Biotin-responsive basal ganglia disease-linked mutations inhibit thiamine transport via hTHTR2: biotin is not a substrate for hTHTR2

Veedamali S. Subramanian,1,2* Jonathan S. Marchant,3* and Hamid M. Said1,2,4

Departments of 1Medicine, 2Physiology and Biophysics, University of California, Irvine, California; 3Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota; and 4Department of Veterans Affairs Medical Center, Long Beach, California

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Subramanian, Veedamali S., Jonathan S. Marchant, and Hamid M. Said. Biotin-responsive basal ganglia disease-linked mutations inhibit thiamine transport via hTHTR2: biotin is not a substrate for hTHTR2. Am J Physiol Cell Physiol 291: C851–C859, 2006—The water-soluble micronutrient thiamine is required for normal tissue growth and development in humans. Thiamine is accumulated into cells through the activity of two cell surface thiamine transporters (hTHTR1 and hTHTR2), which are differentially targeted in polarized tissues. Mutational dysfunction of hTHTR1 is associated with the clinical condition of thiamine-responsive megaloblastic anemia: the symptoms of which are alleviated by thiamine supplementation. Recently, two hTHTR2 mutants (G23V, T422A) have been discovered in clinical kindreds manifesting biotin-responsive basal ganglia disease (BBGD): the symptoms of which are alleviated by biotin administration. Why then does mutation of a specific thiamine transporter isoform precipitate a disorder correctable by exogenous biotin? To investigate the suggestion that hTHTR2 can physiologically function as a biotin transporter, we examined J) the cell biological basis of hTHTR2 dysfunction associated with the G23V and T422A mutations and 2) the substrate specificity of hTHTR2 and these clinically relevant mutants. We show that the G23V and T422A mutants both abrogate thiamine transport activity rather than targeting of hTHTR2 to the cell surface. Furthermore, biotin accumulation was not detectable in cells overexpressing either the full length hTHTR2 or the clinically relevant hTHTR2 mutants, yet was demonstrable in the same assay using cells overexpressing the human sodium-dependent multivitamin transporter, a known biotin transporter. These results cast doubt on the most parsimonious explanation for the BBDG phenotype, namely that hTHTR2 is a physiological biotin transporter.

THIAMINE (vitamin B1) is a water-soluble micronutrient that cannot be synthesized de novo by humans and is accumulated in target tissues by transport across the cell membrane using carrier-mediated mechanisms. The crucial role played by thiamine in cellular metabolism is underscored by the plethora of disorders that result from impaired thiamine accumulation (1, 5, 24). One such condition, thiamine-responsive megaloblastic anemia (9, 12, 22, 26), was shown to be an inherited disorder rather than a result of dietary deficiency, which catalyzed the identification of the first human thiamine transporter (hTHTR1, as the product of the SLC19A2 gene). A second hTHTR (hTHTR2, as the product of the SLC19A3 gene) was subsequently identified via homology cloning (~48% identity at the amino acid level (11, 30)), and these two transporters have consequently been shown by several different laboratories to be saturable, high-affinity thiamine transporters with often overlapping tissue distribution but divergent targeting in polarized cells (6, 30, 34, 37). Both thiamine transporters belong to the major facilitator superfamily of transport proteins, which have a predicted topology of 12 transmembrane (TM) domains between cytoplasmic NH2- and COOH-termini, together with a large cytoplasmic loop between TM6 and TM7, connecting the pseudosymmetrical TM1–6 and TM7–12 domains (10, 11).

Recently, two clinically relevant mutants have been identified in hTHTR2 [glycine-23 to valine (G23V) and threonine 422 to alanine (T422A); see Ref. 41], as the first naturally occurring mutants in this transporter (compared with >16 for hTHTR1; Ref. 26, 31). Intriguingly, these patients manifest biotin-responsive basal ganglia disease (BBGD), a recessive disorder characterized by a brain-specific pathology of childhood onset [subacute encephalopathy progressing through severe cogwheel rigidity, dystonia, and quadriparesis to eventual lethality (27)]. Nothing is known about the impact of these mutations on hTHTR2 cell biology, i.e., whether these point mutations impair functionality through effects on protein stability, targeting, or transport activity. More broadly, why does dysfunction of an experimentally verified thiamine transporter result in a disorder alleviated by high-dose (≥10 mg·kg⁻¹·day⁻¹) biotin supplementation? As suggested by Zeng et al. (41), the simplest explanation is that hTHTR2 is also capable of transporting biotin, and these specific mutations independently impair this transport activity.

To address these issues, we have investigated the cell biological basis of hTHTR2 dysfunction using confocal microscopy to image the targeting of the two “clinical” mutants, G23V and T422A, as well as a series of “experimental” mutants to probe the functional organization of the hTHTR2 protein. These latter mutants encompass three anionic residues potentially important for hTHTR2 function (E120A, E320A, and E346A), as well as mutants which ablate potential glycosylation sites (N45Q, N166Q). By using live cell microscopy to image the cellular distribution of these mutants, as well as [¹H]thiamine uptake assays to assess transport functionality in the same cell, we have resolved the basis of mutational dysfunction and speculate on these results in the context of the functional-domain structure of the hTHTR2 polypeptide. Furthermore, we find no evidence that 1) hTHTR2 can transport [¹H]biotin under our physiological assay conditions, 2) biotin can impair [¹H]thiamine transport in cells overexpressing...
hTHTR2 and reciprocally, and 3) that [3H]thiamine is a substrate for transport on the human intestinal sodium-dependent multivitamin biotin transporter (hSMVT). We discuss these results in the context of the powerful evidence from the BBGD kindreds (41) that correlate hTHTR2 mutation with impaired biotin homeostasis.

**Materials and Methods**

**Materials.** Green fluorescent protein (GFP)-N3 (for fusions to the NH2 terminus of GFP) was from BD Biosciences (Palo Alto, CA). Cell lines were obtained from ATCC (Manassas, VA). DNA oligonucleotide primers (Table 1) were from Sigma Genosys (Woodlands, TX). [3H]Thiamine (specific activity of 555 GBq/mmol) was obtained from ARC (St. Louis, MO) and [3H]biotin (specific activity of 51 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

**Generation of hTHTR2-GFP mutants.** The Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce insertions or deletions of nucleotides into the open reading frame of hTHTR2 (the product of SLC19A3 gene), cloned from a human colon cell line (Caco-2). The hTHTR2-GFP fusion protein was engineered by performing PCR using the gene specific primers: 5’/H11032-CCGCTCGAGATGGATTGTTACAGAACTTCACTAAG-3’ (forward) and 5’/H11032-CGGGATCCGAGTTTTGTTGACATGATGATATTGC-3’ (reverse). PCR products and the GFP-N3 vector were digested with XhoI and BamHI restriction enzymes, and the products were isolated from the gel and ligated to generate an in-frame fusion protein (hTHTR2-GFP), with GFP fused to the COOH terminus of hTHTR2. The GFP-N3 vector is driven by human cytomegalovirus promoter. A neomycin resistance cassette allows stably transfected eukaryotic cells to be selected in the presence of G418. Paired sense and antisense primer oligonucleotides encompassing the specified mutation sites (Table 1), as well as a plasmid hTHTR2-GFP were used as a template for PCR mutagenesis. Nucleotide changes in all constructs were confirmed by sequencing (Laragen, Los Angeles, CA).

**Cell culture, transient, and stable transfections.** Madin-Darby canine kidney cells (MDCK) and human duodenal cells (HuTu-80) were used for transient transfections. MDCK and HuTu-80 cells were seeded in 96-well plates and transient transfections were performed in triplicate in 96-well plates the day before a reading was taken.

**Table 1. DNA oligonucleotide primers**

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<thead>
<tr>
<th>Amino Acid</th>
<th>Forward and Reverse Primers (5’-3’)</th>
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<tr>
<td>G23V</td>
<td>GTGATCTCCCTGCTTTTTTTTTTTTTCCATAGGCA: TCTCAGATCGAGA AAAAACAAATAAAGAGGATGAC</td>
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<tr>
<td>N45Q</td>
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<td>E346A</td>
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</tr>
<tr>
<td>T422A</td>
<td>GTGATCTGAGAGATCATGACGAGGGCTGCTGCTGCTGCTGCTG</td>
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Gene-specific primers used for generating mutations in SLC19A3, showing the nucleotide changes in forward and reverse primers (in bold) used for generating specific point mutations. G23V, glycine 23 to valine; T422A, threonine 422 to alanine.

hTHTR2 and reciprocally, and 3) that [3H]thiamine is a substrate for transport on the human intestinal sodium-dependent multivitamin biotin transporter (hSMVT). We discuss these results in the context of the powerful evidence from the BBGD kindreds (41) that correlate hTHTR2 mutation with impaired biotin homeostasis.

**Fig. 1. Locations of clinical and experimental mutants in the second human thiamine transporter 2 (hTHTR2).** A: amino acid sequence of hTHTR2 highlighting the sites of clinical mutants (G23V, T422A; black shading), experimental mutants (N45Q, N166Q, N256Q, E120A, E320A, and E346A; boxed) as well as the putative transmembrane spanning segments sequences (gray shading), as proposed in Ref. 11. Underlined residues indicate extracellular loops of the polypeptide. B: topological plot showing the approximate localization of the mutated residues and clinical mutants (stars) in hTHTR2.
were maintained in minimal essential medium (MEM). Human colon adenocarcinoma cells (Caco-2) and human glioma cells (U87) were maintained in Dulbecco’s modified Eagle’s medium (DMEM). Media was supplemented with 10% fetal bovine serum, glutamine (0.29 g/l), sodium bicarbonate (2.2 g/l for MEM and 3.7 g/l for DMEM), penicillin (100,000 U/l), and streptomycin (10 mg/l). For transient transfection, cells were grown on sterile glass-bottomed petri dishes (MatTek) and transfected at 90% confluency with 1 μg plasmid DNA using LipofectAMINE 2000 (Invitrogen). After 24–48 h, cells were analyzed by confocal microscopy. For generation of stable cell lines, transiently transfected cells were selected using G418 (0.8 mg/ml) for 6–8 wk.

Confocal imaging of hTHTR2 mutants. Cell monolayers grown on coverslip dishes were imaged for construct expression using a Nikon C-1 confocal scanner head attached to Nikon inverted phase-contrast microscope. Fluorophores were excited using the 488-nm line from an argon ion-laser, and emitted fluorescence was monitored with a 530/20 nm band-pass GFP. Measurements of construct targeting polarity were made using MDCK cells and fluorescence distribution quantified using the IDL analysis package (Research Systems, Boulder, CO). Measurements of transporter distribution were analyzed using one-way ANOVA and subsequent Dunnett’s tests to compare experimental data with control constructs of known targeting polarity (6).

Vitamin uptake assays. [3H]Thiamine and [3H]biotin uptake assays were performed on confluent monolayers using established procedures (34). Protein concentrations were estimated on parallel wells using a Bio-Rad protein assay kit.

Real-time PCR. Total RNA (5 μg) was isolated from U87 cells and primed with oligo-dT primers to synthesize first-strand cDNA (Superscript first-strand synthesis RT-PCR kit, Invitrogen). To amplify the coding region of hTHTR1, hTHTR2, hSMVT, and β-actin, we used the following gene-specific primers (hTHTR1: forward 5′-AGCCAGACCGTCTCCCTTGTA-3′, reverse 5′-TAGAGAGGGCCACACACAC-3′; hTHTR2: forward 5′-TTCTCTGATTTACCCT-CCTG-3′, reverse 5′-GTATGTGCACGGGGAAGA-3′; hSMVT: forward 5′-CGATGACATTTACCAAGC-3′, reverse 5′-GGACAGCCACAGATCAAAGC-3′; β-actin: forward 5′-CATCCT-GGGTCTGGACCT-3′, reverse 5′-AAATGTCAGCCACAGTTTCC-3′). Real-time PCR conditions were performed as described previously, and data are from at least three separate experiments; the level of transporter mRNA normalized to β-actin and then quantified using a relative relationship method as detailed previously (32, 34).
Subcellular targeting of clinical hTHTR2 mutants. To resolve the cellular targeting of G23V and T422A, we transfected cDNA encoding these mutants into confluent monolayers of MDCK and Caco-2 cells (Fig. 2A), two polarized epithelial cell lines that endogenously support vectorial thiamine transport (6, 34, 37). Lateral ("xy") and axial ("xz") confocal images captured 24–48 h later showed that, in both cell lines, the wild-type hTHTR2-GFP protein predominantly targeted to the apical plasma membrane, consistent with previous results (6, 34, 37). Appropriate protein targeting under our assay conditions was also confirmed using other proteins of established targeting polarity [p75 (21), V1aR (8)] as controls (Fig. 2B). Quantification of the polarized distribution of hTHTR2 between the apical and basolateral membrane domains was made from measurements of the fluorescence profile in axial section ("xz" section). These data showed that 90.7 ± 4.0% of cell surface hTHTR2 was present at the apical membrane domain (n = 32 cells, 3 independent transfections; Fig. 2B). Both the hTHTR2 [G23V]-GFP and hTHTR2[T422A]-GFP clinical mutants targeted to the plasma membrane, and were predominantly expressed at the apical cell surface domain (Fig. 2A). However, the polarized phenotype of the G23V mutant was less pronounced than wild-type hTHTR2 (76.9 ± 3.0% expressed apically, n = 30 cells) and circumstantially, we noticed that cells expressing the T422A mutant appeared less bright than wild-type controls. To better quantify this observation at the population (cf. single cell) level, we performed flow cytometric analyses using transiently transfected MDCK populations. Measurements of the mean fluorescent intensity of MDCK cells positively transfected for T422A revealed that this mutation decreased the steady-state level of hTHTR2 55% of wild type; Fig. 2C). Given identical cDNA transfection protocols and promoter expression, this difference likely reflects differences in cellular processing of the mutant protein (e.g., relative rates of synthesis/degradation of a conformationally perturbed protein, see Discussion). Finally, we examined the effect of the two BBGD-related hTHTR2 mutations on the functionality of hTHTR2. Stable overexpression of either hTHTR2 or hTHTR2-GFP in MDCK cells resulted in an enhanced rate of [3H]thiamine accumulation (≥2-fold; P < 0.01, Fig. 2D). In contrast, [3H]thiamine uptake in cells overexpressing either the G23V or the T422A mutant was similar to untransfected cells, or cells transfected with GFP alone (Fig. 2D). These data suggest that both BBGD-related mutations in hTHTR2 are associated with
increase the rate of \(^{3}\)H]thiamine uptake (Fig. 3). Overexpression of hTHTR2 did, however, have no effect on the rate of \(^{3}\)H]thiamine accumulation (Fig. 3A). Overexpression of hTHTR2 did, however, increase the rate of \(^{3}\)H]thiamine uptake (~4.8-fold; Fig. 3B), and this enhanced rate of thiamine uptake was not inhibited by addition of 100 \(\mu\)M cold biotin to the medium. Reciprocally, overexpression of hSMVT had no effect on the rate of \(^{3}\)H]thiamine accumulation (Fig. 3B). In MDCK cells, where we performed targeting analyses and \(^{3}\)H]thiamine uptake assays (Fig. 2), similar results were obtained (Fig. 3C): the rate of \(^{3}\)H]biotin uptake was unaffected by overexpression of GFP alone, hTHTR2, hTHTR2-GFP, hTHTR2 [G23V]-GFP, or hTHTR2[T422A]-GFP. Collectively, these nutrient uptake data do not support the putative role of hTHTR2 as a biotin transporter (39, 41).

Endogenous expression and functionality of transporters in U87 cells. To investigate the expression and functionality of hTHTR1, hTHTR2, and hSMVT in a central nervous system cell line, we performed real-time PCR and nutrient uptake assays in U87 cells, which are derived from a human glioma (20). Figure 4A shows that mRNA for all three transporters was detectable in this cell line, with a relative ratio of ~4.6:2:1 for hTHTR1, hTHTR2, and hSMVT respectively, normalized to \(\beta\)-actin. Similarly to the experiments shown in Fig. 3, the rate of \(^{3}\)H]thiamine accumulation in these cells was unaffected by addition of 100 \(\mu\)M biotin, or by the removal of sodium from the uptake solution (Fig. 4B). In contrast, uptake of \(^{3}\)H]biotin was inhibited by removal of sodium, consistent with the established signature of hSMVT as a Na\(^{+}\)-dependent biotin uptake pathway (Fig. 4B).

Experimental point mutations. To probe the functional-domain structure of the hTHTR2 protein, we made point mutations of (1) three glutamic acid residues conserved within the transmembrane domains of hTHTR1 and hTHTR2 (E120A, E320A, E346A) and (2) two potential glycosylation sites in hTHTR2 (N45Q, N166Q). In polarized MDCK and Caco-2 cells, the E120A (TM4) and E320A (TM8) mutations were associated with slightly decreased expression at the apical domain (Fig. 5, A and B) but a significantly lower expression efficiency as judged by FACS (Fig. 5C). In contrast, the E346A (TM9) mutation showed comparable expression and targeting as wild type. All three mutations decreased the average rate of \(^{3}\)H]thiamine uptake, with the effects of the E120A and E320A mutations being the most pronounced (Fig. 5D).

Finally, we examined the effects of mutation of the two consensus sites for glycosylation predicted in the extracellular domain of hTHTR2 (N45Q, N166Q; Fig. 1). As a control, we also mutated N256Q, located in the TM6-TM7 cytoplasmic loop. This mutation proved to have no effect on the targeting (Fig. 5, A and B), expression (Fig. 5C), or rate of \(^{3}\)H]thiamine accumulation (Fig. 5D). N45Q, in contrast to N166Q, showed somewhat variable targeting in polarized cells: with some cells exhibiting little apical bias, and others strong apical expression. However, the expression level of both N45Q and N166Q was equivalent to hTHTR2 and the \(^{3}\)H]thiamine uptake rate was similar to wild type (Fig. 5D).

**DISCUSSION**

The aim of this study was to investigate the effect of specific hTHTR2 mutations on the functionality of this transporter in live cells. These mutants encompassed newly discovered clinical mutants in patients suffering from biotin-responsive basal ganglia disease (27, 41), as well as several experimental mutants, designed to probe the structure-function relationship of the hTHTR2. Results from these analyses are discussed in turn below.

**Clinical hTHTR2 mutants.** Two distinct clinical mutations in hTHTR2 have been recently identified using linkage analysis from BBGD patients in four families with Middle Eastern ancestry (G23V and T422A) (41). Our functional uptake data...
demonstrate that both mutations ablate the [3H]thiamine transport activity of hTHTR2 (Fig. 2D), although both mutants have significant presence at the cell surface (Fig. 2B). Consequently, in vivo, one would predict these mutants to be associated with a thiamine deficiency in cells that utilize hTHTR2 as the sole thiamine uptake pathway. Notably, both residues are conserved in hTHTR2 sequences from all species cloned to date, as well as in the other members of the SLC19A gene family (hTHTR1 (7, 11, 41) and hRFC (35)), supporting an essential role in transporter functionality (41). From hydropathy analyses (9, 11), these residues are proposed to lie within transmembrane (TM) spanning segments (G23V in TM1, T422A in TM11) of the hTHTR2 protein (Fig. 1B). Crystallographic data from other major facilitator superfamily transporters (2, 16, 18) shows that both TM1 and TM11 contribute to the central hydrophilic cavity of the transporter, underscoring the likelihood that mutation of residues within these regions could impair transporter function.

Gly-23 is situated in TM1, which is one of the four tilted central helices (TM1, -4, -7, and -10) that shape the substrate-translocation pore (2, 16, 18). In transmembrane proteins, glycine residues are found at high frequency at the points of closest packing between transmembrane helices (19). The lack of a side chain facilitates packing against other amino acids with bulkier side groupings, as well as against other glycines to promote helix interactions. Possibly, increased residue volume associated with the G23V mutation promotes steric clashes that impair protein topology or conformational changes responsible for substrate translocation as shown for glycine-to-valine substitutions in other pumps/transporters (3, 13), such as observed here with this particular mutation in hTHTR2 (Fig. 2D). More generally, this initial region of SLC19A polypeptides seems to have low functional tolerance to mutation: a P51L mutation in hTHTR1 (>P33 in hTHTR2, distal to TM1) is associated with thiamine-responsive megaloblastic anemia (23, 36).

Fig. 5. Roles of conserved transmembrane (TM) glutamic acid residues and glycosylation in hTHTR2 targeting. A: confocal images of the targeting of experimental mutations in MDCK and Caco-2 cells. Lateral xy and axial xz images for each construct were captured ~24–48 h after transfection for several cells. B: quantification of polarized expression of constructs measured from the fluorescence profile of axial scans in MDCK cells grown on a glass coverslip. C: flow cytometry analysis of the mean fluorescence intensity of populations of MDCK cells transfected with the indicated constructs. D: measurements of [3H]thiamine uptake by stable MDCK cell lines expressing the indicated constructs. Results are expressed as means ± SE of uptake values obtained from 4 independent experiments. Control and hTHTR2 data are replotted from Fig. 2D. *P < 0.05, statistical comparisons of paired data (control vs. experimental group) were made via one-way ANOVA and subsequent Dunnett’s tests.
Thr-422 lies in TM11, likely one of the four pore-forming transmembrane regions of the hTHTR2 polypeptide (TM8, TM10, TM11, and TM12). For the related hRFC transporter, Hou et al. (17) have recently demonstrated that residues within this TM11 domain play a critical role in substrate translocation. Their analyses, supported experimentally by scanning cysteine accessibility methods, suggested that a region of TM11 (Val-402 to Thr-415) in hRFC forms an amphipathic α-helical structure with certain residues from the hydrophilic face contributing to the aqueous translocation channel (17). Similar topological prediction methods (PredictProtein; Ref. 33) applied to the same region in hTHTR2 suggest that Thr-422 (analogous to Thr-415 of hRFC) forms part of an α-helical secondary structure (Fig. 6A), and is positioned on the hydrophilic face of this amphipathic helix (Fig. 6B). Obviously, direct experimental tests of aqueous accessibility and substrate protection would be a first step to test this prediction: our major inference here is that T422 is located in a crucial region of the hTHTR2 polypeptide for functional activity.

Experimental mutations. There are three glutamic acid residues (E120, E320, and E346) in the TM domains of hTHTR2, any of which may potentially contribute to transport of the positively charged thiamine molecule. Glutamic acid residues at positions E120 and E346 are replaced by positively charged residues at equivalent locations in hRFC (R133 and K336), which transports negatively charged folates, highlighting a similar mutual role for these residues in substrate binding/translocation. Our data show that the E120A mutation is essential for transporter functionality, expression and targeting, whereas the E346A mutation also inhibited thiamine uptake (4). The negative charge at position E320 in TM8 is preserved in hTHTR1 and hRFC (E337 and D310, respectively) and may be important in forming a salt bridge with the positive charged residues conserved at K380 in neighboring TM10 (R373 in hRFC and R298 in hTHTR1). Potentially, mutational ablation of this residue may destabilize transporter conformation thereby preventing functionality.

Fig. 6. Theoretical analysis of TM11 structure in hTHTR2. A: predicted secondary structure of TM11 (residues 402–426) of hTHTR2 from PredictProtein (35), highlighting helical (H) and random coil (E) regions. B: helical wheel plot (left), revealing amphipathic nature of the helical region of TM11 (hydrophobic residues, blue; region likely exposed to hydrophilic central cavity). Right, speculative schematic of the TM topology of hTHTR2 viewed from the cytoplasm, based on crystal structures of other members of the major facilitator superfamily (16–18). The 12 transmembrane helices compose 3 functional groups: pore forming (solid, TM2, 5, 8, and 11), central (dashed, TM1, 4, 7, and 10), and peripheral (gray, TM3, 6, 9, and 12), which assemble to surround a central hydrophilic cavity for substrate binding and translocation. The putative hydrophilic region of TM11, predicted in the helical wheel plot, is shown in red.

Finally, we show that mutation of the putative glycosylation sites of hTHTR2 have little effect on transporter functionality or expression level (Fig. 5), consistent with previous results from the other members of this transport family, namely hRFC and hTHTR1 (4, 35). However, it was noticeable that mutation of the NH2-terminal N-glycosylation site (N45Q) did dysregulate apical hTHTR2 targeting in MDCK cells (Fig. 5), with some cells exhibiting little apical bias in hTHTR2 expression. Although the role of N-glycosylation in apical targeting is controversial as a generalized mechanism for apical sorting (7, 14, 29), the observation that (a subset of) glycosylation sites may impact apical targeting is not without precedent (14, 29).

Relevance to BBGD. Evidence that hTHTR2 is a functional thiamine transporter derives from both overexpression (6, 30, 37), as well as silencing approaches (34). Indeed, hTHTR2 shows high structural identity to hTHTR1 (~48%), a known thiamine transporter, compared with only ~17% to hSMVT, a known biotin transporter. Consequently, the fact that the G23V and T422A mutations in hTHTR2 precipitate a condition alleviated by biotin, rather than thiamine, is an intriguing observation (41). The simplest explanation for this conundrum is that hTHTR2 has broader substrate specificity such that both biotin and thiamine can be translocated via this carrier. Consequently, the mutational dysfunction that was mapped in BBGD kindreds (41) causes cellular biotin deficiency. However, our measurements of [3H]thiamine and [3H]biotin accumulation do not support this explanation (Figs. 3 and 4): cells overexpressing hTHTR2 showed enhanced [3H]thiamine but not [3H]biotin uptake. Furthermore, addition of unlabeled biotin to the extracellular medium did not inhibit the rate of the [3H]thiamine transport. Positive control experiments underscored our ability to detect [3H]biotin transport mediated by hSMVT, which reciprocally did not translocate [3H]thiamine. These data suggest that the hTHTR2 does not transport biotin under normal physiological conditions, such that the most direct interpretation of the BBGD phenotype (that hTHTR2 is a biotin transporter) is not supported by our data. A clear caveat to this conclusion is that we could not perform our experiments in neurons derived from the brain regions (caudate nucleus, putamen) directly implicated in BBGD, owing to the difficulty of obtaining such primary tissue. However, similar results (namely that addition of exogenous biotin failed to inhibit endogenous [3H]thiamine transport) were obtained using U87 cells (Fig. 4). Nevertheless, it remains possible that the substrate specificity of hTHTR2 is modulated in a highly tissue-specific manner in BBGD. If this were true, one must also infer that these brain regions are deficient in hSMVT expression relative to their biotin usage, as the expression of this transporter in many brain regions clearly provides an alternative route for cellular biotin uptake [Fig. 4 (28, 40)]. We also suggest that ablation of [3H]thiamine uptake via hTHTR2 caused by the G23V and T422A mutations (Fig. 2), which could potentially lead to cellular thiamine deficiency, is offset...
by the functional presence hTHTR1. BBDG patients do not suffer from a generalized thiamine deficiency, nor show amelioration following exogenous thiamine administration (27). Therefore, the functional redundancy between these two thiamine transporter isofoms likely shields these patients from macroscopic thiamine deficiency, in a reciprocal manner to the limited tissue pathologies resulting from clinical mutations in hTHTR1 (31). What alternative hypotheses could then explain the BBDG phenotype? Perhaps the functionality of biotin and thiamine transporters in basal ganglia is closely coupled, such that decreases in hTHTR2 functional activity (caused by G23V/T422A) somehow effects a downregulation of biotin accumulation pathways, e.g., hSMVT. However, no direct evidence is yet available to support such a linkage, although thiamine deficiency is known to evoke a variety of changes in accumulation pathways, e.g., hSMVT. However, no direct evidence is yet available to support such a linkage, although thiamine deficiency is known to evoke a variety of changes in

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