N-Glycosylation Is Crucial for Folding, Trafficking, and Stability of Human Tripeptidyl-peptidase I*

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Tripeptidyl-peptidase I (TPP I) is a lysosomal serine-carboxyl peptidase that sequentially removes tripeptides from polypeptides. Naturally occurring mutations in TPP I are associated with the classic late infantile neuronal ceroid lipofuscinosis. Human TPP I has five potential N-glycosylation sites at Asn residues 210, 222, 286, 313, and 443. To analyze the role of N-glycosylation in the function of the enzyme, we obliterated each N-glycosylation consensus sequence by substituting Gln for Asn, either individually or in combinations, and expressed mutated cDNAs in Chinese hamster ovary and human embryonic kidney 293 cells. Here, we demonstrate that human TPP I in vivo utilizes all five N-glycosylation sites. Elimination of one of these sites, at Asn-286, dramatically affected the folding of the enzyme. However, in contrast to other misfolded proteins that are retained in the endoplasmic reticulum, only a fraction of misfolded TPP I mutant expressed in Chinese hamster ovary cells, but not in human embryonic kidney 293 cells, was arrested in the ER, whereas its major portion was secreted. Secreted proenzyme formed non-native, interchain disulfide bridges and displayed only residual TPP I activity upon acidification. A small portion of TPP I missing Asn-286-linked glycan reached the lysosome and was processed to an active species; however, it showed low thermal and pH stability. N-Glycans at Asn-210, Asn-222, Asn-313, and Asn-443 contributed slightly to the specific activity of the enzyme and its resistance to alkaline pH-induced inactivation. Phospholabeling experiments revealed that N-glycans at Asn-210 and Asn-286 of TPP I preferentially accept a phosphomannose marker. Thus, a dual role of oligosaccharide at Asn-286 in folding and lysosomal targeting could contribute to the unusual, but cell type-dependent, fate of misfolded TPP I conformer and represent the molecular basis of the disease process in subjects with naturally occurring missense mutation at Asn-286.

Tripeptidyl-peptidase I (TPP I)† (EC 3.4.14.9) is a lysosomal aminopeptidase that sequentially removes tripeptides from polypeptides with an unsubstituted N terminus and uncharged amino acid in the P1 position (1–3). The enzyme also has a minor endopeptidase activity (4).

Mutations in TPP I gene cause the classic late infantile form of neuronal ceroid lipofuscinosis (CLN2, Jansky-Bielschowsky disease) (5, 6). This fatal autosomal recessive lysosomal storage disorder starts at the age of 2–4 years and leads to severe damage of the central nervous system with accumulation of autofluorescent material in lysosomes, massive neuronal loss, and gliosis (7, 8). In humans, TPP I is broadly distributed in various tissues and organs, so its deficiency also produces lysosomal storage at extraneuronal sites, but without significant deficit of function (9). More than 50 disease-associated mutations have been described so far in TPP I gene (www.ucl.ac.uk/NCL). All mutations produce loss or, in rare cases, profound deficiency of TPP I activity and either absence, or only trace amounts, of the protein by immunoblotting (6, 10, 11).

Natural substrates of TPP I are unknown. It was reported that in vitro, TPP I cleaves peptide hormones such as angiotensin II, glucagon (2), substance P (12), sulfated cholecytokinin-8 (3), angiotensin III, and neuromedin B (13) as well as synthetic amyloid-β peptide 1–42 and 1–28 (13) and probably collagen (1) and subunit c of mitochondrial ATP synthase (13, 14), a proteolipid that constitutes about 85% of the protein content of the CLN2 storage and also accumulates in other forms of neuronal ceroid lipofuscinoses, except CLN1 (15).

Human TPP I is synthesized as a preproenzyme that contains a 19-amino acid signal peptide cleaved off cotranslationally and a 176-amino acid propeptide removed during the maturation process to yield a mature enzyme of 386 amino acid residues (5, 16, 17). By SDS-PAGE, the apparent molecular mass of the proenzyme was determined to be 66–68 kDa and that of the mature enzyme, 46–48 kDa (12, 14, 17–19). Similar to various other proteases, TPP I proenzyme is able to autoactivate at acidic pH in vitro (17, 18). However, our studies suggest that in vitro 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-sensitive serine protease assists in the processing of the proenzyme into the mature form in lysosomes (19). The tripeptide analog of the substrate, Ala-Ala-Phe-chloromethyl ketone, efficiently inhibits both exopeptidase and endopeptidase activity of TPP I at nanomolar or low micromolar concentrations (1, 2, 4, 12, 20).

Earlier inhibition studies allowed for classification of TPP I as a serine protease (1). Later, on the basis of the significant similarity of the TPP I sequence to those of unusual bacterial proteases from Pseudomonas and Xanthomonas, which are insensitive to pepstatin like TPP I, Sleat and colleagues (6) endoglycosidase H; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FCS, fetal calf serum; Hek, human embryonic kidney; LAMP, lysosome-associated membrane protein; mAb, monoclonal antibody; MPR, mannose 6-phosphate receptor; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PNGase F, peptid N-glycosidase F; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)glycine; wt, wild-type.

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proposed that TPP I belongs to a family of pepstatin-insensitive carboxylic peptides (6). Enzymes in this family, recently re- named sedolisins or serine-carboxylic peptides, are mostly of bacterial and fungal origin (21). High-resolution crystal structures available to date for two members of this family, sedolisin-B from *Pseudomonas sp.* 101 (22) and kumamolysin from a thermophile, *Bacillus novo* sp. MN-32 (33), revealed a subtilisin-like fold, a unique catalytic triad, Ser-Glu-Asp, and an aspartic acid residue in the oxyanion hole (21). Mutational analysis of TPP I identified Ser-475 as the active site nucleophile and two acidic residues, Asp-360 and Asp-517, as important for catalytic activity (17), supporting the concept that TPP I is the first sedolisin identified in mammalian cells.

TPP I has five potential N-glycosylation consensus sequences (Asn-Xaa-Ser/Thr), with glycan accepting Asn at amino acid positions 210, 222, 286, 313, and 443. Our deglycosylation experiments with endoglycosidase F and H suggested that all of these potential sites are utilized and that N-linked glycans add around 10 kDa to the molecular mass of TPP I, indicating that the enzyme is extensively glycosylated (19). In the present study, we characterized more closely the role of N-glycosylation in the function of TPP I.

N-Linked glycans are essential for several biological processes, which collectively ensure proper function of lysosomal hydrolases. The best characterized role is that of lysosomal targeting of acid hydrolases. The binding of mannose 6-phosphate marker(s) on N-linked oligosaccharides of acid hydrolases by the mannose 6-phosphate receptors (MPRs) in the trans-Golgi apparatus enables their transport to the late endosomes and then, after dissociation from MPRs, to lysosomes (24). Under normal conditions, only a few lysosomal enzymes such as acid phosphatase (25), glucocerebrosidase and prosaposin (26), and partially, α-glucosidase (27), reach lysosomes via an MPR-independent pathway. According to our data and those of others (18, 19), TPP I is targeted to lysosomes in an MPR-dependent manner.

Apart from mediating lysosomal sorting and transport, N-linked glycans play a prominent role in the folding of newly synthesized lysosomal enzymes in the endoplasmic reticulum (ER) (28, 29). Mutational analyses also disclosed that obliteration of N-glycosylation sites may alter the activity of lysosomal enzymes (29, 30) and enhance their degradation in the ER (28, 29). Mutational analyses also disclosed that obliteration of N-glycosylation sites may alter the activity of lysosomal enzymes (29, 30) and enhance their degradation in the ER (28, 29). Mutational analyses also disclosed that obliteration of N-glycosylation sites may alter the activity of lysosomal enzymes (29, 30) and enhance their degradation in the ER (28, 29).

To investigate the role of each N-glycosylation site in the function of TPP I, we generated N-glycosylation-deficient mutants by substituting glutamine for asparagine in each N-glycosylation consensus sequence of human TPP I, either individually or in combination, by site-directed mutagenesis using PCR. Normal and mutated cDNAs were expressed in Chinese-hamster ovary (CHO) cells and human embryonal kidney (HEK293) cells. Our study shows that all five potential N-glycosylation consensus sites are utilized by human TPP I in *vivo* and that Asn-286-linked glycan is essential for both folding and lysosomal targeting of the enzyme. We also demonstrate that misfolded enzyme expressed in CHO cells is only partially retained in the ER, whereas its significant portion is secreted efficiently by using a constitutive secretory pathway. To the best of our knowledge, TPP I is the first lysosomal enzyme to be identified in which a single N-linked glycan is involved in both folding and lysosomal targeting and the first protein to be identified which is secreted abundantly in a misfolded state when expressed in mammalian cells.

**EXPERIMENTAL PROCEDURES**

*Reagents—*Cell culture media and reagents were purchased from Invitrogen. Enhanced Chemiluminescence (ECL) kit reagents were purchased from Amersham Biosciences. The biocinchonic acid (BCA) kit was from Pierce. [35S]Methionine/cysteine (>1,000 Ci/nmol, Tran[35S]-label) and [32P]Orthophosphoric acid were purchased from ICN. The Vectashield mounting medium was from Vector. pcDNA3.1Hygro vector was from Invitrogen.

Protease inhibitor mixture (Complete), Endoglycosidase H (Endo H), peptide-N-glycosidase F (PNGase F), neuraminidase (from Clostridium perfringens), and FuGENE 6 transfection reagent were from Roche Applied Science. Restriction enzymes and ligase were from New England Biolabs. All other chemicals were from Sigma.

*Antibodies—*Monoclonal antibodies (mAbs BC4 and 2E12) and affinity-purified polyclonal antibodies (pAbs RAS307), which we raised to human recombinant TPP I expressed and purified from *Escherichia coli*, have been described previously (9, 19). mAbs to lysosome-associated membrane protein (LAMP) I, hamster- and human-specific, were from the Developmental Studies Hybridoma Bank, University of Iowa. pAbs against calreticulin were from Affinity Bioreagents, and pAbs against protein disulfide isomerase were from Stressgen. Peroxidase-conjugated secondary antibodies for ECL were from Amersham Biosciences. Secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 555 were from Molecular Probes, and secondary antibodies conjugated to Cy3 were from Jackson ImmunoResearch.

*Site-directed Mutagenesis—*First, we prepared five individual cDNAs so that each was missing one N-glycosylation consensus sequence, Asn-210Ser/Thr, by creating codons for Gin instead of Asn. The cDNA encoding wild-type (wt) human TPP I cloned into the KpnI/NotI site of pcDNA3.1Hygro was described earlier (19). Mutations were generated using the overlap PCR technique according to Ho et al. (32). In the first step, two DNA fragments were amplified: one by using forward TPP I-KpnI primer and antisense mutant primer (F), and the second by using sense mutant primer (R) and reverse TPP I-NotI primer. The two gel-purified fragments were mixed and reamplified using forward TPPI-KpnI and reverse TPP-I-NotI primers. Forward primers were as follows: F-N210Q, 5'-GGCTGTTTTGGCTGGTGCCAGAGCC-3'; F-N222Q, 5'-GGCTGTTTTGGCTGGTGCCAGAGCC-3'; F-N313Q, 5'-GGCTGTTTTGGCTGGTGCCAGAGCC-3'; and F-N443Q, 5'-GGCTGTTTTGGCTGGTGCCAGAGCC-3'. Reverse primers were as follows: R-N210Q, 5'-CCACGTTCTGTTGGAAGGTAATGATTATCTTACG-3'; R-N222Q, 5'-GGAACCGCTTGGTCCACCTTCACAAGAAGC-3'; R-N313Q, 5'-GGAACCGCTTGGTCCACCTTCACAAGAAGC-3'; and R-N443Q, 5'-GGAACCGCTTGGTCCACCTTCACAAGAAGC-3'.

cDNAs containing single mutations at individual N-glycosylation sites served as templates for further mutagenesis until a construct was obtained which contained all five N-glycosylation sites mutated (double site, triple site, quadruple site mutants and a mutant in which all N-glycosylation sequons were obliterated). In all of these constructs, codons for Gin were replaced by codons for Gin. Full-length TPP I cDNAs containing desired mutation(s) were subcloned into the KpnI/NotI site of pcDNA3.1Hygro. The structural integrity of each mutated cDNA was verified by PCR-mediated dideoxy sequencing of the entire insert.

*Cell Culture and Transfection—*Cells were maintained in F-12 medium (CHO cells, ATCC CCL-61) or Dulbecco's modified Eagle medium (HEK293 cells, ATCC CCL-137, and Neuro-2a cells, ATCC CCL-131), both supplemented by 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics at 37 °C in a humidified atmosphere with 5% CO2. One day before transfection, CHO and HEK293 cells were seeded on 35-mm culture dishes. Cells were transfected by using FuGENE 6, according to the manufacturer's recommendation, either with circular (transient transfectants) or linearized plasmid (for stably transfected cells). 2–3 days after transfection, cells were either collected or subcultured and maintained in selection medium supplemented by 200 μg/ml hygromycin B. Single colonies were picked up, expanded, and tested for TPP I using Western blotting and enzymatic activity assay. The highest expressers (at least two different cell lines of stably transfected cells) were used for further experiments.

*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 (Complete) (lysis buffer) or in a buffer containing 20 mM ammonium formate, pH 3.5, 0.1% Triton X-100, and protease inhibitor mixture (Complete) (acidic lysis buffer). Protein concentration was de-
termined by using the BCA assay and bovine serum albumin as a standard. 10–40 μg of protein/lane was loaded onto 10% Tris-Tricine PAGE. Before running on SDS-PAGE, cell culture media were cleared by centrifugation at 14,000 × g for 5 min. Electrophoretically separated proteins were electrotransferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST), incubated overnight with primary antibodies, washed extensively in PBST, incubated with peroxidase-conjugated secondary antibodies diluted 1:5,000, and developed by using the ECL.

Immunofluorescence Microscopy—For double immunostaining, cells grown in LabTek chamber slides (Nunc International) 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 diluted in 10% FCS in PBS overnight at 4 °C, cells were washed with PBS and incubated for 1 h at room temperature with species-specific secondary antibodies conjugated with fluorescent dyes: Alexa Fluor 488 (for TPP I; green fluorescence) and either Cy3 or Alexa Fluor 555 (for LAMP I, protein disulfide isomerase, and calreticulin; red fluorescence). The coverslips were mounted with Vectashield medium and viewed with a Nikon Eclipse E600 laser scanning confocal microscope. Omission of the primary antibodies was used as a control of the method.

In Vivo Labeling and Immunoprecipitation—Cells grown to 80–90% confluence either in 35-mm or 60-mm dishes were starved for 1 h either in methionine- and cysteine-free Dulbecco’s modified Eagle medium with 5% dialyzed FCS or in PO4-free Dulbecco’s modified Eagle medium with 5% non-fat dry milk in PBS for 1 h. After incubation with primary antibodies diluted in 10% FCS in PBS overnight at 4 °C, cells were washed with PBS and incubated for 1 h at room temperature with species-specific secondary antibodies conjugated with fluorescent dyes: Alexa Fluor 488 (for TPP I; green fluorescence) and either Cy3 or Alexa Fluor 555 (for LAMP I, protein disulfide isomerase, and calreticulin; red fluorescence). The coverslips were mounted with Vectashield medium and viewed with a Nikon Eclipse E600 laser scanning confocal microscope. Omission of the primary antibodies was used as a control of the method.

N-Glycosylation of Human TPP I

Human wtTPP I expressed in CHO cells appears on immunoblots of cell lysates as two species: a minor band with a molecular mass of ~68 kDa corresponding to a proenzyme and a major band with a molecular mass of ~48 kDa corresponding to the processed, mature form (Fig. 1A, lane 7). A portion of the proenzyme is also secreted into the culture medium (Fig. 1B, lane 7). The molecular mass of secreted proenzyme is slightly, by ~2 kDa, higher than the molecular mass of the cellular proenzyme, which is because of the heterogeneous glycosylation pattern (19). As shown in Fig. 1, A and B, all mutated proteins with a single N-glycosylation site abrogated demonstrated increased mobility on immunoblots of cell lysates and conditioned media compared with wtTPP I. This finding indicates that the human enzyme in vivo utilizes all five potential N-glycosylation sites, which is consistent with our earlier data from deglycosylation experiments (19).

Apart from the difference in molecular masses, one mutant protein, ΔGS3 (N286Q), also demonstrated an altered ratio of the proenzyme-mature form compared with wtTPP I and other N-glycosylation-deficient proteins (Fig. 1A, lane 4). By densitometry analysis of immunoblots (Fig. 1C), the proenzyme constituted about 78% of the total ΔGS3 protein in cell lysates, whereas the proenzyme of wtTPP I and other N-glycosylation mutants did not exceed 7% of the total TPP I in cell lysates. A similar pattern of ΔGS3 mutant processing was observed in transiently transfected CHO (not shown) and HEK293 cells (see Fig. 11). These data indicated that the oligosaccharide at Asn-286 is important for the maturation of human TPP I.

In addition, as shown in Fig. 1A, the molecular mass of the cellular proenzyme of ΔGS3 mutant, but not of its processed form or secreted proenzyme (Fig. 1B), was slightly smaller than that of other mutant proteins. This finding suggested that in the absence of glycan at Asn-286, the structure of oligosaccharides at other Asn residues of the cellular TPP I proenzyme may be modified. Proenzyme of TPP I visualized on immunoblots of cell lysates is fully sensitive to Endo H and represents the pool of newly synthesized TPP I still residing in the ER (Ref. 19 and see Fig. 9B); thus, it contains only core glycans (Glc3Man1GlcNAc2) (33).

To investigate whether a shift in electrophoretic mobility of ΔGS3 mutant resulted from oligosaccharide modification, CHO cells expressing ΔGS3 mutant were metabolically radiolabeled in the presence of 1-deoxymannojirimycin (inhibitor of ER mannosidases) or castanospermine (inhibitor of ER glucosidases) (34). As shown in Fig. 2A, after only 15 min of pulse, cellular proenzyme of ΔGS3 mutant appeared as a broad band migrating faster on SDS-PAGE than the proenzyme of ΔGS3 mutant, which indicates that the structural modification responsible for altered SDS-PAGE mobility of ΔGS3 mutant occurred in the ER, early during its biosynthesis. Deoxymannojirimycin blocked this electrophoretic mobility shift and led to the generation of two species of ΔGS3 proenzyme, one showing mobility similar to ΔGS1 mutant in untreated cells, and the other migrating slightly slower. After castanospermine treatment, the major species of ΔGS3 proenzyme were also produced; however, both of them had higher molecular masses than that of untreated proenzyme of ΔGS1 and ΔGS3 mutants. Together, these experiments demonstrate that soon after biosynthesis, core glycans of TPP I mutant protein with an eliminated glycosylation site at Asn-286 undergo modifications producing forms with heterogeneous content of glucose residues

RESULTS

Human TPP I Contains Five N-Linked Glycans, of Which One at Asn-286 Is Involved in Maturation of the Enzyme— Human TPP I has five potential N-glycosylation sites at Asn residues 210, 222, 286, 313, and 443; thus, they are all located in the mature enzyme. To determine which of these sites are utilized in vivo, we eliminated each potential N-glycosylation site in TPP I cDNA by converting the Asn codon to a Gln codon.

cDNAs encoding the TPP I with one individual N-glycosylation site abrogated (ΔGS1, N210Q; ΔGS2, N222Q; ΔGS3, (N286Q; ΔGS4, N313Q; and ΔGS5, N443Q) were stably expressed in CHO cells. As a control, we used CHO cells stably transfected with cDNA encoding wtTPP I, and mock-transfected cells.

Human wtTPP I expressed in CHO cells appears on immunoblots of cell lysates as two species: a minor band with a molecular mass of ~68 kDa corresponding to a proenzyme and a major band with a molecular mass of ~48 kDa corresponding to the processed, mature form (Fig. 1A, lane 7). A portion of the proenzyme is also secreted into the culture medium (Fig. 1B, lane 7). The molecular mass of secreted proenzyme is slightly, by ~2 kDa, higher than the molecular mass of the cellular proenzyme, which is because of the heterogeneous glycosylation pattern (19). As shown in Fig. 1, A and B, all mutated proteins with a single N-glycosylation site abrogated demonstrated increased mobility on immunoblots of cell lysates and conditioned media compared with wtTPP I. This finding indicates that the human enzyme in vivo utilizes all five potential N-glycosylation sites, which is consistent with our earlier data from deglycosylation experiments (19).

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(most likely nonglucosylated and monoglucosylated forms) and extensively trimmed mannose residues. Castanospermine and deoxymannojirimycin treatment also increased the amount of ΔGS3 proenzyme secreted to the culture medium, by around 1.5- and 3-fold, respectively (Fig. 2B). Decreased molecular mass of secreted proenzyme of ΔGS3 mutant after deoxymannojirimycin treatment compared with that of ΔGS3 proenzyme in untreated cells reflects the altered completion of complex sugars on untrimmed core glycans in the Golgi apparatus.

**Lack of Asn-286-linked Glycan Partially Arrests TPP I in the ER**—According to our earlier study, maturation of TPP I proenzyme takes place in lysosomes (19). Thus, the presence of the mature TPP I species on immunoblots of lysates of CHO cells expressing ΔHG9004 GS1, ΔHG9004 GS2, ΔHG9004 GS4, and ΔHG9004 GS5 mutants suggested that these polypeptides were correctly targeted to lysosomes. In contrast, the striking predominance of the proenzyme over the mature form in lysates of ΔHG9004 GS3 mutant pointed to altered intracellular trafficking of this mutant protein. To determine
whether, and which, glycosylation-deficient mutant proteins reach lysosomes, we examined the subcellular distribution of mutated polypeptides in stably transfected CHO cells. Laser scanning confocal microscopy analysis showed that ∆GS1, ∆GS2, ∆GS4, and ∆GS5 mutant proteins and likewise wtTPP I colocalized with LAMP I, a lysosomal marker; thus, they were indeed transported to lysosomes (Fig. 3). However, ∆GS3 mutant protein was detected in very few vesicular structures immunopositive to LAMP I, whereas the bulk of immunoreactivity showed a pattern resembling the ER distribution. Colocalization studies with antibodies to calreticulin (Fig. 4) and protein disulfide isomerase (not shown), the ER markers we used, confirmed that a significant portion of ∆GS3 mutant protein is retained in the ER.

To elaborate further the role of N-linked glycans in TPP I maturation and lysosomal targeting, we created several constructs encoding human TPP I with combinations of two N-glycosylation sites eliminated as well as constructs in which three, four, and all five N-glycosylation consensus sequences were obliterated. Immunoblot analysis of lysates of CHO cells transiently transfected with mutated cDNAs demonstrated that the ratio of the proenzyme to the mature enzyme was altered, and that the level of the processed form was severely reduced or undetectable in all expressed double site mutant proteins if one of the mutated sites was Asn-286 (Fig. 5A, lanes 4, 7, 10, and 11; Fig. 5B). In addition, the molecular mass of the proenzyme of all proteins in which Asn-286-linked oligosaccharide was missing was slightly, but distinctly, lower than that of other double site mutants and thus, similar to data we obtained for ∆GS3 mutant protein.

The ratio of proenzyme to mature enzyme was also altered in some other mutant proteins with two or three oligosaccharides missing compared with wtTPP I, but to a lesser extent than in polypeptides with Asn-286 mutated (Fig. 5, A and B). Mutated protein in which all N-glycosylation sites were abrogated was not processed. Interestingly, mutant protein in which all N-glycosylation sites except Asn-286 were removed (∆GS1, 2, 4, 5) was also unprocessed (Fig. 5A, lane 14). Immunofluorescence study of CHO cells transiently transfected with cDNA encoding ∆GS1, 2, 4, 5 mutant protein demonstrated its mostly ER localization (Fig. 5C).

Data presented above indicate that Asn-286-linked glycan is critical for lysosomal targeting and processing of human TPP I. However, although Asn-286-linked glycan fully compensated for each single N-linked oligosaccharide that was missing, it could not ensure proper maturation of TPP I when multiple other oligosaccharides were eliminated simultaneously. This observation demonstrates that sugar moieties at Asn-210, Asn-222, Asn-313, and Asn-443, collectively, assist Asn-286-linked glycan in TPP I maturation in vivo. Because tunicamycin affected trafficking and maturation of human TPP I expressed in CHO cells in a similar way (19), it appears that elimination of N-glycosylation consensus sequences per se, and not introduction of several mutations into TPP I molecule, was responsible for the ER retention of multiple N-glycosylation site mutants which we observed.

Prolonged Half-life and Dimerization of TPP I Lacking Asn-286-linked Oligosaccharide—Correct folding is necessary for lysosomal hydrolases as well as membrane and secretory proteins to exit the ER (35). Our initial experiments showing that a significant portion of ∆GS3 mutant protein is retained in the ER suggested that this mutant protein did not reach a native conformation, implying that Asn-286-linked glycan may participate in the folding of TPP I. To address this issue, first we analyzed the biogenesis of the single N-glycosylation-site mutant proteins.

Stably transfected CHO cells expressing wtTPP I and single N-glycosylation site mutant proteins were pulse labeled with [35S]methionine/cysteine and harvested after the indicated periods (Fig. 6). Autoradiographic images showed that wtTPP I is synthesized as a precursor protein with a molecular mass of ~68 kDa, which disappears gradually as the ~50-kDa species corresponding to the mature enzyme increases in intensity (Fig. 6, bottom right panel). At ~8 h of chase, mature enzyme appears on autoradiogram as a broad band with a ~48-kDa or as a 48–50-kDa doublet, which is caused by the trimming of its oligosaccharides by lysosomal exoglycosidases (19). Biogenesis of ∆GS1, ∆GS2, ∆GS4, and ∆GS5 mutant proteins was similar to that of wtTPP I, except that the molecular masses of the proenzyme and mature enzyme were lower by around 2 kDa than that of wtTPP I. In contrast, ∆GS3 mutant protein showed an altered ratio of the proenzyme to the mature form at all chase periods studied (Fig. 6, bottom left panel). Because of the extensive lysosomal trimming of oligosaccharides generating multiple species of processed polypeptides (especially prominent in ∆GS1, ∆GS3, and ∆GS5), we were not able to estimate...
reliably the half-life of the mature form of mutant proteins. The estimated half-life of the proenzyme to the mature form for wtTPP I and each mutant protein was calculated based on densitometry analysis of immunoblots. C, laser scanning confocal microscopy analysis of CHO cells transiently expressing ΔGS1, 2, 4, 5 mutant protein. Upper panel, cells were immunostained with pAbs to TPP I (green fluorescence of secondary antibodies labeled with Alexa Fluor 488) and mAbs to LAMP I, a lysosomal marker (red fluorescence of Alexa Fluor 555-conjugated secondary antibody). Lower panel, cells were immunostained with mAbs to TPP I (green fluorescence of secondary antibodies labeled with Alexa Fluor 488) and pAbs to protein disulfide isomerase, an ER marker (red fluorescence of Alexa Fluor 555-conjugated secondary antibody). The yellow color on merged images shows colocalization. Note that ΔGS1, 2, 4, 5, thus TPP I mutant protein with a sole N-glycosylation site at Asn-286 remaining intact, was localized mostly to the ER and absent in lysosomes. Original magnification, ×1,000.

Fig. 5. Elimination of multiple N-glycosylation sites affects maturation and lysosomal targeting of human TPP I. A, immunoblots of lysates of CHO cells transiently expressing wtTPP I and TPP I with multiple N-glycosylation sites obliterated grown in culture medium supplemented with 5 mM mannose 6-phosphate are shown. Immunoblots were developed with mAbs to TPP I. B, the ratio of the proenzyme to the mature form for wtTPP I and each mutant protein was calculated based on densitometry analysis of immunoblots. C, laser scanning confocal microscopy analysis of CHO cells transiently expressing ΔGS1, 2, 4, 5 mutant protein. Upper panel, cells were immunostained with pAbs to TPP I (green fluorescence of secondary antibodies labeled with Alexa Fluor 488) and mAbs to LAMP I, a lysosomal marker (red fluorescence of Alexa Fluor 555-conjugated secondary antibody). Lower panel, cells were immunostained with mAbs to TPP I (green fluorescence of secondary antibodies labeled with Alexa Fluor 488) and pAbs to protein disulfide isomerase, an ER marker (red fluorescence of Alexa Fluor 555-conjugated secondary antibody). The yellow color on merged images shows colocalization. Note that ΔGS1, 2, 4, 5, thus TPP I mutant protein with a sole N-glycosylation site at Asn-286 remaining intact, was localized mostly to the ER and absent in lysosomes. Original magnification, ×1,000.

SDS-PAGE analysis (not shown). However, when we analyzed cell lysates and conditioned medium of CHO cells expressing wtTPP I and N-glycosylation mutants by the nonreduced SDS-PAGE, an additional strong band of ~140 kDa appeared in lysates and secretions of CHO cells expressing ΔGS3 protein (Fig. 7A). As was revealed by pulse-chase experiments analyzed under nonreducing conditions (Fig. 7B), this band was visible in immunoprecipitates of ΔGS3 expressing cells already at 0 time of chase; thus, it appeared early during the biosynthesis of the mutant protein and persisted, although it was less intense, until the last time point examined—24 h of chase. In
conditioned medium of CHO cells expressing ΔGS3 mutant examined by the nonreduced SDS-PAGE, the ~140 kDa band predominated at both time points evaluated (2 and 24 h). The appearance of this band under nonreducing conditions, its resistance to SDS boiling, its molecular mass, and its presence in cell secretions all indicated that the 140-kDa species represents a homodimer of ΔGS3 proenzyme stabilized by non-native, interchain disulfide bonds rather than a complex of ΔGS3 mutant with another protein.

Thus, both prolonged half-life with ER retention and formation of non-native, interchain disulfide bonds by ΔGS3 protein support the idea that a major portion of ΔGS3 mutant protein did not acquire a native conformation.

N-Linked Glycans Affect Autoactivation, Activity, and Stability of TPP I—TPP I proenzyme is able to autoactivate rapidly in acidic pH in vitro. This process leads to removal of the prodomain, generation of the 48-kDa species, and acquisition of the enzymatic activity toward a reporter substrate (18, 19). To determine whether N-glycans participate in generation of the active enzyme, first we investigated the ability of the N-glycosylation mutants to autoprocess in vitro. Under the experimental conditions employed, wtTPP I was fully processed into the mature form in both cell lysates and cell secretions (Fig. 8A and B, lane 6, compare with Fig. 1, A and B). In lysates of CHO cells expressing ΔGS1, ΔGS2, ΔGS4, and ΔGS5 mutants, also only mature TPP I species were present (Fig. 8A, compare with Fig. 1A). Similarly, in cell secretions, the mature species of ΔGS1, ΔGS2, ΔGS4, and ΔGS5 mutants distinctly prevailed, and only trace amounts of these polypeptides remained in the unprocessed forms (Fig. 8B, lanes 1, 2, 4, and 5, compare with Fig. 1B). However, activation in pH 3.5 was not able to induce the processing of ΔGS3 precursor noticeably. The level of the mature form of ΔGS3 polypeptide did not increase in cell lysates upon incubation at pH 3.5 compared with non-incubated lysates, and only minute amounts of the mature ΔGS3 polypeptide could be seen in cell secretions upon prolonged exposure of immunoblots (Fig. 8, A and B, lane 3), even after incubation at pH 3.5 for longer than 1 h at 37 °C (not shown). Of note, although the level of the mature form of ΔGS3 mutant did not increase after incubation of cell lysates at pH 3.5, the amount of the proenzyme was reduced under these conditions (compare Fig. 8A with Fig. 1A). This observation suggests that at this acidic pH, ΔGS3 proenzyme either was degraded in cell lysates or it aggregated.

To assess whether N-glycans directly affect the enzymatic properties of TPP I, we examined the specific activity of mutant proteins with single N-glycosylation sites obliterated. As depicted in Figs. 8, C and D, specific TPP I activity toward a reporter substrate in lysates of cells expressing ΔGS1, ΔGS2, ΔGS4, and ΔGS5 was 5–15% lower than that of wtTPP I. In cells expressing ΔGS3 mutant, specific TPP I activity did not exceed ~17% of the values obtained for wtTPP I (Fig. 8C). Specific TPP I activity in cell secretions of ΔGS1, ΔGS2, ΔGS4, and ΔGS5 mutants was 20–35% lower than that of wtTPP I. Conditioned medium of CHO cells expressing ΔGS3 mutant showed only residual activity (~2% of wtTPP I values) (Fig. 8D). The higher specific activity of ΔGS3 mutant protein in cell lysates than in culture medium was most likely caused by the presence of higher amounts of mature form of ΔGS3 mutant in cell lysates than in cell secretions exposed to pH 3.5 in vitro. However, when we expressed the specific activity of ΔGS3 mutant as the ratio of the total activity measured to the amount of mature form of ΔGS3 polypeptide in cell lysate, the values obtained were higher (by ~60%) than those of wtTPP I (not shown). In conjunction with the colocalization studies, this result suggests that a small portion of the proenzyme of ΔGS3 mutant protein was able to reach a native or near native conformation, was targeted to the lysosomes, and processed to an active form.

One of the cardinal features of correctly folded proteins is their thermal stability. Therefore, we analyzed whether N-glycans contribute to the thermal stability of human TPP I. As shown in Fig. 8E, only elimination of the glycosylation site at Asn-286 (ΔGS3 mutant) significantly reduced the thermal stability of human TPP I, with the remaining activity not exceeding ~25% of the initial values after a 30-min incubation at 50 °C.

Finally, to investigate whether N-linked glycans also contribute to protection against alkaline pH-induced TPP I inactivation (13), we measured specific TPP I activity in lysates of CHO cells expressing mutant proteins and wtTPP I that were preincubated for various periods at pH 7.0. As shown in Fig. 8F, mutant proteins ΔGS1, ΔGS2, ΔGS4, ΔGS5, and wtTPP I showed a gradual loss of specific TPP I activity which was mild (up to ~10%) after a 5-min incubation and reached ~45–50% for mutant proteins and ~30% for wtTPP I at a 30-min incubation. Thus, at all time points, the remaining activity of ΔGS1, ΔGS2, ΔGS4, and ΔGS5 mutant proteins was only slightly or moderately (up to 20%) lower than the remaining activity of wtTPP I. In contrast, ΔGS3 mutant showed a dramatic loss of
specific TPP I activity already after a 5-min incubation at pH 7.0, with the remaining activity not exceeding ~20% of starting values after a 30-min incubation.

Thus, we conclude that Asn-286-linked glycan is critical for autoprocessing and stability of TPP I, whereas glycans at Asn-210, 222, 313, and 443 contribute only slightly to the specific activity of the enzyme and its resistance to alkaline pH-induced inactivation.

Increased Secretion of Misfolded TPP I Lacking Asn-286-linked Glycans—The data presented above indicated that the major fraction of ΔGS3 mutant protein produced by CHO cells is misfolded. Despite this, as we showed in Figs. 1, 2, 6, A and B, and 8B, only some part of incorrectly folded ΔGS3 mutant protein was arrested in the ER, whereas its significant portion was secreted into the culture medium. To characterize more closely the mechanism of secretion of misfolded protein, first we examined the level of secretion of wtTPP I and mutant proteins stably expressed in CHO cells by pulse-chase experiments and densitometry scanning analysis. As shown in Fig. 9A, the amount of secreted proenzyme of ΔGS3 mutant was severalfold higher than that of wtTPP I and ΔGS1, ΔGS2, ΔGS4, and ΔGS5 mutants at both time points of chase studied. Of note, the amount of ΔGS3 proenzyme recovered from cell secretions was distinctly higher at 7 than at 24 h of chase, in contrast to wtTPP I and other glycosylation-deficient mutants, which showed higher levels of secretion at 24 h than at 7 h of chase. This finding indicates that secreted ΔGS3 proenzyme was not stable and underwent degradation in the extracellular milieu. ΔGS1 mutant was also secreted more abundantly than was wtTPP I (by ~2-fold); however, its secretion was lower than that of ΔGS3 mutant.

To investigate whether secreted ΔGS3 mutant passed the Golgi apparatus and entered the constitutive secretory pathway, we performed deglycosylation studies under steady-state conditions. Cellular proenzyme of wtTPP I and proenzyme of ΔGS3 mutant were fully sensitive to Endo H treatment (Fig. 9B); thus,
both were only “core-glycosylated,” pointing to ER localization. Mature forms of wtTPP I and ΔGS3 mutant protein were partially resistant to Endo H, indicating that they acquired complex/hybrid-type sugars on their way to lysosomes. However, in cell culture media, both wtTPP I proenzyme and ΔGS3 proenzyme were partially resistant to Endo H treatment (Fig. 9C), which evidenced that not only wtTPP I proenzyme but also proenzyme of incorrectly folded ΔGS3 mutant must have entered at least the medial Golgi compartment, where formation of complex-type sugars on polypeptides is initiated. Treatment of cell secretions with neuraminidase showed a slight but distinct reduction in size of the proenzyme of both wtTPP I and ΔGS3 mutant protein (Fig. 9D), which indicates that these polypeptides contained sialylated oligosaccharides. Because sialyltransferase acts in the medial Golgi compartment (37), this last observation allows us to conclude that although misfolded, the proenzyme of ΔGS3 mutant was able to reach and pass the trans-Golgi compartment on its secretory pathway.

Incomplete Degradation of Misfolded ΔGS3 Mutant by the Proteasome—Note in Fig. 9B, left panel (asterisk), an additional band with an apparent 35-kDa of lysates of CHO cells expressing ΔGS3 mutant protein. This TPP I species that is fully sensitive to Endo H appeared in variable amounts in lysates of CHO cells expressing ΔGS3 mutant as well as in transiently transfected HEK293 cells expressing ΔGS3 mutant protein (see Fig. 11). Treatment of cells with β-lactone, a specific and irreversible inhibitor of the proteasome (38), reduced the intensity of this band by ~80%, concomitantly increasing the amount of the unprocessed form (Fig. 9E). This finding evidenced that the 35-kDa species represents a product of incomplete degradation of ΔGS3 mutant by the proteasome.

Asn-210- and Asn-286-linked Sugars Are Preferentially Phosphorylated in Human TPP I—According to our studies and those of others, human TPP I utilizes an MPR-dependent pathway for lysosomal targeting (18, 19). Thus, we investigated which of the five N-linked sugar moieties of human TPP I are phosphorylated on mannose residues. Altered maturation of ΔGS3 protein might hamper unequivocal identification of phosphorylated mannose residues in 32PO4-labeled cells expressing this mutant. Thus, on the basis of our data showing that wtTPP I and all single N-glycosylation site mutant proteins were secreted efficiently, we decided to use cell secretions for these experiments.

As is demonstrated in Fig. 10A, both wtTPP I and all single N-glycosylation site mutants contained 32PO4 label. This finding shows that human TPP I is phosphorylated on more than one oligosaccharide. Because Endo H treatment completely released the label from the immunoprecipitated enzymes, phosphorylation occurred on mannose residues of the oligosaccharides (not shown). Adjustment of the values obtained after phospholabeling according to the densitometry scanning of immunoblots (Fig. 10B) showed that the amount of the label was reduced significantly (by ~50%) in ΔGS1 and ΔGS3 mutant protein (Fig. 10C), which suggests that mannose residues at Asn-210 and Asn-286 are phosphorylated preferentially in the TPP I molecule.
The secretions of CHO cells stably expressing wtTPP I and maintained in 6-well plates were exposed continuously for 24 h to age of values obtained for wtTPP I proenzyme (Fig. 9A). The level of phosphorylation was calculated as a ratio of pixel density of each corresponding band on phosphorimager and immunoblot and expressed as a percentage of values obtained for wtTPP I proenzyme (C). Neuro-2a cells maintained in 6-well plates were exposed continuously for 24 h to secretions of CHO cells stably expressing wtTPP I and ΔGS1, ΔGS2, ΔGS3, ΔGS4, and ΔGS5 mutant proteins that were grown on filter inserts in the absence or presence of mannose 6-phosphate (Man-6-P). The level of phosphorylation was calculated as a ratio of pixel density of each corresponding band on phosphorimager and immunoblot and expressed as a percentage of values obtained for wtTPP I proenzyme (C). Neuro-2a cells maintained in 6-well plates were exposed continuously for 24 h to secretions of CHO cells stably expressing wtTPP I and ΔGS1, ΔGS3, and ΔGS4 mutant proteins that were grown on filter inserts in the absence or presence of mannose 6-phosphate (Man-6-P). Conditioned media of co-cultured CHO cells (upper panel) and lysates of Neuro-2a cells (lower panel) were analyzed afterward by immunoblotting with mAbs to TPP I. Con, control Neuro-2a cells grown in standard medium. Upper panel, 20 μl of medium conditioned for 24 h; lower panel, 40 μg of protein/lane. CHO cells expressing ΔGS4 mutant were used for these studies as an additional control.

To investigate the role of phosphomannose residues of TPP I in endocytosis, we used a co-culture system to ensure the continuous availability of ΔGS3 precursor to recipient cells (Neuro-2a), given that secreted ΔGS3 mutant is prone to degradation, as demonstrated in Fig. 9A.

As is shown in Fig. 10D, upper panel, at the end point of the experiment, the highest amounts of TPP I were present in the conditioned medium of CHO cells expressing ΔGS1 and ΔGS4 mutants. However, Neuro-2a cells internalized and processed most abundantly the proenzymes of wtTPP I and ΔGS4 mutant (Fig. 10D, lower panel, lanes 2 and 8). Uptake of ΔGS1 proenzyme by Neuro-2a cells was distinctly less efficient and did not exceed 30% of the value obtained for wtTPP I. Secreted proenzyme of ΔGS3 mutant was not taken up by Neuro-2a cells at all (Fig. 10D, lower panel, lane 6). Endocytosis of wtTPP I and of ΔGS1 and ΔGS4 mutant proteins was MPR-dependent, given that it was completely blocked by the presence of mannose 6-phosphate in cell culture medium.

This experiment shows that misfolded TPP I mutant protein cannot bind to MPR at the plasma membrane. In addition, striking difference in endocytosis efficiency between wtTPP I and ΔGS1 mutant, together with the data presented earlier, indicate that phosphorylation of both sugar moieties, at Asn-210 and Asn-286, is needed to ensure the most effective MPR-dependent trafficking of human TPP I.

Altered Folding but Reduced Secretion of Human TPP I

Missing Asn-286-linked Glycan Expressed in HEK293 Cells—Misfolded TPP I mutant missing the Asn-286-linked sugar expressed in CHO cells was secreted abundantly into the culture medium. Given that secretion of misfolded proteins is either strongly diminished or completely arrested because these proteins are usually excluded from ER exit sites and destined for ER-associated degradation (ERAD), secretion of misfolded TPP I proenzyme suggested either that structural properties of this particular mutant protein allowed export from the ER or that it was because of the specific properties of CHO cells.

To address these issues, we analyzed the intracellular processing and secretion of ΔGS3 mutant transiently expressed in HEK293 cells. By laser scanning confocal microscopy analysis, ΔGS3 mutant was localized mostly to the ER, and only a small portion of the mutant protein could be visualized in lysosomes (not shown); thus, similar to what we observed in CHO cells. On immunoblots of lysates of HEK293 cells (Fig. 11, left panel), also similar to what we found in CHO cells, ΔGS3 mutant appeared mostly as an unprocessed precursor, and a minor portion of the enzyme (less than 15%) was present as a mature ~46-kDa species. Electrophoretic mobility shift displayed by the cellular proenzyme of ΔGS3 mutant expressed in CHO cells and a 35-kDa incomplete degradation product were also present in HEK293 cells. However, in HEK293 cells, the amount of ΔGS3 proenzyme released to the culture medium was distinctly lower than that of wtTPP I (by 90%) and ΔGS1 mutant that was used as an additional control (Fig. 11, right panel). This observation indicates that the cellular fate of misfolded TPP I proenzyme may be substantially determined by cell type-specific factors.

**Fig. 10.** Glycans at Asn-210 and Asn-286 preferentially accept phosphomannose marker. A–C, 32PO₄-labeled TPP I was immuno-precipitated from secretions of cells stably expressing wtTPP I and ΔGS1, ΔGS2, ΔGS3, ΔGS4, and ΔGS5 mutant proteins after a pulse of 7 h, then resolved on SDS-PAGE, transferred onto nitrocellulose membrane, and analyzed by phosphorimager (A). Afterward, the membrane was developed with mAbs to TPP I to assess the total amount of TPP I proenzyme recovered in each immunoprecipitate to estimate the 32PO₄-labeled fraction of secreted polypeptides (B). The level of phosphorylation was calculated as a ratio of pixel density of each corresponding band on phosphorimager and immunoblot and expressed as a percentage of values obtained for wtTPP I proenzyme (C).

**Fig. 11.** Altered processing and reduced secretion of TPP I mutant with eliminated glycosylation site at Asn-286 (ΔGS3) expressed in HEK293 cells. HEK293 cells were transiently transfected with wtTPP I, ΔGS1, and ΔGS3 or mock-transfected. 48 h post-transfection, cell lysates (left panel) and cell secretions (right panel) that were conditioned for the last 24 h in serum-free medium supplemented with 5 mM mannose 6-phosphate were analyzed by immunoblotting with mAbs to TPP I, 20 μg of protein of cell lysates and 30 μl of cell secretions/lane. Because of low levels, endogenous TPP I in HEK293 cells was visualized only after a long exposure of immunoblots.
N-Glycosylation of Human TPP I

DISCUSSION

N-Glycans Direct Folding of Human TPP I—All lysosomal hydrolases identified to date are N-glycosylated and carry from one (cathepsin L) (39) to seven (α-glucosidase) (40) N-linked oligosaccharide side chains. However, similar to other glycoproteins (41), the dependence of lysosomal enzymes on N-glycans for folding varies. Thus, although elimination of only one N-glycosylation site, out of several utilized, affected folding of human α-galactosidase A (28), acid ceramidase (42), and murine sphingomyelinase (29), elimination of multiple N-glycosylation sites was necessary to prevent tetramerization of human N-galactosidase A (28), acid ceramidase (42), and murine sphingomyelinase (29), elimination of multiple N-glycosylation sites was necessary to prevent tetramerization of human β-glucuronidase (43), and only the removal of all N-glycosylation sites led to misfolding of β-hexosaminidase A (44). Mouse procathepsin L folded correctly, even if its unique functional N-glycosylation site was obliterated (45).

Our study showed that human TPP I in vivo utilizes all five potential N-glycosylation sites; thus, it belongs to heavy N-glycosylated acid hydrolases. Elimination of a single N-glycosylation consensus sequence at Asn-286 profoundly affected the TPP I folding pathway, whereas obliteration of any other individual N-glycosylation consensus sequence had no effect on folding. This finding indicates that Asn-286-linked sugar is critical for reaching a native conformation by human TPP I. However, TPP I also folded incorrectly when N-glycosylation sites other than Asn-286 were eliminated simultaneously, demonstrating that other N-glycans collectively play a complementary role in this process.

Only a small portion of TPP I that was missing Asn-286-linked sugar was able to reach the native or near native conformation because only ~5% of newly synthesized proenzyme was delivered to the lysosomes and was processed to an enzymatically active species. The major portion of TPP I proenzyme missing Asn-286-linked sugar remained misfolded.

However, unexpectedly, the cellular fate of misfolded TPP I proenzyme expressed in CHO cells differed significantly from that of other misfolded glycoproteins investigated to date. As a general rule, newly synthesized glycoproteins that are not yet folded correctly are retained in the ER, where chaperone proteins bind to them and assist in their folding. Those proteins that attain a native conformation are then exported from the ER to their target organelles, whereas terminally misfolded proteins are eventually degraded by ERAD (35). In contrast, only a portion of the misfolded TPP I proenzyme expressed in CHO cells was retained in the ER, whereas a large fraction was able to exit the ER, pass the trans-Golgi compartment, and enter the constitutive secretory pathway. This unusual behavior of mutated TPP I, not yet reported for other misfolded glycoproteins, including lysosomal enzymes, could result from a dual role of Asn-286-linked oligosaccharide: its involvement in both folding and intracellular transport to lysosomes. In this respect, human TPP I is the first identified acid hydrolase in which the same oligosaccharide is involved in these two essential biological processes.

However, when TPP I with the Asn-286 glycosylation site eliminated was expressed in HEK293 cells, the proportion of misfolded proenzyme secreted into the culture medium was distinctly lower than in CHO cells. This finding suggests that mechanisms ensuring the efficiency of ER retention and the secretion competency of potentially detrimental non-native TPP I may operate in a cell type-specific and/or species-specific manner.

Detailed analysis of molecular mechanisms leading to the folding of human TPP I was not the goal of this study, and it is being addressed in our ongoing research. Here, we would like to comment on some of our data suggesting that the action of ER mannosidases could significantly contribute both to ER retention and to mistargeting of misfolded TPP I mutant.

In mammalian cells, folding is initiated cotranslationally and cotranslationally when a nascent polypeptide chain enters the translocon complex in the ER membrane, which allows separate domains of growing polypeptide to fold independently and in a sequential manner as they emerge from the translocon complex (41, 46, 47). Relatively, the best characterized primary quality control system operating in the ER in higher eukaryotes is the calnexin/calreticulin cycle (33, 41, 47). Transfer of N-linked core oligosaccharides (GlcManGlcNAc) to a nascent polypeptide occurs cotranslationally during the translocation of the protein to the ER lumen, and immediately after the transfer glucose residues are trimmed by glucosidases I and II to generate nonglycosylated forms (ManGlcNAc). If glycoprotein is not yet folded, the UDP-Glc:glycoprotein glucosyltransferase adds a single glucose unit in an α(1→3) bond to the terminal mannos of the α(1→3)–α(1→2) branch to generate monoglucosylated forms, which are recognized by and bound to calnexin/calreticulin, lectin chaperones, and the thiol-disulfide oxidoreductase, ERP57, assisting in folding. Glucosidase II releases glucose residue and lectin chaperone from the substrate glycoprotein, which, if still unfolded, is reglucosylated to a monoglucosylated form by glucosyltransferase, the folding sensor, and enters another calnexin/calreticulin cycle. Rounds of binding and release of chaperones and glycoproteins are repeated until glycoproteins reach their native conformation, which allows them to exit the ER and enter the secretory pathway.

Accumulation of incorrectly folded proteins in the ER of mammalian cells causes transcriptional induction of numerous genes, including those encoding the ER chaperones (48), which retain the unfolded proteins in the ER, prevent their aggregation, and facilitate the folding of conformers that are caught in low energy kinetic traps (49). However, the mechanisms by which terminally misfolded protein is recognized, folding attempts are stopped, and protein is diverted for degradation are still not entirely understood.

One of the mechanisms proposed recently infers that permanently misfolded proteins undergo a trimming of the terminal α1,2-linked mannose residue from the middle branch of the core oligosaccharide by a slow acting ER α1,2-mannosidase I to generate ManαGlcNAc isomer B (50–52), which is recognized by the ER degradation-enhancing 1,2-mannosidase-like protein (53), whose overexpression, indeed, led to faster release of folding-incompetent proteins from the calnexin cycle and earlier onset of degradation (54, 55). Nevertheless, ER α1,2-mannosidase I overexpressed in HEK293 cells, enhancing ERAD of the misfolded genetic variant-null Hong Kong α1-antitrypsin, not only greatly increased the formation of ManαGlcNAc but also induced the formation of GlcαManαGlcNAc and increased trimming of core glycans to ManαGlcNAc (56), which suggested that carbohydrate recognition determinant triggering ERAD is not restricted to ManαGlcNAc isomer B. Although in vitro, class I ER α1,2-mannosidases were able to generate even ManαGlcNAc1,2, suggesting that the specificity of these enzymes is not as strict as reported previously (57), it is still uncertain whether also in vivo generation of ManαGlcNAc and ManαGlcNAc2 could be caused by the action of ER mannosidase I. The participation of Manα-mannosidase in this process was proposed recently (58).

According to our data, this portion of misfolded TPP I proenzyme that was retained in the ER underwent extensive trimming of mannose residues on its core glycans. Thus, given that this oligosaccharide modification represents a signal for the disposal of permanently misfolded proteins and that mannose trimming on TPP I proenzyme missing the Asn-286 glycosylation site was already present after only a 15-min pulse, it is reasonable to assume that a portion of newly synthesized TPP
I mutant protein was predestined for ERAD early, either during or shortly after completion of its translation. Because the activity of glucosidase II is distinctly reduced against oligosaccharides lacking one or more mannose residues (i.e. by 80% for Glc$_3$Man$_5$GlcNAc compared with Glc$_6$Man$_{10}$GlcNAc) (58), misfolded TPP I proenzyme with reduced mannose content on core glycans could dissociate less efficiently from calnexin/calreticulin, which could be responsible, at least partially, for its ER retention. However, core glycans lacking the terminal mannose residues poor substrates for glucosyltransferase, thereby bringing the calnexin cycle to an end. Thus, similar to what was proposed for other glycoproteins, i.e. misfolded α$_1$-antitrypsin (51), removal of mannose residues from multiple attached oligosaccharides could couple intracellular retention and the degradation process of incorrectly folding human TPP I mutant.

In this respect, secretion of misfolded proenzyme could be attributed to the exhaustion of the ER quality control system causing ER mannosiase I, as a slow acting enzyme, to be incapable of processing all misfolded TPP I produced in the ER. This assumption is supported by data showing that misfolded TPP I mutant was degraded inefficiently by the proteasome, its removal from the ER was delayed, and deoxymannojirimycin treatment further increased its secretion. It was noted that deoxymannojirimycin treatment also significantly increased the secretion of a fraction of secretion-impaired PI Z, the most common severe deficiency variant of human α$_1$-antitrypsin (51, 60). In lights of these findings, we propose that the extent of mannose trimming on core oligosaccharides represents one of the factors determining the cellular fate of misfolded TPP I mutant.

Recent studies indicate that cells can dispose of incorrectly folded polypeptides using several degradative pathways, of which the ubiquitin-proteasome pathway is the best characterized and probably the most commonly used for ERAD (35). Because β-lactone, an inhibitor of the 26 S proteasome, significantly reduced the degradation of both wtTPP I and ΔGS3 mutant, it appears that human TPP I utilizes the ubiquitin-proteasome pathway for ERAD. However, the prolonged half-life of misfolded TPP I mutant and the generation of its 35-kDa degradation intermediate suggest that the disposal of misfolded TPP I mutant was not very efficient. Interestingly, although before degradation by the proteasome, glycoproteins are deglycosylated in the cytosol because of the action of PNGase (61, 62), the 35-kDa species still contained Endo H-sensitive oligosaccharides. Thus, it appears that the conformational rearrangements made the glycans of misfolded TPP I mutant inaccessible for the action of cytosolic N-glycanase.

It is still not entirely understood how soluble proteins exit the ER. Although proteins that did not reach a native conformation are generally excluded from the ER exit sites, some misfolded proteins are retrieved in coatomer protein II-mediated vesicular transport, even from as far as the trans-Golgi compartment (35, 41). In some instances, transport to the Golgi apparatus and retrieval to the ER are necessary for misfolded proteins to be degraded, as has been shown for carboxypeptidase Y and proteinase A in yeast (63). Because the Golgi apparatus is less restrictive in terms of quality control than the ER, it appears that once released from the ER, the misfolded TPP I mutant could be transported further to the cell membrane and released by using the constitutive secretory pathway.

Preferential Phosphorylation in Human TPP I of N-Glycans at Asn-210 and Asn-286.—Acquisition of mannose 6-phosphate markers by lysosomal hydrolases that utilize MPRs for lysosomal sorting begins 15–20 min after their synthesis, when they are still in the pre-Golgi compartment (24). Selected mannose residues in N-linked high mannose oligosaccharides accept N-acetylglucosamine 1-phosphate from UDP-GlcNAc to generate phosphodiesters because of the action of the UDP-N-acetyl-D-glucosamine:lysosomal-enzyme N-acetylglucosamine phosphotransferase (phosphotransferase). When lysosomal enzymes move to the Golgi apparatus, α-mannosiase I excises the terminal mannose residue from the α1,3 branch of the core glycan, allowing for phosphotransferase to act on this branch, which leads to formation of diphosphorylated species. Then, in the trans-Golgi, N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase, the “uncovering enzyme” removes the N-acetylglucosamine residues generating phosphomonoesters, which show high affinity binding to MPRs. MPR-lysosomal enzyme complexes formed in the trans-Golgi are then transported in clathrin-coated vesicles to the late endosomes from which, after dissociation from the receptors, acid hydrolases are delivered to the lysosomes.

Our study showed that two oligosaccharides, at Asn-210 and Asn-286, are preferentially phosphorylated in human TPP I. Elimination of either of these N-glycosylation sites altered intracellular trafficking of the enzyme, indicating that phosphorylation of glycans at both Asn-210 and Asn-286 is needed for efficient sorting of human TPP I in the trans-Golgi compartment. Of note, although folded incorrectly, TPP I mutant protein lacking oligosaccharide at Asn-286 was phosphorylated. It has still not been entirely elucidated how phosphotransferase can distinguish lysosomal hydrolases from other secretory glycoproteins. Available data suggest that the enzyme recognizes a protein determinant in native conformation of acid hydrolases, which involves a broad surface patch encompassing critical lysine residues (64–67). Our study implies that even incorrectly folded acid hydrolase can be substrate for phosphotransferase.

Both MPRs, the 46-kDa cation-dependent MPR and the 300-kDa insulin-like growth factor II/cation-independent MPR (IGFI/C1-MPR), participate in sorting and lysosomal targeting of acid hydrolases in trans-Golgi compartment, but only IGFI/CI-MPR participates in endocytosis of lysosomal enzymes (68, 69). Although the affinity of IGFI/C1-MPR to acid hydrolases is generally higher than that of cation-dependent MPR, they both participate in lysosomal targeting of largely overlapping complement of lysosomal enzymes, and neither receptor can fully compensate for the absence of the other (70). Of note, in contrast to wtTPP I, misfolded TPP I mutant protein expressed in CHO cells, although phosphorylated by phosphotransferase, was not recognized by MPRs either in the trans-Golgi or at the plasma membrane, as judged by its increased secretion and lack of internalization by cultured cells. This could be the result of at least two factors. First, misfolded TPP I mutant might not be recognized and processed by uncovering enzyme in the trans-Golgi, which could affect its binding affinity to MPRs. Second, phosphomannose marker on Asn-210-linked oligosaccharide of misfolded mutant, even if uncovered, might not be exposed appropriately on the surface to allow for interaction with the ligand binding site of MPR. Elucidation of these phenomena is open for further study; however, these data suggest that the Golgi apparatus may function in the quality control machinery of acid hydrolases by excluding from lysosomal sorting those incorrectly folded acid hydrolases that were able to escape the ER.

The Role of N-Glycans for Enzymatic Activity of TPP I.—Although N-glycosylation is required for the folding of the majority of lysosomal hydrolases, the direct role of N-glycans in the regulation of their enzymatic activity is less evident. Thus, although a lack of individual N-glycans affected catalytic activity of murine acid sphingomyelinase (29), N-glycans could be removed from correctly folded human β-glucuronidase without significant loss of activity (43). As our study revealed, only the elimination of the glycosyla-
tion site at Asn-286 in human TPP I dramatically affected autoprocessing, catalytic activity, and stability of the enzyme; however, this site-specific effect was mostly secondary and resulted from altered folding of the enzyme missing Asn-286-linked sugar. This is supported by the finding that a small fraction of TPP I mutant missing Asn-286 sugar that was targeted to the lysosome, thus folded correctly, showed specific activity towards a low-molecular-weight substrate even higher than wtTPP I. This last observation suggests that oligosaccharide attached at Asn-286 in TPP I may impose some steric hindrance on the catalytic cleft of the enzyme in that its absence could facilitate interaction between the substrate and catalytic pocket. Analysis of the tertiary structure of the human TPP I is necessary to validate this possibility. However, the thermal and pH-dependent stability of the mature form of TPP I missing glycan at Asn-286 was distinctly lower than that of wtTPP I and other mutant proteins, suggesting that Asn-286-linked oligosaccharide may stabilize the structure of an already folded enzyme. Elimination of glycosylation sites other than that at Asn-286 did not significantly affect autoprocessing, and it only slightly decreased the enzymatic activity and stability of correctly folded enzyme.

Misfolding and Mismatching Represent the Molecular Basis of CLN2 Disease Process in Subjects with Mutation at Asn-286 in Human TPP I—A naturally occurring missense mutation at glycosylation site Asn-286 (N286S) was recently reported in two subjects with CLN2. Affected homozygotes for this mutation showed a clinical course only slightly milder than that observed in the classic late infantile form of NCL (31). The results of our study suggest that misfolding and mismatching of TPP I represent the molecular basis of the disease process in these individuals. Although a small fraction of the enzyme missing the glycosylation site at Asn-286 was targeted to the lysosomes in transfected CHO and HEK293 cells, this could reflect an overexpression effect, and the amount of mutated enzyme that could reach the lysosomes in patients’ cells might be distinctly lower, if any at all, thus leading to the CLN2 disease process. Also, the steric effect of Glu that replaced Asn in our mutagenesis analyses may be more benign than the less conservative replacement of Asn by Ser in naturally occurring mutant.

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REFERENCES

1. Page, A. E., Fuller, K., Chambers, T. J., and Warburton, M. J. (1993) Arch. Biochem. Biophys. 307, 354–359
2. Vines, D., and Warburton, M. J. (1998) Biochim. Biophys. Acta 1384, 233–242
3. Bernardini, P., and Warburton, M. J. (2001) Eur. J. Pediatr. Neurol. 5, 69–72
4. Ezaki, J., Takeda-Ezaki, M., Oda, K., and Kominami, E. (2000) Biochem. Biophys. Res. Commun. 285, 904–908
5. Sleat, D. E., Donnelly, R. J., Luckland, H., Liu, C. G., Sohar, I., Pullarkat, R. K., and Lobel, P. (1997) Science 277, 1802–1805
6. Sleat, D. E., Gin, R. M., Sohar, I., Wisniewski, K. E., Sklower Brooks, S., Pullarkat, R., Palmer, D. N., Lerner, T. J., Boettiger, R. M., Udall, P., Skakotos, A. N., Donnelly, R. J., and Lobel, P. (1999) Am. J. Hum. Genet. 64, 1511–1522
7. Wisniewski, K. E., Kida, E., Golabek, A. A., Kaczmarski, W., Connell, F., and Kornfeld, S. (2001) Eur. J. Pediatr. Neurol. 5, 69–72
8. Ezaki, J., Takeda-Ezaki, M., Oda, K., and Kominami, E. (2000) Biochem. Biophys. Res. Commun. 285, 904–908
9. Sleat, D. E., Donnelly, R. J., Luckland, H., Liu, C. G., Sohar, I., Pullarkat, R. K., and Lobel, P. (1997) Science 277, 1802–1805
10. Kida, E., Golabek, A. A., Wisniewski, K. E. (2001) Adv. Genet. 45, 1–34
11. Kida, E., Golabek, A. A., Wisniewski, K. E. (2001) Adv. Genet. 45, 35–68
12. Kida, E., Golabek, A. A., Walu, M., Wu, P., Kaczmarski, W., and Wisniewski, K. E. (2001) J. Neurochem. Exp. Neurol. 60, 280–292
13. Junaid, M. A., Sklower Brooks, S., Wisniewski, K. E., and Pullarkat, R. K. (1999) Clin. Chem. 45, 169–176
14. Wisniewski, K. E., Kida, E., Golabek, A. A., Kaczmarski, W., and Pullarkat, R. K. (2001) Eur. J. Biochem. 281, 66–97
15. Junaid, M. A., Sklower Brooks, S., Wisniewski, K. E., and Pullarkat, R. K. (1999) Clin. Chem. 45, 169–176
16. Ezaki, J., Tanida, I., Kamehagi, N., and Kominami, E. (1999) J. Neurochem. 72, 2573–2582
17. Palmer, D. N., Jolly, R. D., van Mil, H. C., Tynnela, J., and Westlake, J. V. (1997) Neuropeptides 28, 45–58
18. Liu, C.-G., Sleat, D. E., Donnelly, R. J., and Lobel, P. (1998) Genomics 50, 206–212
19. Lin, L., Sohar, I., Luckland, H., and Lobel, P. (2001) J. Biol. Chem. 276,