the other inhibitors studied affected the level of mature enzyme (Fig. 6, A, lanes 5–8, and B, lanes 4–6). Thus, it appears that AEBSF inhibits the process of maturation of hTPP I proenzyme but does not induce any detectable deleterious effect on enzyme already processed.

Nonlinear regression fit of the inhibition data of hTPP I maturation in vivo versus AEBSF concentration produced a hyperbolic curve with an estimated IC_{50} of ~144 μM (Fig. 6C).

We next compared the effect of AEBSF on proteolytic processing of hTPP I with that exerted by another serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), as well as AAF-CMK, a specific inhibitor of tripeptidyl peptidases. In contrast to AEBSF (at 0.4 mM), neither PMSF (at 0.6 mM) nor AAF-CMK (at 1 μM) significantly affected the intracellular processing of hTPP I (Fig. 7A). The level of secretion of hTPP I was not changed by AEBSF treatment, which indicates that the absence of the mature form of the enzyme in cells upon AEBSF administration was not caused by its increased secretion. AEBSF shows higher stability at physiological pH value and superior effectiveness in inhibiting a broad range of serine proteases in comparison with PMSF. Furthermore, specific inhibitory activity of AEBSF only partially overlaps with leupeptin (e.g., leupeptin does not inactivate chymotrypsin and related proteases). These factors can explain the lack of inhibitory effect of PMSF and leupeptin on the proteolytic processing of hTPP I we observed. The lack of AAF-CMK effect on this process suggests that the mature hTPP I already present in lysosomes is not involved in transactivation of hTPP I proenzyme in vivo.
As an inhibitor of serine proteases, AEBSF could potentially also interact directly with TPP I and affect its proteolytic processing in vivo by inhibiting its autoactivation. To investigate this possibility, we examined the effect of AEBSF on hTPP I autoactivation under in vitro conditions and compared it with that exerted by PMSF and AAF-CMK. Inhibitors were first preincubated with the immature hTPP I at pH 7.0 for 30 min and then incubated at pH 3.5 for 30 min at 37 °C, and the samples were analyzed by SDS-PAGE and Western blotting by using mAb against hTPP I. Neither PMSF (at 0.6 mM) nor AEBSF (at 0.4 mM) inhibited self-activation of the hTPP I zymogen in vitro (Fig. 7B). AAE-CMK (at 1 μM) inhibited autoactivation of hTPP I by ~5%. We also analyzed the ability of AEBSF to inhibit the activity of hTPP I in vitro toward a reporter substrate. hTPP I proenzyme was preincubated at pH 3.5 for 20 min to allow for self-activation and then incubated with various concentrations of AEBSF for an additional 30 min. Afterward, TPP I activity was measured, as described under “Experimental Procedures.” As illustrated in Fig. 7C, AEBSF did not inhibit activity of hTPP I in vitro toward the reporter substrate at the concentrations used for in vivo studies (0.4 mM). Slight inhibition (below 20%) of hTPP I activity was found only at higher AEBSF concentrations. Thus, the inhibition of hTPP I processing by AEBSF we observed in vivo could not be attributed either to its effect on autoactivation or activity of hTPP I.

As an additional control, we examined the effect of 4-(2-aminoethyl)-benzene-sulfonamide (AEBSNH₂) on maturation of endocytosed hTPP I proenzyme in N2a cells. AEBSNH₂ is a structural analogue of AEBSF, which is inactive in proteolysis, in which the fluoride group is substituted with -NH₂ (49). AEBS significantly inhibited the proteolytic processing of endocytosed proenzyme in N2a cells, similar to its effect on bio-synthetically labeled hTPP I in CHO cells. In contrast, AEBSNH₂ did not affect the maturation of internalized proenzyme (Fig. 7D). We obtained similar results for CLN2 lymphoblasts and fibroblasts (not shown). This observation further substantiates our thesis that the effect of AEBSF on hTPP I processing in cultured cells is associated with its antiproteolytic activity.

Of interest, AEBSF treatment was not followed by cellular accumulation of the unprocessed proenzyme, which we observed after inhibition of hTPP I maturation by tunicamycin, BFA, monensin, or 3MA. In some experiments, we even observed decreased amounts of the proenzyme in cells treated with AEBSF. This finding suggested that if not processed in lysosomes, hTPP I zymogen was rapidly degraded by lysosomal proteases. To examine this possibility, N2a cells were incubated with hTPP I proenzyme in the presence of AEBSF and inhibitors of all major classes of proteases. As illustrated in Fig. 7E, both E64 and pepstatin A co-incubated with AEBSF significantly increased the amount of hTPP I proenzyme in N2a cells in comparison with that observed in cells incubated with AEBSF alone. This experiment confirms that unprocessed hTPP I proenzyme is rapidly degraded in lysosomes and that cysteine and aspartic proteases participate in this process.

It was recently reported that Ser475 represents the active site nucleophile of hTPP I (15). Thus, we reasoned that this mutation should disturb the normal processing of TPP I, assuming that it is an autocatalytic process. Hence, we analyzed the processing and specific activity of TPP I in primary fibroblasts from a CLN2 subject with a missense mutation in the active site serine (S475L) in one allele and a splice site junction mutation (3556G → C) on the other allele (7). A splice site junction mutation at this position is one of the most common mutations in CLN2 subjects, and according to our data, homozygotes for this mutation display no TPP I activity and no detectable protein on Western blot (27). By enzymatic assay, specific TPP I activity in control fibroblasts was 1.51 nmol-min⁻¹·mg⁻¹. In fibroblasts from the compound heterozygote toward S475L mutation, TPP I activity was undetectable, as in another CLN2 cell line studied (homozygote toward R208STOP) (Fig. 8A). However, when we analyzed fibroblast lysates from the subject with the active site Ser475L mutation (S475L (lane 1), fibroblasts from the CLN2 subject homozygous toward the R208STOP mutation (lane 2), and control fibroblasts (lane 3). Thirty μg of protein are shown per lane (mAb 8C4).

**DISCUSSION**

**Biosynthesis and Intracellular Transport of hTPP I—**The present study demonstrates that hTPP I is synthesized as a zymogen with an apparent mass of 68 kDa, which is converted within a few hours to a ~50-kDa species and then to a fully processed mature enzyme with a mass of 48 kDa. The mature hTPP I expressed in CHO cells is a stable protein with a half-life of ~20 h. In an attempt to identify the subcellular compartment in which the proenzyme of hTPP I matures, we used compounds that interfere with ER to Golgi transport (BFA), Golgi structure and function (monensin), and pH of intracellular acidic organelles (bafA, monensin) as well as inhibitors of fusion between late endosomes and lysosomes (bafA, temperature block, 3MA). All of these treatments inhibited the conversion of hTPP I zymogen to the mature form. Thus, although some acid hydrolases acquire enzymatic activity and function already in the prelysosomal compartments, such as late endosomes (28, 50), early endosomes (51), or even ER (52), our data suggest that maturation of hTPP I takes place in the lysosomes.

We also observed trimming of oligosaccharides on the maturing enzyme in the lysosomes, producing a ~2-kDa reduction in mass, as a late event of hTPP I maturation in biosynthetically labeled CHO cells and after endocytosis of the proenzyme by CLN2 fibroblasts, lymphoblasts, and N2a cells. The role of this late posttranslational modification of hTPP I function is unclear at present. A detailed analysis of the potential functional significance of oligosaccharide removal by lysosomal exoglycosidase in another lysosomal enzyme, glucocerebrosidase, led to the conclusion that oligosaccharide removal simply reflects further maturation of the enzyme and is of no importance to its function (29).

![Fig. 8. hTPP I in fibroblasts from a CLN2 subject with a missense mutation in the active site Ser475L.](image)
It is well documented that overexpressed acid hydrolases are enzymatically active; thus, they are correctly targeted and processed into mature forms. However, production of large amounts of overexpressed enzyme in cells may affect some steps of its normal biosynthetic pathway. One of the biological effects associated with overexpression of acid hydrolases is their increased or induced secretion. Secretion of hTPP I expressed in the CHO cells we observed was also reported by others (22), although under standard conditions, endogenous hTPP I was not released by cultured primary cells (18). Thus, selective secretion of hTPP I appears to result from its overexpression. The cellular mechanisms responsible for secretion of overexpressed hydrolases are still not understood. An aggregation-secretion model proposed in the past (53) suggested that the majority of overexpressed lysosomal enzymes aggregates in the trans-Golgi network, becomes inaccessible for MPRs, and is released from cells by default via the constitutive secretory pathway. However, both our data and the results of others (22) indicate that even if overexpressed hTPP I aggregates in the trans-Golgi network, this is a temporary and reversible process. Another example of posttranslational modifications related to the overexpression of lysosomal hydrolase that we also observed is the different structure of oligosaccharides on the secreted and intracellular glycoforms of lysosomal enzymes (24–26). It is most likely caused by the relative inefficiency of the glycosylation machinery in the trans-Golgi network to completely modify the oligosaccharide chains on enzymes synthesized at a high rate (25). Because overexpressed hydrolases retain the Man-6-P label and are active after endocytosis, they represent a good source of enzyme for the development of enzyme replacement therapy (22, 23, 53).

Of interest, bafA and, to a lesser extent, also monensin not only increased secretion of hTPP I proenzyme, which is a well documented effect of these compounds on acid hydrolases (37–39) but also led to the appearance of the mature, fully processed 48-kDa species in the culture media. Two possible mechanisms could be responsible for this phenomenon. First, bafA and monensin could induce secretion of mature hTPP I from lysosomes by initiating regulated exocytosis of lysosomal content. In support of this hypothesis, release of mature cathepsin D and β-hexosaminidase from lysosomes of human fibroblasts after monensin treatment (37) as well as induction of N-succinyl-β-glucosaminidase secretion by mouse macrophages after bafA treatment (43) has already been reported. Second, hTPP I proenzyme secreted after bafA and monensin treatment could be processed into the mature form in the conditioned media, either autocatalytically or enzymatically (see below). At present, neither of these mechanisms can be definitively confirmed or excluded on the basis of data we have collected. However, because hTPP I precursor is synthesized at a relatively slow rate, the amount secreted during the 3-h chase period is small, and its steady-state level in cells is distinctly lower than that of the mature enzyme, it appears that the first scenario is more probable.

Until recently, the release of acid hydrolases into the extracellular environment was believed to be confined to specialized secretory cells such as mast cells, neutrophils, or cytotoxic T lymphocytes. A compelling line of evidence indicates that numerous other types of cells such as fibroblasts, myoblasts, and epithelial cells including CHO cells release lysosomal acid hydrolases after the fusion of conventional lysosomes with plasma membrane in a Ca\(^{2+}\)-dependent manner (54). The molecular mechanisms of this process, termed regulated exocytosis, are still not entirely understood (for a review, see Ref. 55). Recent data suggest that the lysosomal synaptotagmin isoform Syt VII, a member of the synaptotagmin family of Ca\(^{2+}\)-binding proteins, is involved in regulated exocytosis of lysosomal enzymes mediating plasma membrane repair (56). Monensin facilitates the entry of Ca\(^{2+}\) to the cell by a Na\(^{+}\)-out/ Ca\(^{2+}\)-in exchange (35). Thus, by increasing the influx of Ca\(^{2+}\) into the cell, monensin could potentially induce Ca\(^{2+}\)-regulated exocytosis of lysosomal hydrolases. The results of the studies presented below suggest that bafA also could alter calcium homeostasis in cells. First, at least in certain types of cells, the vacuolar H\(^{-}\)-ATPase is expressed not only on intracellular vesicular structures but also on the cell membrane (for a review, see Ref. 57). Second, experiments in yeast (58) and mammalian cells (59) suggest that vacuolar H\(^{-}\)-ATPase activity may be essential for proper Ca\(^{2+}\) homeostasis. Third, it appears that at least some subunits of vacuolar H\(^{-}\)-ATPase interact with calcium channels (60). However, more experiments are needed to determine whether, indeed, inhibition of activity of vacuolar H\(^{-}\)-ATPase by bafA could affect intracellular ionic gradients and facilitate Ca\(^{2+}\) entry to the cell, thus allowing initiation of a series of events leading to exocytosis of lysosomal enzymes.

Glycosylation of hTPP I—hTPP I has five potential N-glycosylation sites (14). Treatment of cell immunoprecipitates with both endo H, which removes only the high mannose type of asparagine-linked oligosaccharides, and PNGase F, which cleaves off all asparagine-linked oligosaccharides, immediately after pulse with \[^{35}S\]methionine/cysteine reduced the mass of hTPP I proenzyme by ~10 kDa. This finding indicates that newly synthesized hTPP I is modified only by high mannose-type glycans. The same reduction in mass of hTPP I was observed after tunicamycin treatment, which, by inhibiting UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase, precludes N-glycosylation in vivo. The apparent molecular mass of the completely deglycosylated hTPP I we observed is in agreement with the estimated molecular mass of hTPP I deduced from the cDNA sequence. Thus, taking into account that the molecular mass of a high mannose N-glycosylation residue is 1,800–2,000, all potential N-glycosylation sites are utilized in hTPP I. Mature hTPP I, after 3 h of chase, was found to be partially resistant to endo H treatment, which indicates that some N-linked glycoconjugates are of the complex/hybrid type. Glycosylation is a common feature of lysosomal proteins. Carbohydrates contribute to the proper folding and assembly of newly synthesized nascent proteins in the lumen of the ER and promote interaction(s) with the components of the quality control machinery (for a recent review, see Ref. 61). Oligosaccharides also ensure stability and resistance to protease digestion of some lysosomal hydrolases and lysosomal membrane proteins (62–64) as well as intrinsic enzyme activity (65) and solubility (66), although the removal of the carbohydrate moiety from a variety of glycoproteins had no apparent effect on their biological activity or chemical properties (67).

The formation of Man-6-P residues on N-linked carbohydrate moieties on lysosomal hydrolases is also essential for their binding to the MPRs in the trans-Golgi and further transport to the lysosome via the MPR-dependent pathway. According to our data, the lack of N-linked carbohydrates on hTPP I after tunicamycin treatment inhibited intracellular trafficking and the maturation process of hTPP I. This observation demonstrates the importance of oligosaccharide modification for proper lysosomal transport and subsequent maturation of hTPP I. Of interest, upon tunicamycin treatment, the level of unglycosylated hTPP I precursor migrating at ~58 kDa was distinctly higher than the level of hTPP I proenzyme in un-

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\[^2\] A. A. Golabek, E. Kida, M. Walus, P. Wujek, P. Mehta, and K. E. Wisniewski, unpublished data.
treated cells. By using confocal microscopy, we observed that in cells exposed to tunicamycin, a portion of hTPP I is present in large vesicular structures associated with the ER compartments, colocalizing with calreticulin, an ER-resident protein involved in protein folding. It is well documented that inhibition of glycosylation may alter the proper folding of proteins in the ER, leading to their aggregation (for a recent review, see Ref. 68). Tunicamycin is able to trigger the unfolded protein response associated with induction of numerous ER chaperone proteins such as calreticulin, BiP, or GRP94 (69), leading to stabilization of ER-retained proteins (70). Thus, both aggregation in the ER and association with ER chaperone proteins could contribute to the increased stability of the ~58-kDa unglycosylated kTPP I species we observed in cells exposed to tunicamycin. It should be noted that a portion of unglycosylated immature hTPP I was still secreted into the media after tunicamycin treatment. This observation indicates that unglycosylated immature hTPP I was released from the cells by using the MPR-independent, constitutive secretory pathway.

Proteolytic Processing of hTPP I—Proteolytic processing leading to the removal of prodomains of zymogens is a part of the maturation pathway of most, if not all, lysosomal proteases. Prodomains of proteases assist in protein folding and inhibition of enzymatic activity; thus, they prevent nonspecific protein degradation and enable spatial and temporal regulation of proteolytic activity (71, 72).

Previous studies showed that in vitro, hTPP I zymogen is capable of self-activating at acidic pH (15, 22). A detailed analysis of this process at pH 4.0 showed that the zymogen was rapidly ($t_{1/2} = 7$ min) converted to a mature form with a transient appearance of lower molecular mass species, which disappeared at longer time points (15). However, because autoactivation of TPP I in vitro is most efficient in a narrow pH range of 2.5–4 (15), thus at lower pH values than those reported for lysosomes (46–48), we reasoned that in vivo, another protease could be involved in this process.

The results of our studies strongly suggest that in vivo, hTPP I is indeed proteolytically processed by a serine-type protease that is sensitive to AEBSF. According to our data, AEBSF, a potent serine protease inhibitor, did not inhibit hTPP I activity toward a reporter substrate or its autoactivation in vitro. However, it was capable of inhibiting cleavage of hTPP I proenzyme into the mature form in cultured cells with $IC_{50}$ of $-144 \mu M$. Inhibition of maturation of both newly synthesized hTPP I and endocytosed hTPP I by AEBSF in lysosomes caused degradation of unprocessed proenzyme by aspartic and cysteine proteases.

AEBSF is an irreversible and cell-permeable inhibitor of a broad range of serine proteases. Its charged aminomethyl moiety acts as a substrate analogue, forming ionic complexes with proteases, whereas the reactive sulfonil fluoride group enables formation of a stable covalent bond with the enzyme (73). Experiments with AEBSNH₂, a structural analogue of AEBSF, provided further support to our thesis that inhibition of hTPP I maturation in cells treated with AEBSF resulted specifically from its antiproteolytic action. As we have shown, AEBSNH₂, which is inactive in inhibiting serine proteases, was unable to prevent proteolytic cleavage of hTPP I proenzyme in cultured cells.

It was shown previously that a S475A mutant expressed in CHO cells was catalytically inactive, despite its being proteolytically processed, although less efficiently than wild-type protein (15). This suggested that either other protease(s) or CHO cell TPP I could be involved in the cleavage of the catalytically inactive mutant. We had the opportunity to analyze proteolytic processing and activity of hTPP I in fibroblasts from a CLN2 subject heterozygous toward a missense point mutation in the active site nucleophile Ser^475. A splice site junction mutation present in the other allele of the cln2 in this individual produces a frameshift after Phe^689, which precludes production of the active enzyme (27). As we demonstrated, mutant TPP I was processed normally in cultured fibroblasts, despite the fact that mutation of the Ser^475 produced inactive enzyme. This finding clearly illustrates that catalytically inactive TPP I could not be responsible for its self-activation, emphasizing that the cleavage must have been accomplished by an enzyme other than TPP I.

All of these data strongly suggest that although TPP I is capable of self-activating in vitro, a serine-type protease that is sensitive to AEBSF is involved in proteolytic processing of TPP I proenzyme into the mature form in vivo. It should be noted, however, that even if our study implicates a serine protease in TPP I maturation in vivo, it does not preclude the possibility that under certain conditions, such as sufficiently low pH, maturation of TPP I could also proceed via autoactivation in vivo.

TPP I is up-regulated under various pathological conditions such as malignancy, neurodegeneration, ischemia, or inflammation (11, 74); its deficiency causes a fatal lysosomal storage disorder, and to date, it is the only identified tripeptidyl peptide acting in lysosomes, which emphasizes the important role of TPP I for the biology of cells. It is tempting to postulate that participation of another serine protease in TPP I maturation could additionally control the functional availability of TPP I for the cell, apart from its potential regulation at the transcriptional level (14), and ensure a cell type-specific response adequate to the metabolic requirements of the cell under normal and pathological conditions.

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