The human colonic thiamine pyrophosphate transporter (hTPPT) is a glycoprotein and N-linked glycosylation is important for its function

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Abstract

The recently identified human thiamine pyrophosphate transporter (hTPPT; product of the *SLC44A4* gene) is responsible for absorption of the microbiota-generated TPP in the large intestine. The hTPPT is highly expressed in the colon, but not in other regions of the intestinal tract and is localized exclusively at the apical membrane domain of epithelia. The hTPPT protein is predicted to have multiple TM domains with a number of putative N-glycosylation sites, but it is not known if the protein is actually glycosylated, and if so at which site, and their role in the functionality of the transporter. Using several approaches including inhibiting *de novo* N-glycosylation in human colonic epithelial NCM460 cells with tunicamycin as well as enzymatic de-glycosylation, we show that the hTPPT protein is, indeed, a glycoprotein. Glycosylation of hTPPT was shown, by mean of site-directed mutagenesis, to occur at Asn^{69}, Asn^{155}, Asn^{197}, Asn^{393}, and Asn^{416}. However, only N-glycosylation at Asn^{69}, Asn^{155}, and Asn^{393} appeared to be important for transporter functionality possibly through an effect on protein conformation and/or interaction with its ligand (but not through changes in expression at the cell membrane as determined by live cell confocal imaging). Results of this study showed, for the first time, that the hTPPT is glycosylated and that N-linked glycosylation occurs at multiple sites with some of them being important for function. The results also provide an indirect support for a membrane topology for hTPPT with 10 transmembrane domains as predicted by the TMHMM transmembrane helixes prediction program.
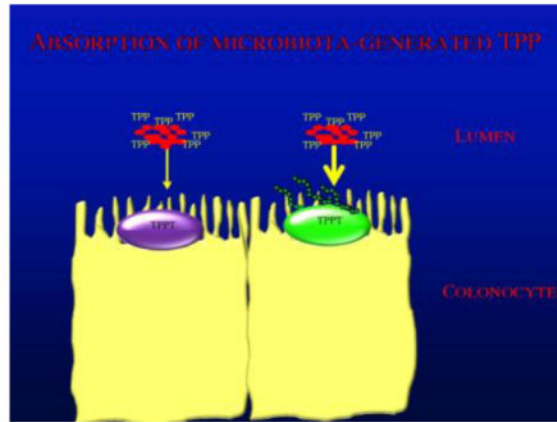
Graphical abstract

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

ABSORPTION OF MICROBIOTA-GENERATED TPP

Keywords

Thiamine pyrophosphate; glycosylation; transport; uptake; mutation

1. INTRODUCTION

Thiamine (vitamin B1) is indispensable for oxidative energy (sugar) metabolism and ATP production in the mitochondria via its role as a cofactor (mainly in the form of thiamine pyrophosphate; TPP) for multiple enzymes (transketolase, pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, and branched-chain ketoacid dehydrogenase) [reviewed in 1, 2, 3]. The vitamin is also important in reducing cellular oxidative stress and maintaining the normal cellular redox state [4, 5, 6]. Recent studies have attributed additional roles to other forms of the vitamin, namely to thiamine triphosphate, in regulating the function of membrane chloride channels and in acting as a phosphate group donor to proteins [7]. With such important metabolic roles, it is not surprising that low intracellular levels of this micro-nutrient leads to impairment in a variety of cellular metabolic events including oxidative energy metabolism, management of oxidative stress [4, 5, 6, 8], and mitochondrial function/ structure [9]. Deficiency of thiamine in humans leads to distinct abnormalities that include beriberi and Wernicke's encephalopathy. Such a deficiency represents a significant nutritional problem in both developed and underdeveloped countries, and occurs in a variety of conditions including chronic alcoholism, diabetes mellitus, and inflammatory bowel disease [reviewed in 1, 10–14]. In contrast to the negative effects of thiamine deficiency, optimizing thiamine body homeostasis has the potential to prevent diabetes-associated nephropathy, retinopathy and related tissue damage [15, 16].

Humans and other mammals cannot synthesize thiamine endogenously, and thus, must obtain the vitamin from exogenous sources via intestinal absorption. The intestine is exposed to two sources of thiamine: a dietary source and a bacterial source [the latter is in reference to the thiamine generated by the large intestinal microbiota; refs. 17–20]. The dietary source of thiamine is processed and absorbed in the small intestine; the latter occurs via a specific carrier-mediated process [21–23] that involves the human thiamine transporter -1 and -2 [products of the *SLC19A2* and *SLC19A3* genes, respectively; refs. 24, 25]. A

significant amount of the thiamine generated by the microbiota of the large intestine exists in the phosphorylated form of TPP [17]. Recent studies from our laboratory have shown that human colonocytes possess a highly efficient and specific carrier-mediated mechanism for TPP uptake [26]. We have also cloned the colonic TPP uptake system and showed the transporter to be the product of the *SLC44A4* gene [27]. The ORF of human TPP transporter encodes a protein of 710 amino acids, having a predicted molecular mass of around 79 kDa, is highly conserved among mammals, and is predicted to have multiple TM domains with a number of putative glycosylation sites. Expression of the human TPP transporter was found to be restricted to the colon and is negligible in other parts of the GI tract [27]; also, the protein was found to be expressed exclusively at the apical membrane domain of epithelia [27].

Currently there is little known about the structure-function relationship of the colonic TPP transport system. We are interested in addressing this issue, and in this study we have focused on determining the glycosylation status of the protein, which of its putative N-glycosylation sites are actually glycosylated, and on the role of N-glycosylation in its function. Our results showed that the hTPPT protein is N-glycosylated at Asn^{69}, Asn^{155}, Asn^{197}, Asn^{393}, and Asn^{416}; however, only glycosylation at Asn^{69}, Asn^{155}, and Asn^{393} appear to be important for functionality of the membrane transporter.

2. MATERIALS AND METHODS

2.1. Materials

Custom-made ^{3}H-labeled thiamine pyrophosphate ([^{3}H]-TPP; specific activity, 1.0 Ci/mmol; radiochemical purity, 98.3%) was obtained from Moravek Biochemicals, Inc., (Brea, CA). Unlabeled TPP and all other chemicals and reagents used in the study were obtained from commercial vendors and were of analytical/molecular biology grade.

2.2. Cell culture and transfection

The human colonic NCM460 cell line was obtained from INCELL (San Antonio, TX). The human retinal pigment epithelial ARPE19 cells and Madin-Darby canine kidney (MDCK) cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (ARPE19 cells) or Minimal Essential Medium (MEM) (MDCK cells) media supplemented with 10% (vol/vol) FBS, penicillin (100,000 U/l), and streptomycin (10 mg/l). The NCM460 cells were maintained in Ham's F-12 culture medium supplemented with 20% (vol/vol) FBS and antibiotics. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) following manufacturer's protocol.

2.3. Uptake assay

Initial rate of uptake (5 min) of ([^{3}H]-TPP was examined at 37°C in Krebs-Ringer (K-R) buffer in cells maintained in 12-well plates as previously described [26, 27]. Protein concentrations of cell digest were measured in parallel wells using Dc protein assay kit (Bio-Rad, Hercules, CA). In transfected cells, uptake of TPP by the induced system was calculated by subtracting uptake by ARPE19 cells transiently transfected with hTPPT from

uptake by cells transfected with empty vector; carrier-mediated TPP uptake was determined by subtracting the passive diffusion component from total uptake.

2.4. Tunicamycin and PNGase F treatment

NCM460 cells were treated with 2μg/ml tunicamycin for 48 hrs followed by TPP uptake and Western blot analysis. Total membrane protein (~100 μg) was prepared from NCM460 cells and then incubated with Peptide-N- Glycosidase F (PNGase F) (10 μg protein/μl of PNGase F; NEB Inc., MA) for 1 hr at 37° C followed by Western blot analysis.

2.5. Site-directed mutagenesis

Quick Change II Site-directed mutagenesis kit from Stratagen (La Jolla, CA) was used for mutating individual residues using full-length hTPPT (accession number KU175229) construct cloned into pFLAG-CMV-2 or YFP vector [27] and specific mutant primers (Table 1) following manufacturer's instruction. The mutated clone was then identified by sequencing of the isolated plasmid DNA (Laragen, CA).

2.6. Cell surface biotinylation and Western blotting

Whole cell lysates or plasma membrane proteins isolated with a cell surface protein isolation kit (Pierce Biotechnology, Rockford, IL) were prepared as described [27]. Proteins were separated in NuPAGE 4–12% Bis-Tris gradient minigels (Invitrogen), transferred onto immobilon polyvinylidene difluoride membrane (Fisher Scientific), and subjected to Western blot analysis. The primary antibodies were goat polyclonal anti-hTPPT antibodies [CTL4 (D-14) antibody, catalog # sc-68049; Santa Cruz Biotechnology, Santa Cruz, CA] at 1:200 dilution or monoclonal anti-FLAG M2 antibody (Sigma) at 1:1000 dilution. The secondary antibodies were anti-goat IRDye-800 antibody or anti-mouse IRDye-800 antibodies (LI-COR Bioscience, Lincoln, NE) at 1:30,000 dilutions. Immunoreactive bands were visualized using the Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE).

2.7. Confocal imaging

hTPPT-YFP (WT) and mutant constructs were transiently transfected into MDCK cells and transfected cells were imaged after 48 hrs using an inverted Nikon C-1 confocal microscopy as described before [27–29].

2.8. Statistical Analysis

Data on uptake of TPP are presented as mean ± SE of at least three independent experiments and are expressed as pmol/milligram protein/5 min. Level of significance was determined by the Student's t-test, and a p-value of < 0.05 was considered as being significant. Carrier-mediated ^{3}H-TPP uptake was determined by subtracting the diffusion component (uptake in the presence of 1 mM TPP) from total uptake. Western blot and confocal imaging were all performed on two or more separate occasions, and representative blots/images are presented in this paper.

3. RESULTS

Topology prediction for membrane transporter can be achieved using various predictive algorithms, of which the SOSUI transmembrane helixes prediction program, i.e. SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui) and the TMHMM transmembrane helixes prediction program (http://www.cbs.dtu.dk/services/TMHMM-2.0) are most commonly used. Hydrophobicity analysis using the SOSUI transmembrane helixes program predicts the hTPPT protein to have 13 putative transmembrane domains (TMD), with an extracellular amino terminus and an intracellular carboxyl terminus, and is potentially N-glycosylated (http://www.cbs.dtu.dk/services/NetNGlyc) at Asn[29]. This topographical model has been reported by us previously [27] but has not been verified experimentally.

The TMHMM transmembrane helixes program predicts the presence of 10 TMD for hTPPT with both N- and C-terminal ends oriented inside (Fig. 1). Based on this topographical model, the hTPPT has seven putative N-linked glycosylation sites (N-X-S/T) located in the extracellular loops between TMD: Asn[69], Asn[155], Asn[197] (a large loop between TMDs 1 and 2), Asn[298] (a loop between TMDs 3 and 4), and Asn[393], Asn[409], Asn[416] (a loop between TMDs 5 and 6). While Asn[69], Asn[197], Asn[298], Asn[393], and Asn[416] sites are conserved across species (human, bovine, mouse and rat), the Asn[155] potential glycosylation site (N-M-T) is replaced by Asp in the mouse and rat TPPT; on the other hand, the potential site Asn[409] (N-P-T) exists only in human.

Thus, both of the prominent transmembrane helixes prediction programs suggest that the hTPPT is glycosylated but there is no experimental evidence in support of that; also no information exists as to which site(s) is actually glycosylated and whether glycosylation influences function.

3.1. hTPPT is glycosylated in human colonic epithelial cells and glycosylation is important for transport function

Our previous studies with the human colonic epithelial NCM460 cells and human proximal colonic apical membrane (AM) preparations have shown that the colonic hTPPT protein is post-translationally modified [27]. This observation is based on the decreased electrophoretic mobility of the hTPPT protein band immuno-detected by Western Blot using specific anti-SLC44A4/hTPPT polyclonal antibodies. Indeed, the molecular mass of the prominent immunoreactive band was ~ 110 kDa for plasma-membrane associated hTPPT protein in NCM460 cells and ~90–110 kDa for the hTPPT in the proximal colonic AM. This experimentally determined molecular mass of hTPPT is higher than the predicted molecular mass of ~ 79 kDa estimated for the predominant colonic hTPPT isoform. Since the hTPPT polypeptide is predicted to have potential N-linked glycosylation sites(s), we hypothesized that the post-translational modification is occurring at these glycosylation sites. To test this hypothesis, we used two experimental approaches to assess the glycosylation status of the hTPPT protein in colonic epithelial cells. In the first approach, we inhibited the *de novo* N-glycosylation in the colonic epithelial NCM460 cells by tunicamycin, and analyzed the migration pattern of hTPPT. NCM460 cells were treated with tunicamycin (2 μg/ml) for 48 hrs followed by Western blot analysis of the cell lysate using specific anti-SLC44A4 polyclonal antibodies. As shown in Figure 2A, after the tunicamycin treatment: (i) the

intensity of the ~ 110 kDa band [mature plasma-membrane associated hTPPT; 27] dramatically decreased, and (ii) it was associated with the appearance of new prominent immunoreactive band of lower molecular mass (~95–100 kDa). Importantly, there was no change in intensity of the ~75–79 kDa band (which is most likely non-glycosylated core protein), indicating that the tunicamycin treatment does not lead to a complete de-glycosylation of hTPPT. These data indicate that in colonic epithelial NCM460 cells the hTPPT protein is N-glycosylated and suggest that post-translational modifications other than N-linked glycosylation are also involved in protein maturation.

In the second approach, we used enzymatic de-glycosylation approach to assess the N-linked glycosylation status of hTPPT in NCM460 cells. A crude membrane protein fractions of NCM460 cells were treated with PNGase F, an amidase that cleaves between the innermost N-acetyl glucosamine and asparagine residue in high mannose, hybrid, and complex oligosaccharides [30], followed by Western Blot analysis. Western blot analysis (Fig. 2B) showed that in samples treated with PNGase F the hTPPT immunoreactive band of ~110 kDa (control cells) was shifted to a lower size band of ~100 kDa. These results further confirm the above observations with tunicamycin that the hTPPT is, in fact, N-glycosylated in colonic epithelial cells.

To determine the impact of N-linked glycosylation on functionality of the colonic hTPPT, we examined the effect of tunicamycin (2 μg/ml; for 48 hrs) treatment of colonic epithelial NCM460 cells on uptake of ^{3}H-TPP (0.3 μM). The results (Fig. 2C) showed that tunicamycin treatment causes a significant (p < 0.01; ~56%) inhibition in carrier-mediated TPP uptake compared to untreated control cells.

3.2. Mutagenesis of the potential N-glycosylation sites and the effect on hTPPT function

To further verify the role of N-linked glycosylation in hTPPT function, we examined the contribution of the individual potential N-glycosylation sites in transport function. This was achieved by introducing mutations into the individual putative N-glycosylation sites and examining the effect of such modifications on the migration pattern of the hTPPT protein, and on its transport function. These experiments were performed using ARPE19 cells (after transient transfection with the hTPPT) because these cells are easier to transfect than NCM460 cells, and have been used in previous studies to characterize hTPPT [27]. First, we showed that the hTPPT expressed in ARPE19 cells is also the subject of post-translational modifications likely via N-glycosylation, as it was found in the case of NCM460 cells. This was done by mean of Western Blot analysis (using anti-SLC44A4/hTPPT polyclonal antibodies) of the total cell lysates and plasma membrane proteins that showed that the predominant plasma membrane associated form of hTPPT has an apparent molecular weight of ~85 kDa (Fig. 3). We then moved to mutate all the eight putative N-glycosylation sites predicted for the hTPPT by the above-described SOSUI and TMHMM topographical prediction models. Individual mutations were introduced resulting in the replacement of the individual asparagine residues with aspartate (an amino acid that has similar structure to asparagine but cannot be glycosylated) followed by examining the effect of the particular mutation on the migration pattern of hTPPT. In these studies, wild-type or the mutant forms of hTPPT (FLAG- tagged) were transiently expressed in ARPE19 cells, followed by cell

lysates preparation and immunoblotting using anti-FLAG monoclonal antibodies. The results showed an increase in electrophoretic mobility of the prominent immunoreactive band of ~85 kDa (seen in control, i.e. cells transfected with the wild-type hTPPT) in cells transfected with the hTPPT mutated at Asn^{69}, Asn^{155}, Asn^{197}, Asn^{393}, and Asn^{416} (Fig. 4A). In contrast, in cells transfected with the protein mutated at Asn^{29}, Asn^{298}, Asn^{409}, the hTPPT migrated at a molecular weight of ~85 kDa, i. e., the same as the control protein. These observations indicate that Asn^{69}, Asn^{155}, Asn^{197}, Asn^{393}, and Asn^{416} residues of hTPPT are glycosylated in ARPE19 cells. These data are consistent with the topographical model of hTPPT predicted by the TMHMM transmembrane helixes prediction program but not that predicted by the SOSUI program.

To examine the effect of mutations in N-linked glycosylation sites on the functionality of hTPPT, we measured carrier-mediated TPP uptake by the induced system in ARPE19 cells transiently expressing mutants as compared to wild-type hTPPT. The results (Fig. 4B) showed that mutating Asn^{69}, Asn^{155}, and Asn^{393} of the hTPPT protein led to a significant (p < 0.01) decrease in TPP uptake. In contrast, no change in TPP uptake was detected in cells transfected with the hTPPT mutated at Asn^{197} and Asn^{416}. We also generated double mutants of hTPPT by disrupting, in pairs, three glycosylation sites observed to be important for transporter function, i.e. N69D/N155D, N69D/N393D, N155D/N393D and tested their effect on TPP uptake upon transient transfection of ARPE19 cells. Among them, only the mutant N155D/N393D (data not shown) resulted in further reduction in TPP uptake compared to mutants in single glycosylation site, indicating that the effect of disrupting Asn^{155} and Asn^{393} glycosylation sites was cumulative.

To examine whether the decrease in TPP uptake by cells with hTPPT disrupted N-glycosylation sites at Asn^{69}, Asn^{155}, and Asn^{393} was due to impaired expression of the protein at the cell membrane, we determined the cell surface expression level of hTPPT. This was done using live cell confocal imaging of transiently expressing MDCK cells with hTPPT-YFP (WT) and mutant constructs (hTPPT[N69D]-YFP, hTPPT[N155D]-YFP, and hTPPT[N393D]-YFP (cells were transfected with equal amount of DNA and imaged with similar laser intensity for all constructs). The results (Fig. 5) showed that cell surface expression of hTPPT was comparable in cells transfected with the different mutants and with the wild-type construct. Two additional controls were used, mutants (hTPPT[N197D]-YFP and hTPPT[N416D]-YFP) (glycosylated residue; no effect on uptake) and both showed comparable level of hTPPT expression at the cell membrane. These data indicate that lack of glycosylation of Asn^{69}, Asn^{155}, and Asn^{393} does not affect delivery of the transporter to the plasma membrane, and suggest that other mechanism(s) is involved in the decrease of transport function of hTPPT due to de-glycosylation.

4. DISCUSSION

Many proteins, including those at the cell surface, are known to be N-glycosylated, and this post-translation modification plays a role in protein functioning through regulation of protein folding, maintenance of protein stability, proper trafficking to subcellular compartments, and modulation of biological activity [31–33]. hTPPT, a transporter that plays an important role in the absorption of the microbiota-generated TPP in the large

intestine, appears to be post-translationally modified since its predicted molecular size of ~ 79 kDa differs from its apparent molecular size of ~110 kDa detected on a Western blot [27]. Knowing that the hTPPT polypeptide has a variety of the putative N-linked glycosylation sites, we assessed the N-glycosylation status of hTPPT in colonic cells using two independent approaches: the *de novo* inhibition of N-glycosylation by tunicamycin, and, enzymatic removal of N-linked carbohydrate chains by PNGase F. The results of both approaches showed that the hTPPT is, indeed, N-glycosylated in colonic cells. The observations that the molecular weight of the N-de-glycosylated protein did not decrease to its expected de-glycosylated core protein of ~79 kDa suggest that other post-translational modifications might contribute to hTPPT maturation. O-linked glycosylation might be of particular interest since O-glycosylation has been reported to play a role in apical sorting of membrane transporter [34]. This issue, however, will be addressed in future investigations.

N-linked glycosylation is known to affect functionality of many transporters, including those involved in vitamin uptake [28, 35, 36]. Here, we found that the tunicamycin-induced inhibition of N-glycosylation of hTPPT resulted in impairment of TPP uptake by colonic NCM460 cells suggesting an important role for N-glycosylation in the function of the transporter. To understand which of the hTPPT putative N-glycosylation sites were actually glycosylated, and the contribution of that glycosylation on function, we mutated each of the putative N-glycosylation sites and analyzed the consequences of such mutations on migration pattern and function of hTPPT. We utilized the ARPE19 cells in the latter studies as they have better transfection efficiency then NCM460 cells and have been used previously in characterizing many features of the hTPPT. We observed that in ARPE19 cells, the mature plasma membrane associated form of hTPPT also has a decreased electro-mobility (as it was previously found in the case of NCM460 cells) and migrated as a band of ~85 kDa (band of ~110 kDa in NCM460 cells). This suggests that the hTPPT protein is glycosylated in both cell lines, but to a different extent in colonic and retinal cells, and points to tissue-specificity in protein maturation via glycosylation. Our studies also showed that while the Asn^{69}, Asn^{155}, Asn^{197}, Asn^{393}, and Asn^{416} residues of hTPPT are all glycosylated in ARPE19 cells, only the mutations at Asn^{69}, Asn^{155}, and Asn^{393} have functional consequences. Since mutations at the latter sites did not affect cell membrane expression of the mutated hTPPT, it is possible that glycosylation at these sites affects the conformation of the protein and/or its interaction with its ligand. Further studies are needed to address these issues.

In addition to clarifying the glycosylation status of the hTPPT protein, determining which residues are actually glycosylated, and the consequence of that glycosylation on functionality, our studies also provide support for the membrane topology predicted by the TMHMM transmembrane helixes prediction program over the SOSUI program as the protein was found to be glycosylated at sites predicted to be in the extracellular domains of the membrane protein in the former but not the latter model. Similar topology and N-glycosylation status has been described for other members of the *SLC44A* family (37, 38, 39). In summary, our studies show that the hTPPT is a glycoprotein and that N-glycosylation is important for its function.

Acknowledgments

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Highlights

- hTPPT is the glycosylated protein.

- N-glycosylation occurs at Asn^{69}, Asn^{155}, Asn^{197}, Asn^{393}, and Asn^{416}.

- Glycosylation at Asn^{69}, Asn^{155}, and Asn^{393} is important for hTPPT functionality.

- This study provides a support for a 10 TMD topology for the hTPPT.
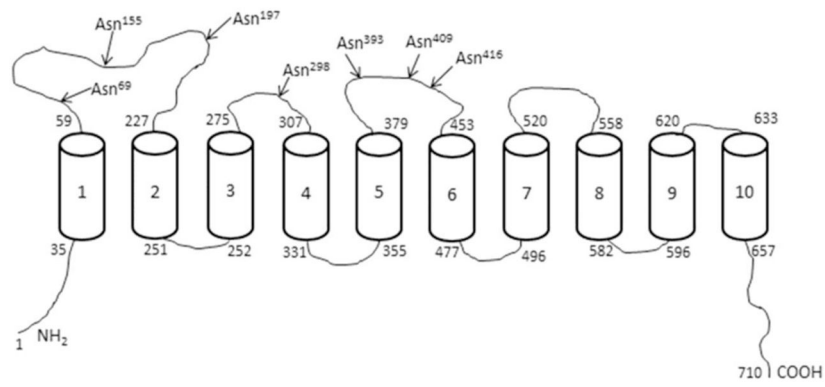
Fig. 1. Structural model of the hTPPT1

According to TMHMM, hTPPT1 is predicted to have 10 TMD with both N- and C-terminal ends oriented in the cytoplasm. The arrows indicate the location of potential *N*-glycosylation sites by the NetNGlyc1.0 server.
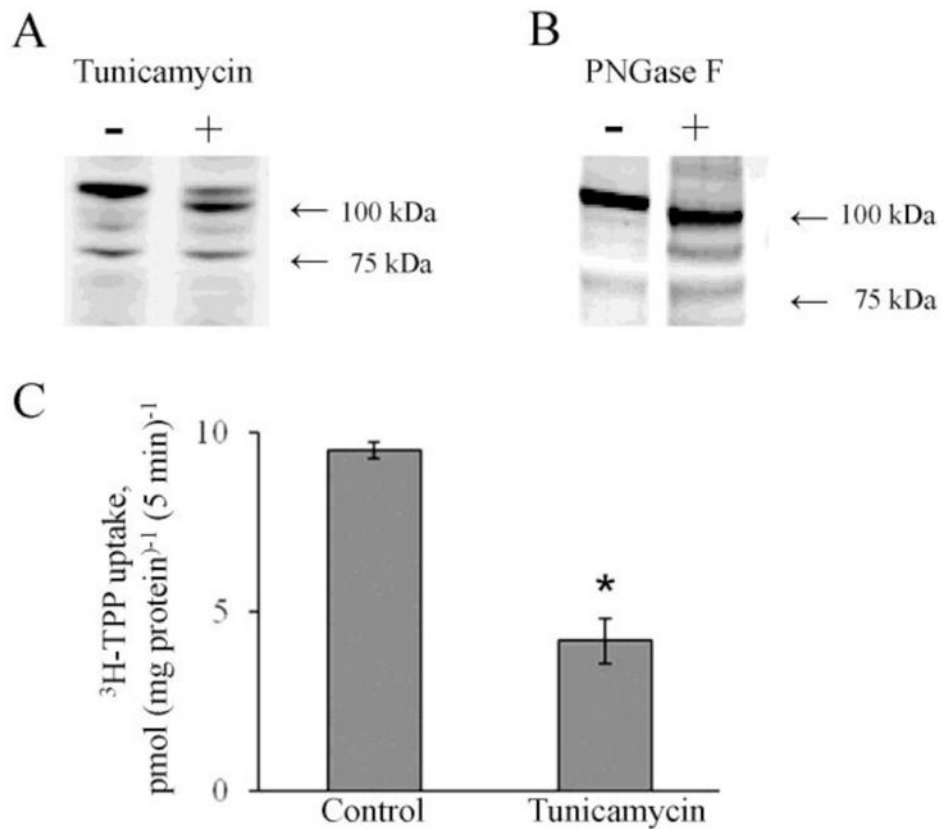
Fig. 2. The hTPPT is glycosylated in NCM460 cells and N-linked de-glycosylation affects TPP uptake

A & B, Western Blot analysis was performed after the tunicamycin treatment (2 μg/ml, 48 hrs) of NCM460 cells (A) and PNGaseF treatment of crude membrane protein fraction from NCM460 cells (B) as described in "Materials and Methods" using polyclonal anti-SLC44A4/hTPPT antibody. In both cases, a shift in an apparent molecular size of hTPPT is shown. The images are representatives of two independent experiments with similar results. **C**, Effect of tunicamycin on carrier-mediated TPP uptake by NCM460 cells. Cells were preincubated with tunicamycin (2 μg/ml, 48 hrs). [^{3}H]-TPP (0.3 μM) was added to the incubation medium (K-R buffer of pH 7.4) at the onset of incubation, and uptake was measured after 5min of incubation. Control cells - no tunicamycin pretreatment. Data are mean ± S.E. of six to eight separate uptake determinations. *$p < 0.01$.
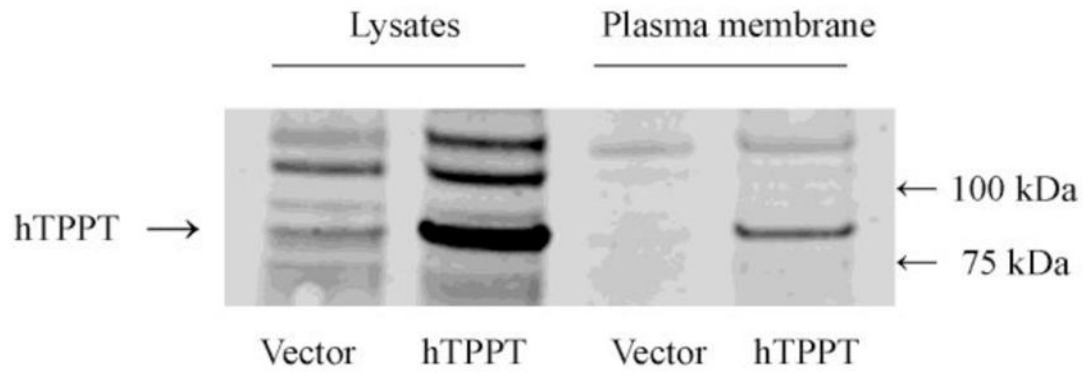
Fig. 3. Plasma membrane expression of hTPPT in ARPE19 cells transiently transfected with hTPPT

ARPE19 cells were labeled with EZ-Link Sulfo-NHS-SS-Biotin, and biotinylated proteins were isolated with NeutrAvidin-agarose followed by Western blot analysis using the polyclonal anti-SLC44A4/hTPPT antibodies. Control cells, transiently transfected with the vector. The image is representative of two separate sets of experiments with similar results.
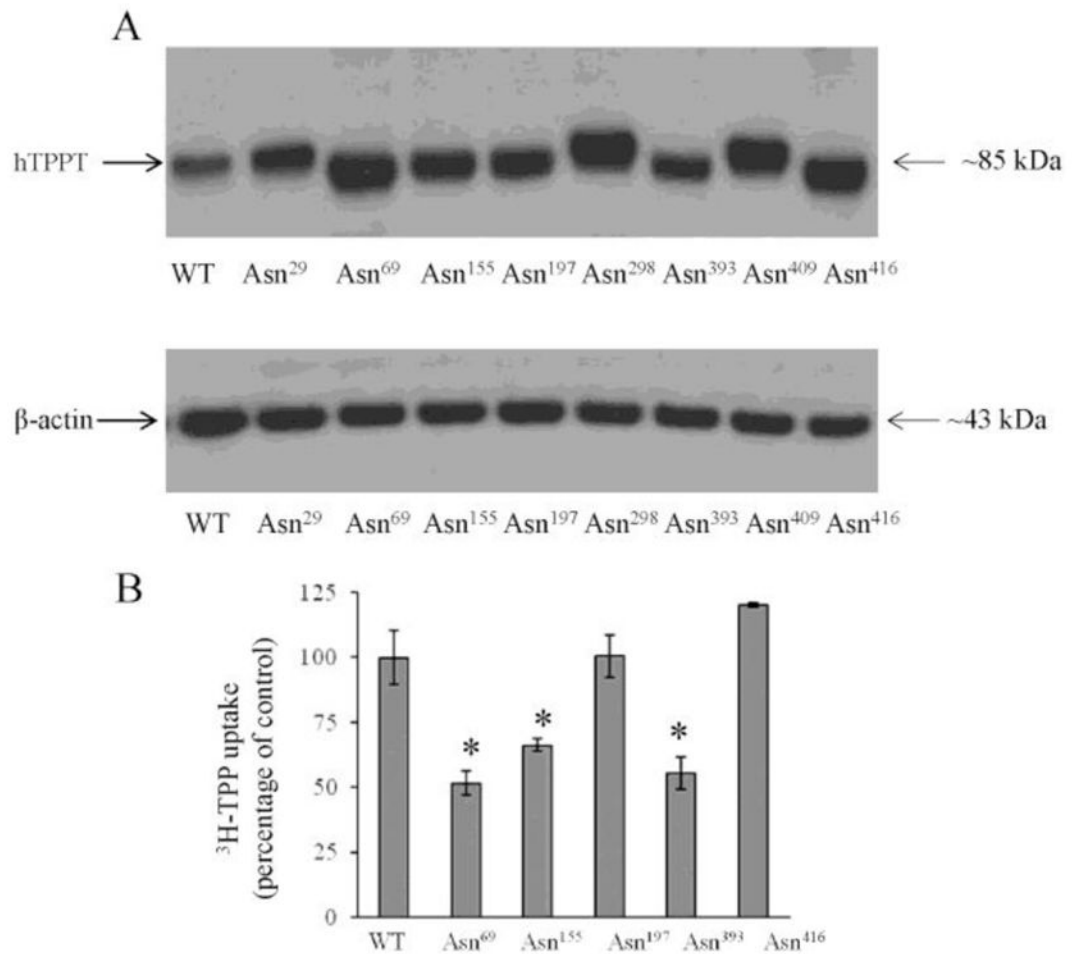
Fig. 4. The effect of mutations in the putative N-glycosylation sites of hTPPT on the migration pattern of the hTPPT protein and its transport function

A, Western blot analysis of cell lysates prepared from ARPE19 cells transiently transfected with the wild-type (WT) or the mutant forms of hTPPT (FLAG - tagged everywhere) was performed using monoclonal anti-FLAG antibody (upper panel) or monoclonal anti-β-actin antibody (lower panel). The images are representatives of two independent experiments with similar results. **B,** Effect of mutations in N-linked glycosylation sites of hTPPT on carrier-mediated TPP uptake. ARPE19 cells transiently expressing mutants (or control, wild-type hTPPT, WT) were used for TPP uptake measurements. [^{3}H]-TPP (0.3 μM) was added to the incubation medium (KR buffer of pH 7.4) at the onset of incubation, and uptake was measured after 5min of incubation. Carrier-mediated TPP uptake by the induced system was calculated as described in "Materials and Methods". Data are mean ± S.E. of six to eight separate uptake determinations. *p < 0.01.
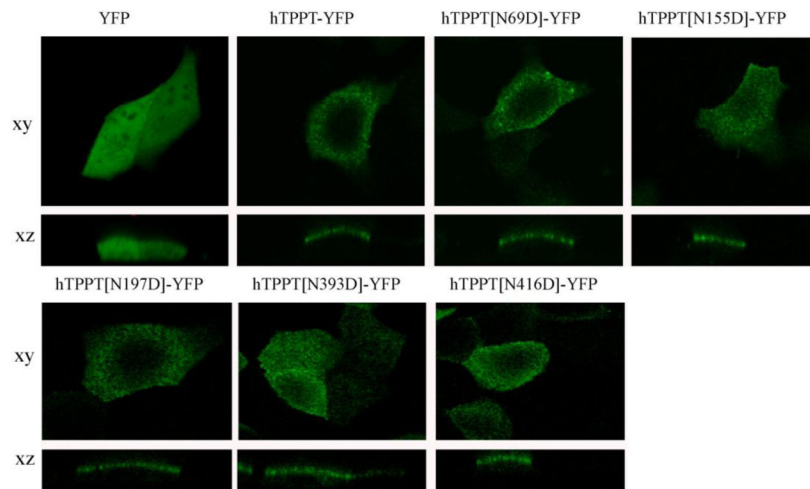
Fig. 5. Effect of mutations in the putative N-glycosylation sites of hTPPT on the cell surface expression of hTPPT

Lateral (xy) and axial (xz) images were obtained from MDCK cells transiently expressing YFP, wild-type (hTPPT-YFP) and mutant constructs after 48 hrs of post-transfection. Image shown represents 3 separate sets of experiments with comparable results.

Table 1

Primers used for site-directed mutagenesis of hTPPT.

Mutation	Forward and Reverse Primers (5′-3′)
N29D	CGAGGCCCCATCAAG<u>GAC</u>AGAAGCTGCACAGATGTCATC GATGACATCTGTGCAGCTTCT<u>GTC</u>CTTGATGGGGCCTCG
N69D	CAAGTCCTCTACCCCAGG<u>GAC</u>TCTACTGGGGCCTAC GTAGGCCCCAGTAGA<u>GTC</u>CCTGGGGTAGAGGACTTG
N155D	CCAGGGGTACCCTGG<u>GAT</u>ATGACGGTGATCACAAGC GCTTGTGATCACCGTCAT<u>ATC</u>CCAGGGTACCCCTGG
N197D	GCGCTCCCAGGGATCACC<u>GAT</u>GACACCACCATACAGCAG CTGCTGTATGGTGGTGTC<u>ATC</u>GGTGATCCCTGGGAGCGC
N298D	CAGCTGGGTTTCACCACC<u>GAC</u>CTCAGTGCCTACCAG CTGGTAGGCACTGAG<u>GTC</u>GGTGGTGAAACCCAGCTG
N393D	GTGCTCTGGGCATCC<u>GAC</u>ATCAGCTCCCCCGGC GCCGGGGGAGCTGAT<u>GTC</u>GGATGCCCAGAGCAC
N409D	CCAATAAATACATCATGC<u>GAC</u>CCCACGGCCCACCTTGTG CACAAGGTGGGCCGTGGG<u>GTC</u>GCATGATGTATTTATTGG
N416D	ACGGCCCACCTTGTGG<u>GAC</u>TCCTCGTGCCCAGGGCTG CAGCCCTGGGCACGAGGA<u>GTC</u>CACAAGGTGGGCCGT

The mutated DNA codon is underlined.