Targeting and Trafficking of the Human Thiamine Transporter-2 in Epithelial Cells

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Humans lack biochemical pathways for thiamine synthesis, so cellular requirements are met via specific carrier-mediated uptake pathways. Two proteins from the solute carrier SLC19A gene family have been identified as human thiamine transporters (hTHTRs), SLC19A1 (hTHTR1) and SLC19A2 (hTHTR2). Both of these transporters are co-expressed but are differentially targeted in polarized cell types that mediate vectorial thiamine transport (e.g. renal and intestinal epithelia). It is important to understand the domain structure of these proteins, namely which regions within the polypeptide sequence are important for physiological delivery to the cell surface, in order to understand the impact of clinically relevant mutations on thiamine transport. Here we have characterized the mechanisms regulating hTHTR2 distribution by using live cell imaging methods that resolve the targeting and trafficking dynamics of full-length hTHTR2, a series of hTHTR2 truncation mutants, as well as chimeras comprising the hTHTR1 and hTHTR2 sequence. We showed the following: (i) that the cytoplasmic COOH-tail of hTHTR2 is not essential for apical targeting in polarized cells; (ii) that delivery of hTHTR2 to the cell surface is critically dependent on the integrity of the transmembrane backbone of the polypeptide so that minimal truncations abrogate cell surface expression of hTHTR2; and (iii) video rate images of hTHTR2-containing intracellular vesicles displayed rapid bi-directional trafficking events to and from the cell surface impaired by microtubule-disrupting but not microfilament-disrupting agents as well as by overexpression of the dynamin subunit dynamin (p50). Finally, we compared the behavior of hTHTR2 with that of hTHTR1 and the human reduced folate carrier (SLC19A1) to underscore commonalities in the cell surface targeting mechanisms of the entire SLC19A gene family.

The water-soluble micronutrient thiamine (vitamin B<sub>1</sub>) is essential for normal cellular functions, growth and development. In its coenzyme form (thiamine pyrophosphate), this vitamin plays an important role in cellular metabolism and energy production reactions. Not surprisingly, thiamine deficiency leads to a number of clinical abnormalities that encompass cardiovascular and neurological disorders. Because humans have lost their ability to synthesize thiamine de novo, they must rely on intestinal transport to meet their metabolic requirements. Furthermore, elimination of thiamine from the body is controlled via re-absorption of filtered thiamine in the renal glomeruli. Both of these systemic routes regulating thiamine uptake and elimination have been demonstrated to be carrier-mediated (9–11).

The molecular identity of the systems involved in these transport processes was revealed following the cloning of two human thiamine transporters, thiamine transporter-1 (hTHTR1<sup>3</sup>); and the product of the SLC19A2 gene (4–7)) and thiamine transporter-2 (hTHTR2; the product of the SLC19A3 gene (12)). These transporters, which share ~48% identity at the amino acid level, are predicted to have 12 transmembrane domains (TM) domains with cytoplasmic NH<sub>2</sub> and COOH termini and a large cytoplasmic loop between TM 6 and TM 7 connecting the pseudo-symmetrical TM1–6 and TM 7–12 domains (4, 12). Both proteins are endogenously expressed within intestinal and renal epithelia and transport thiamine with similar substrate specificity and pH sensitivity (11–14).

The third member of this gene family (the gene product of the SLC19A1 gene) is the human reduced folate carrier (hRFC (15)), which shares a similar topology with an overall ~45% amino acid identity to the human thiamine transporters. Functionally, hRFC constitutes the major pathway for membrane transport of reduced folates as well as a route for uptake of certain antifolate chemotherapeutics, e.g. methotrexate. Therefore, the SLC19A4 gene family encodes micronutrient transporters of similar structural design but divergent substrate specificity. Because mutational dysfunction of the thiamine transporters, hTHTR1 and hTHTR2, has implicated in discrete clinical pathologies (3, 8), it is imperative to understand the structural and cellular mechanisms supporting micronutrient transport, i.e. what features of their polypeptide structure support delivery to the cell surface, targeting to discrete plasma membrane domains as well as micronutrient translocation. To what extent are these features conserved between the different members of the SLC19A family?

Here we have resolved factors controlling the delivery of hTHTR2 to the cell surface in polarized renal and intestinal epithelia, using live cell imaging methods to image the subcellular vesicular trafficking and membrane targeting of a series of hTHTR2 constructs, as well as hTHTR1 and hTHTR2 chimeras, fused to green fluorescent protein (GFP). In contrast to many other nutrient transporters, the cytoplasmic COOH-tail of hTHTR2 is not essential for physiological targeting. Rather, we demonstrate that the cell surface expression of hTHTR2 is critically dependent on the integrity of the transmembrane backbone of hTHTR2.

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the hTHTR2 polypeptide. Delivery to the cell surface was tracked by video rate imaging methods to resolve the rapid dynamics of intracellular hTHTR2-GFP-containing structures, the motion of which was dependent on intact microtubules but not microfilaments. Furthermore, measurements of steady-state targeting of hTHTR2 in the presence of microtubule-disrupting agents, or a cytoplasmic dynein inhibitor, disrupted the polarized expression of the full-length hTHTR2 polypeptide at the apical membrane domain. Comparison of these results with data obtained previously for hRFC and hTHTR1 (16, 17) underscores commonalities shared between all members of the SLC19A gene family in regard to the structural features essential for cell surface delivery, despite their divergent targeting to discrete plasma membrane domains.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**Custom-made [3H]thiamine (specific activity 555 GBq/mmoll) was obtained from ARC (St. Louis, MO). The enhanced green, yellow, and cyan fluorescent protein vectors (GFP-N3, YFP-N1, and CFP-N1) and the subcellular targeted fluorescent protein vectors (DsRed2-ER, mitochondria CFP, and Golgi CFP) were from BD Biosciences. G418 was from Invitrogen. Cytoskeletal disrupting agents were from Calbiochem. N-(3-triethylammoniumpropyl)-4-((6-(4-(diethylamino)phenyl)hexatrienyl)-pyridinium dibromide (FM4-64) and LysoTracker Probes were purchased from Molecular Probes (Eugene, OR). MDCK (NBL-2), HuTu-80, and Caco-2 cells were obtained from ATCC (Manassas, VA). pCB6-HA-p50 was a generous gift from Prof. T. A. Schroer (The Johns Hopkins University). Plasmids for p75 and V1aR were described previously (18). Anti-hTHTR2 polyclonal antibodies were described previously (13, 18). Antibodies to hTHTR1 and hTHTR2 were from Sigma. hTHTR1 and hTHTR2 monoclonal antibodies were described previously (13, 18). Anti-rabbit (TRITC) conjugate (secondary antibodies) and all other reagents were from Sigma.

**Generation of hTHTR2-GFP and Truncated Constructs—**Construction of the full-length cDNA for hTHTR2-GFP has been described previously (18). Truncated constructs were generated by PCR using the primer combinations shown in Table 1 and in conditions described elsewhere (16, 19). The PCR products and the GFP-N3 and YFP-N1 vectors were digested with the restriction enzymes XhoI and BamHI or XhoI and SacII, and the products were gel-separated and then ligated together to generate in-frame fusion proteins with the fluorescent protein (GFP or YFP) fused to the COOH-terminus of each hTHTR-2 construct. To generate hTHTR1 and hTHTR2 chimeras, in which TM12 and COOH-terminal cytoplasmic tails of hTHTR1 and hTHTR2 were interchanged (Fig. 2A), a SalI site was introduced at 1326 and 1276 positions of hTHTR1 and hTHTR2, respectively. Chimeras were generated by PCR using the primer combinations shown in Table 1 and in conditions described above. The PCR products were digested with the restriction enzymes XhoI and SalI or SalI and BamHI, and the products were gel-separated and then ligated to generate chimera. PCR was performed on ligated chimeric products using a combination of forward (XhoI) hTHTR1 and reverse (BamHI) hTHTR2 or vice versa primers to confirm ligation. The PCR products and GFP-N3 were digested with XhoI and BamHI and ligated together to generate in-frame mutant fusion proteins. The resulting nucleotide sequence of each construct was verified by sequencing (Laragen, CA).

**Cell Culture and Transient Transfection—**Human duodenally derived intestinal epithelial cells (HuTu-80) and MDCK were maintained in minimal essential medium. Human adenocarcinoma (Caco-2) cells were maintained in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10–20% fetal bovine serum, glutamine (0.29 g/liter), sodium bicarbonate (2.2 g/liter), penicillin (100,000 units/liter), and streptomycin (10 mg/liter). For transient transfection, cells were grown on sterile glass-bottomed Petri dishes (MatTek, MA) and transfected at 95% confluency with 2 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen). For transient transfection on filters, MDCK or Caco-2 cells were seeded onto collagen-coated filters (Corning Costar, Cambridge, MA) and grown until confluency. Individual filter dishes were transfected ~5 days post-confluency in Opti-MEM medium containing 2 μg of plasmid DNA using Lipofectamine 2000. Transfected cells were visualized using confocal microscopy after 24–48 h.

**Generation of Stable Cell Lines—**HuTu-80 cells expressing hTHTR2-GFP, hTHTR2-YFP, GFP, and YFP were generated by G418 selection (0.8 mg/ml). To determine the level of hTHTR2 expression in the stable cell lines, we performed semi-quantitative RT-PCR (17). Functionality of hTHTR2 was determined by [3H]thiamine uptake assay. Briefly, cell monolayers were incubated for 7 min at 37 °C in Krebs-Ringer buffer (pH 7.4) supplemented with [3H]thiamine (30 nCi). Uptake was terminated by addition of ice-cold Krebs-Ringer buffer, and accumulated radioactivity was measured by scintillation counting.

**Depolymerization of Microtubules—**MDCK cells were treated with nocodazole using minor modifications of the method described by Kreitzer et al. (20). Briefly, cells were incubated in serum-free medium on ice for 30 min to depolymerize the cold-labile, nocodazole-resistant microtubules. Cells were then treated with 20 μM nocodazole (30 min, on ice) and thereafter transfected at room temperature with hTHTR2-GFP cDNA. The monolayer was then maintained in 10 μM nocodazole (37 °C, overnight) until imaging 18–24 h later.

**Immunofluorescence—**Nocodazole-treated MDCK cells (monolayers transfected with hTHTR2-GFP as well as naive controls) were fixed for 10 min in 4% paraformaldehyde solution (Electron Microscopy Sciences, Washington, PA). Cells were permeabilized with 2% Triton X-100 and blocked in 1% bovine serum albumin (30 min at room temperature). Cells were then incubated with hTHTR2 antibodies in phosphate-buffered saline supplemented with blocking solution (2 h, room temperature) and finally incubated with anti-rabbit TRITC-conjugated secondary antibodies in blocking solution (1 h). For visualization of immunofluorescence, cells were mounted using fluoromount reagent (Southern Biotechnology Associate Inc., Birmingham, AL) and imaged using confocal microscopy.

**Live Cell Confocal Microscopy—**Cells were imaged using a Bio-Rad MRC 1024 confocal scanner attached to an Olympus Provis AX70 upright microscope that was equipped with a ×60 water immersion objective for imaging filter-grown MDCK or Caco-2 cells. 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 or a 620 nm long pass filter. For video rate confocal images of vesicular trafficking, hTHTR2-GFP fluorescence was monitored using a homemade confocal microscope (21). Images were captured at 30 Hz (1 frame every 33 ms) and digitized using the VideoSavant processing package (IO Industries, London, Ontario, Canada). For processing the motion of individual vesicles, the frame to frame tracking function in Metamorph was employed (Universal Imaging, Downingtown, PA). Total internal reflection fluorescence (TIRF) microscopy, using a high numerical aperture lens (NA = 1.45, ×60), was used to image near-membrane trafficking events. Fluorescence emission was resolved using a EM-CCD (Hamamatsu, C9100-02). QuickTime videos of trafficking events are provided as supplemental movies 1–6.

**Flow Cytometry—**Flow cytometry was performed using a FACSCalibur benchtop cytometer (BD Biosciences). To obtain sufficient num-
bers of positively transfected cells for these experiments, MDCK cells were grown in T25 tissue culture flasks and transfected using Lipo-fectamine 2000 in serum-free media (Opti-MEM). Monolayers were trypsinized 48 h post-transfection, and cells were pelleted, resuspended, and filtered (35 μm filter) in 2-ml aliquots of Ca²⁺-free media at a density of 1 × 10⁶ cells/ml as described previously (16). In all flow cytometry experiments, samples of untransfected and GFP-transfected cells were run in parallel with experimental samples to calibrate suitable parameters for identifying the intact, transfected cell population.

RESULTS

hTHTR2 Targets to the Apical Cell Surface in Renal and Intestinal Epithelia—To visualize the targeting of hTHTR2 in both renal and intestinal epithelia, the cDNA of the full-length hTHTR2 protein was fused to GFP (hTHTR2-GFP) and transiently transfected into MDCK and Caco-2 cells, so that the resulting fluorescence distribution could be imaged using confocal microscopy. In confluent monolayers of both cell lines grown either on filter or cover glass, hTHTR2-GFP was localized at the apical cell surface (Fig. 1, A and B), as well as within a variety of intracellular structures distal to the apical surface (discussed below). Axial sections (xz) demonstrated that the plasma membrane expression of hTHTR2 was confined to the apical membrane domain, a distribution that contrasted with that of cells transfected with GFP alone, where fluorescence distribution was evident throughout the entire cytoplasmic volume (Fig. 1, A and B). Measurements of fluorescence intensity from axial sections of hTHTR2-expressing cells grown on filter sup-

![FIGURE 1. Apical targeting of hTHTR2-GFP in intestinal and renal epithelial cells. A, targeting of hTHTR2-GFP and GFP in single MDCK cells in lateral (xy, left) and axial (xz, section). B, targeting of hTHTR2-GFP and GFP in single Caco-2 cells in lateral (xy, left) and axial (xz, section). C, cumulative population measurements of axial polarity of hTHTR2 expression, compared with known apical (p75) and basolateral markers (V1aR).]
port showed almost exclusive bias toward expression at the apical cell surface (91.1 ± 3.6% apical in MDCK cells and 84.6 ± 6.2% apical in Caco-2 cells; see Fig. 1C). Appropriate polarization of monolayers was confirmed using constructs of known targeting polarity (p75, apical; V1aR, basolateral in MDCK cells; see Fig. 1C). Finally, functional assays of the [3H]thiamine accumulation using filter-grown MDCK cells revealed that apical accumulation of [3H]thiamine was almost exclusive bias toward expression at the apical cell surface, consistent with previous results (18).

duction mutants of the other members of the SLC19A family (hRFC and hTHTR1), revealed a similar effect, i.e., loss of this cytoplasmic COOH-terminal sequence decreased the overall polarity of expression of hTHTR2 in either MDCK or Caco-2 cells, imaged 24–48 h after transient transfection. Both of these truncation mutants (hTHTR2-(464)-GFP and hTHTR2-(455)-GFP, respectively, in MDCK cells; see Fig. 2A and B). The apical-basal bias of each truncation construct was certainly less pronounced than wild type hTHTR2-GFP-expressing cells. Given identical CDNA transfection protocols and promoter expression, this difference likely reflects differences in cellular processing of the truncated protein (e.g. relative rates of synthesis or degradation). To better quantify these differences at the population (cf. single cell) level, we performed flow cytometric analyses using transiently transfected MDCK populations. Measurements of the mean fluorescent intensity of positively transfected MDCK cells revealed that although partial or complete truncation of the COOH-terminal tail did not influence apical targeting polarity, loss of this cytoplasmic COOH-terminal sequence decreased the steady-state level of construct expression. The mean fluorescence of cell populations expressing hTHTR2-(464)-GFP and hTHTR2-(455)-GFP was only −75 and −25% as intense as MDCK cell populations expressing full-length hTHTR2-GFP (Fig. 2C). Comparison of these results with truncation mutants of the other members of the SLC19A gene family (hRFC and hTHTR1), revealed a similar effect, i.e. truncations

table transmembrane spanning domain were swapped (Fig. 2A). Despite replacement of the entire cytoplasmic COOH-terminal region, each chimera targeted with similar polarity to the parent protein (Fig. 2, A and B), i.e. the hTHTR2:hTHTR1 chimera targeted apically, and the hTHTR1:hTHTR2 targeted more basolaterally. Therefore, we conclude that the polypeptide sequence of the cytoplasmic tail is not essential for cell surface targeting (truncation mutants) nor does it contain determinants that direct the polarized targeting of the full-length polypeptide.

Although the cytoplasmic COOH-terminal region is nonessential for targeting to the cell surface, its presence does influence the amount of hTHTR2 protein expressed as inferred from the fluorescence intensity of transfected cells. For example, although the hTHTR2-(455)-GFP construct was predominantly targeted to the apical cell surface, cells expressing this construct were noticeably dimmer (at equivalent laser power) than wild type hTHTR2-GFP-expressing cells. Given identical CDNA transfection protocols and promoter expression, this difference likely reflects differences in cellular processing of the truncated protein (e.g. relative rates of synthesis or degradation). To better quantify these differences at the population level, we performed flow cytometric analyses using transiently transfected MDCK populations. Measurements of the mean fluorescent intensity of positively transfected MDCK cells revealed that although partial or complete truncation of the COOH-terminal tail did not influence apical targeting polarity, loss of this cytoplasmic COOH-terminal sequence decreased the steady-state level of construct expression. The mean fluorescence of cell populations expressing hTHTR2-(464)-GFP and hTHTR2-(455)-GFP was only −75 and −25% as intense as MDCK cell populations expressing full-length hTHTR2-GFP (Fig. 2C). Comparison of these results with truncation mutants of the other members of the SLC19A gene family (hRFC and hTHTR1), revealed a similar effect, i.e. truncations

### Table 1

**Combination of primers used to prepare the truncated constructs by PCR**

| Construct       | Forward and reverse primers (5′–3′)                                      | Positions | Fragment |
|-----------------|------------------------------------------------------------------------|-----------|----------|
| hTHTR2-GFP      | COCTCGACAGTGATTGTTACAGAAGTTCACTAAG; GTCGACGGTACGTGAACTAGTTATTC;        | 1–1488    | 1488     |
| hTHTR2-YFP      | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1380    | 1308     |
| hTHTR2-(1–464)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1380    | 1308     |
| hTHTR2-(1–455)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1308    | 1308     |
| hTHTR2-(1–446)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1308    | 1308     |
| hTHTR2-(1–436)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1308    | 1308     |
| hTHTR2-(1–423)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1269    | 1269     |
| hTHTR2-(1–396)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1188    | 1188     |
| hTHTR2-(1–190)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–570     | 570      |
| hTHTR2-(456–496)-GFP | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1366–1488 | 122      |

**Chimeras**

| Chimera          | Forward and reverse primers (5′–3′)                                      | Positions | Fragment |
|------------------|------------------------------------------------------------------------|-----------|----------|
| hTHTR2-(1–425)   | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1275    | 1275     |
| hTHTR2-(426–496) | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1276–1491 | 215      |
| hTHTR1-(1–442)   | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1–1325    | 1325     |
| hTHTR1-(443–497) | GTCGACGGTACGTGAACTAGTTATTC; GTCGACGGTTGAGAAGAAGGAAAGCAGG;               | 1326–1491 | 165      |

The Cytoplasmic COOH-terminal Tail of hTHTR2 Is Not Required for Apical Cell Surface Targeting.—To delimit the region(s) within the hTHTR2 polypeptide important for targeting to the cell surface in polarized cells, we designed a series of eight hTHTR2 truncation constructs (Table 1). First, we examined the effect of truncation of the cytoplasmic COOH-terminal tail of hTHTR2, because the polarized targeting of many cell surface transporters is impacted by targeting motifs/sequences within the region. Truncation of the COOH-terminal tail of hTHTR2 (amino acids 456–496), either partially (hTHTR2-(464)-GFP) or completely (hTHTR2-(455)-GFP), did not disrupt the overall polarity of expression of hTHTR2 in either MDCK or Caco-2 cells, imaged 24–48 h after transient transfection. Both of these truncation constructs expressed with predominant apical polarization (e.g. axial fluorescence ratios resolved 84.0 ± 6.2 and 77.2 ± 7.5% apical targeting for hTHTR2-(464)-GFP and hTHTR2-(455)-GFP, respectively, in MDCK cells; see Fig. 2A and B). The apical-basal bias of each truncation construct was certainly less pronounced than wild type (Fig. 2A), but for both mutants the majority of expressed protein was targeted apically (by at least a 3:1 ratio). Next, we engineered two reciprocal chimeras between hTHTR1 (basolateral bias (18)) and hTHTR2 (apical bias; see Fig. 1), in which the entire COOH-terminal tail and the transmembrane spanning domain were swapped (Fig. 2A). Despite replacement of the entire cytoplasmic COOH-terminal region, each chimera targeted with similar polarity to the parent protein (Fig. 2, A and B), i.e. the hTHTR2:hTHTR1 chimera targeted apically, and the hTHTR1:hTHTR2 targeted more basolaterally. Therefore, we conclude that the polypeptide sequence of the cytoplasmic tail is not essential for cell surface targeting (truncation mutants) nor does it contain determinants that direct the polarized targeting of the full-length polypeptide.
into the cytoplasmic tail of hRFC and hTHTR1 decreased the protein expression (Fig. 2C) but not the targeting of the protein to the plasma membrane (16, 17). Therefore, although the COOH-terminal tail does not dictate targeting, it appears to play an important role in the efficiency of expression of all the members of the SLC19A gene family.

FIGURE 2. The cytoplasmic COOH-terminal region is not essential for polarized expression of hTHTR2 at the cell surface. A, left, polarized targeting of truncated constructs (hTHTR2-(1–455)-GFP and hTHTR2-(1–464)-GFP) as well as chimeras (hTHTR2:hTHTR1 and hTHTR1:hTHTR2) in transiently transfected MDCK cells expressed as a proportion at the apical or basal cell surface. Right, schematic representation of individual constructs. hTHTR2 is represented with a cytoplasmic NH2-terminal sequence, 12 transmembrane-spanning regions (amino acids 8–455), and a cytoplasmic COOH-terminal tail (amino acids 456–496). In all cases, GFP was fused to the COOH-terminal of the construct. B, lateral (xy) and axial (xz) sections showing construct targeting in MDCK cells. C, truncation of the cytoplasmic COOH-terminal tail affects transporter expression. FACS analyses of mean total fluorescence of MDCK cells were positively transfected with the indicated hTHTR2 truncation constructs, expressed as a percentage of that obtained with the full-length hTHTR2-GFP protein (n = 3 independent transfections). For comparison, results for hRFC and hTHTR1 are shown. Data are from Refs. 16–18.

Plasma Membrane Expression of hTHTR2 Is Dependent on the Integrity of the Polypeptide Backbone—In contrast to deletion/replacement of the COOH-terminal tail, further truncation into the transmembrane backbone of hTHTR2 resulted in a complete loss of cell surface targeting. Truncation after TM6 (hTHTR2-(190)-GFP), i.e. in the cytoplasmic
A linker between the TM1–6 and TM7–12 domains, resulted in retention of the mutant construct within the endoplasmic reticulum (Fig. 3A) as well as a dramatic decrease in amount of protein expressed as judged by fluorescence intensity (Fig. 3B). Similarly, shorter deletions comprising either the last two TM domains (hTHTR2-(396)-GFP) or the last TM domain (i.e. hTHTR2-(423)-GFP and hTHTR2-(436)-GFP) also abrogated cell surface expression (Fig. 3A) and decreased the steady-state level of protein expression (Fig. 3B). Even deletion of the distal 9 amino acids (hTHTR2-(446)-GFP), predicted to comprise part of the last transmembrane domain (TM12), resulted in a loss of cell surface target-
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First, we generated a stable hTHTR2-YFP and stable HuTu-80 cell line via G418 selection (~70% YFP-positive cells assessed by FACS; see Fig. 5A, i). After ~10 weeks of antibiotic selection, RT-PCR measurements demonstrated that hTHTR2 expression was ~2.2-fold greater in this stable cell line relative to mock-transfected controls. Functional measurements of [3H]thiamine accumulation revealed that [3H]thiamine uptake was ~1.6-fold greater in the stable cell line, confirming functionality of the full-length fusion protein (Fig. 5A, ii). Live cell confocal imaging revealed YFP fluorescence was distributed throughout the entire cell surface, as well as within numerous intracellular structures, as demonstrated by three-dimensional renditions of single cells (e.g. Fig. 5A, iii, and supplemental movie 1). Particle counting functions applied to flattened projections of “xzy” stacks taken from hTHTR2-YFP-expressing cells typically estimated >100 discrete particles per cell (Fig. 5A, iii), although this value may be an overestimate because of the motility of intracellular structures during the time taken for image capture. Co-labeling with organelle specific markers suitable for dual emission studies confirmed expression across the whole cell surface and within the Golgi apparatus (Fig. 5B). However, the intracellular particles were distinct from the mitochondrial and endoplasmic reticulum networks, although a small degree of overlap was demonstrable with the lysosomal network (Fig. 5B).

Visual inspection of confocal images revealed the following two major classes of hTHTR2-YFP-expressing particles: first, large vesicular structures (diameter <0.5 μm, ~5~10 evident throughout a single confocal plane; Fig. 5C, asterisks); and second, numerous smaller vesicles (Fig. 5C, arrows). It was immediately evident that these structures exhibited differential mobility. Although it was possible to track the movement of the larger structures from frame to frame (see below and Fig. 6C), the small structures were sufficiently dynamic so that it was impossible to resolve their trajectories even at room temperature by using the slow frame scan speeds typical of many commercially available confocal microscopes (~1–2 s per frame). For example, Fig. 5C, ii, shows examples of images captured at 2-s intervals, in which small vesicles (arrows) could be observed appearing in new locations from frame to frame, but these individual particles could not be reliably tracked between successive images. In contrast, the larger particles (Fig. 5C, ii, asterisks) could be resolved in similar locations from one frame to the next. The dynamic motion of the small hTHTR2-YFP-containing vesicles is better depicted in Fig. 5C, iii, which is an overlay of successive frame-scan images of a hTHTR2-YFP-expressing cell, and each scan is represented as a specific color. Although the larger particles remained relatively motionless over the 8-s record period (producing a composite white color), the smaller particles were sufficiently motile that they appeared in discrete frames only as individual colors (e.g. red, green, blue, etc.), each relating to a single image frame.

FIGURE 4. Microtubule disruption inhibits steady-state localization of hTHTR2 protein in polarized MDCK cells. Microtubules were depolymerized following the protocol of Kreitzer et al. (20) as described under “Experimental Procedures.” A, in live cells treated with nocodazole prior to transient transfection, hTHTR2-GFP displayed a dysregulated expression profile after 18–24 h (right) compared with control cells (left) in axial section (xz), as shown in the associated fluorescence profiles. B, in fixed cells, axial (xz) confocal images showed colocalization of hTHTR2-GFP fluorescence (top, green) and hTHTR2 (middle, red), as evident in the fluorescence overlay (bottom) in both control cells (left) and nocodazole-treated cells (right).
FIGURE 5. Characterization of a hTHTR2-YFP-expressing stable HuTu-80 cell line. A. i. lateral (xy) scan of hTHTR2-YFP-expressing HuTu-80 stable cell line. ii. top, RT-PCR results comparing hTHTR2 expression in control (C, untransfected, left) and the hTHTR2-YFP-expressing HuTu-80 cell line (S, right). Bottom, \( [3H]\text{thiamine} \) uptake results (expressed as pmol of \( [3H]\text{thiamine} \)/mg protein/7 min) using the HuTu-80 control and hTHTR2-YFP stable cell line. iii. distribution of cytoplasmic hTHTR2-containing structures (red) in a single HuTu-80 cell. Inset, three-dimensional projection of the same cell from an xyz image stack (M; provided as supplemental video 1). B. dual emission experiments comparing hTHTR2 distribution

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Therefore, to better resolve the motion of the small vesicles, we turned to video-rate confocal microscopy (21). However, even with the high temporal resolution of this technique, the trafficking vesicles proved to be challenging to image because their small size (and YFP content) limited their resolvability to short imaging periods due to fluorophore bleaching. Nevertheless, by monitoring the coordinates of individual vesicles at 33-ms intervals over several seconds, the motion of these particles could be represented as color tracks indicating the speed, direction, and frequency of movements. For example, Fig. 6A highlights the movement of a single vesicle in a region of a hTHTR2-GFP-expressing cell maintained at 37 °C, the motion of which was tracked over several seconds (Fig. 6A, ii, and supplemental movie 2). Periods of rapid, approximately linear movements, often bi-directionally over the same region, were interspersed with frequent periods of relative immobility. Single vesicles were observed moving toward and away from the cell surface, as well as circumferentially beneath the plasma membrane.

Quantitation of these linear movements from many different cells (examples in Fig. 6A, iii) demonstrated an average “run length” of 3.9 ± 0.6 μm (41 vesicles). The cumulative velocity histogram suggested a wide distribution of vesicular speeds, with an average velocity of 2.3 ± 0.1 μm s⁻¹ (Fig. 6A, iv). By having established these parameters, we proceeded to examine the effects of cytoskeletal disruption on vesicular trafficking. Cells were maintained at 37 °C on the microscope stage, and 5 s of video rate records were captured at various time points (5, 10, 15, and 30 min) after the addition of either nocodazole (10 μM), colchicine (20 μM), γ-lumicolchicine (75 μM), or cytochalasin D (15 μM). Whereas cytochalasin D and γ-lumicolchicine had little effect on vesicular motility during this period, both nocodazole and colchicine inhibited the movement of hTHTR2-containing particles (Fig. 6A, iv).

Because these data implicated the involvement of microtubule-based transport events (see “Discussion”), we attempted to directly image the movement of single hTHTR2 vesicles on microtubules. To do this we exploited the advantages of TIRF microscopy to image near membrane trafficking events (22, 23). Fig. 6B compares epifluorescence and TIRF images of a HuTu-80 cell expressing a GFP-tagged α-tubulin. Although epifluorescence illumination of the entire cell revealed little detail (Fig. 6B, i), TIRF illumination at the cover glass interface clearly resolved distinct microtubular tracks throughout the “footprint” of the cell (Fig. 6B, ii). Although the emission spectra of GFP and YFP were not separable to allow independent resolution of each fluorophore, co-expression of GFP-tubulin and hTHTR2-YFP resulted in morphological phenotypes sufficiently distinct (compare Figs. 5A, iii, and 6B, ii) to identify vesicles moving on distinct microtubular structures. Supplemental movie 3 shows such an example of an hTHTR2-YFP vesicle visualized moving toward the cell surface on a track dictated by a microtubule (Fig. 6B, iii). Compared with the dynamic behavior of the small hTHTR2-containing particles, the larger particles were relatively motionless when imaged at video-rate (Fig. 6C, i). However, both sets of particles were frequently observed to interact, an example is shown in Fig. 6C, ii, where one vesicle appearing to derive from the plasma membrane rapidly moved into sufficiently close apposition with the larger particle that it became difficult to discriminate the structures as separate entities. The behavior is highlighted via a Prewitt filter applied to the same image.

**DISCUSSION**

The second human thiamine transporter, hTHTR2, was recently identified by homology cloning (12). This transporter is expressed in a number of tissues, including the kidney and along the entire length of the human intestinal epithelium, where it is expressed at the apical plasma membrane domain (12, 13). However, nothing is known about the mechanisms that control the apical delivery of hTHTR2 in these polarized epithelia that are responsible for thiamine (re)absorption. Here, we have investigated the molecular determinants of hTHTR2 targeting in renal and intestinal epithelial cells, as well as the cellular mechanisms that underpin the apically directed motility of hTHTR2. Our findings in both of these areas are discussed in the following two sections and compared with results obtained with the two other SLC19A family members, hRFC (SLC19A1) and hTHTR1 (SLC19A2).

**Molecular Determinants of hTHTR2 Targeting in Polarized Epithelial Cells**—Sequences or conformational motifs critical for plasma membrane targeting have been identified in many cell surface proteins (26–31). Given that regions within the cytoplasmic COOH-terminal region have been shown to dictate the polarized expression of several nutrient transporters in epithelial cells, we were first interested in the role of this region (amino acids 456–496) in influencing the apical targeting of hTHTR2. We hypothesized that the significant divergence in the amino acid sequence and length of the cytoplasmic regions of hTHTR1 and hTHTR2 might contribute to the differential targeting of these transporters in polarized epithelia. However, both the truncation as well as the chimeric approach failed to demonstrate a key role for this region in dictating the polarity of transporter expression (Fig. 2). Therefore, unlike many other nutrient transporters, this gene family does not depend on targeting “motifs” confined exclusively within their NH₂-(31) and COOH-terminal cytoplasmic regions (29, 30), but rather...
FIGURE 6. Vesicular dynamics of hTHTR2-YFP resolved by video-rate confocal microscopy. Characterization of dynamics of individual vesicles containing hTHTR2-YFP in HuTu-80 cells. A, localization (i) and dynamics (ii) of a single hTHTR2-YFP-positive vesicle in a cell maintained at 37 °C. The video sequence from which this image was obtained is available as supplemental material (M indicates supplemental movie 2). iii, tracks of individual vesicles (represented by different colors) derived from similar experiments (scale bar, 5 μm). iv, cumulative velocity histogram of the hTHTR2-YFP small trafficking vesicle population measured during periods of linear movements at 37 °C. v, similar measurements made at various times (x axis) after addition of nocodazole (N; 10 μM), colchicine (C; 20 μM), γ-lumicolchicine (G; 75 μM), and cytochalasin D (Cy; 15 μM). B, TIRF imaging of HuTu-80 cells. i, HuTu-80 cells expressing a tubulin-GFP construct imaged by wide field epifluorescence illumination. ii, same cell imaged by TIRF (λ = 488 nm). iii, video still from a HuTu-80 cell co-expressing hTHTR2-YFP and tubulin-GFP showing motion of a vesicle along a microtubule track (M indicates supplemental movie 3). C, interactions between hTHTR2-YFP-containing structures. i, image from an hTHTR2-YFP-expressing HuTu-80 cell showing the localization (left) and an example of the dynamics (M indicates supplemental movie 4; color track, right) of a larger hTHTR2-YFP-containing particle. ii, an example of a small trafficking vesicle that derived from the vicinity of the cell surface (yellow arrow) interacting with the larger cytoplasmic hTHTR2-YFP-containing particles. Raw (top) and Prewitt-filtered (bottom) images from sequences provided as supplemental material (M indicates supplemental movies 5 and 6).
belongs to a growing group of multispansing membrane proteins where targeting motifs are likely contained within the transmembrane region of the polypeptide (32–34). In contrast, truncation into the polypeptide backbone of hTHTR2 abrogated expression at the cell surface. Truncation within the loop connecting both halves of hTHTR2 (hTHTR2-(1–190) or after TM10 (hTHTR2-(1–396)), TM11 (hTHTR2-(1–423) and hTHTR2-(1–436)), or only a 9-amino acid truncation into TM12 (hTHTR2-(1–446)), all prevented expression of hTHTR2 at the cell surface. These results parallel recent data obtained with hRFC (16) and hTHTR1 (17), which collectively demonstrate the following: (i) a redundancy of both the NH2-terminal and COOH-terminal cytoplasmic regions in cell surface targeting for all three family members, and (ii) a shared intolerability to transmembrane deletions from either the NH2 or COOH terminus for all three family members. Consequently, comparison of the effect of polypeptide truncations analyzed here with predicted clinical mutations that result in premature truncation of the polypeptide (examples shown for hTHTR1; see Fig. 8A) suggests that the clinically relevant truncation mutations identified in hTHTR1 will likely abrogate cell surface expression. Recent crystallographic structures of three bacterial members of the major facilitator superfamily of proteins (GlpT (35), LacY (36), and OxlT (37)) provide structural insight into the likely basis for this result. From all three crystal structures, as well as from biochemical studies (38), it is evident that 12 transmembrane domains divide into three functional groups (see Fig. 8) as follows: the peripherally embedded helices (TM 3, 6, 9, and 12) and two sets of central cavity lining regions, the pore-forming transmembrane domains (TM 2, 5, 8, and 11) and four tilted central helices that contact either the cytoplasmic or extracellular facets of the channel (TM 4, 7, and 10). One helix from each grouping contributes to two three-helix bundles (e.g. TM 7, 11, and 12; TM 8–10) comprising half of the full-length transporter. Therefore, COOH-terminal deletions into TM12, although itself a peripheral helix, likely impact the conformation of the pore-forming TM11, a region recently implicated as a key substrate binding domain in hRFC (39). Similarly, even small NH2-terminal deletions not only impact the structure of a central helix (TM1) but also the conformation of the closely associated pore-forming helix TM5. The crucial role of the helices lining the central hydrophilic cavity is further underscored by the fact that all point mutations identified to date in hTHTR1 or hTHTR2 that are implicated as clinically relevant are closely associated with the transmembrane domains lining the central cavity (Fig. 8B). In summary, therefore, mutagenesis results from all three of the SLC19A transporters, which share ~45% overall amino acid identity, concur that the integrity of the polypeptide backbone is crucial for normal cell surface targeting, albeit targeting to different membrane domains in polarized epithelia (hRFC, basolateral; hTHTR1, biased basolaterally (18); hTHTR2, apical).

**Intracellular Trafficking and the Role of Dynein in Apical Targeting of hTHTR2**—The capacity to image hTHTR2-YFP dynamics using the high temporal and spatial resolution of video-rate confocal microscopy demonstrated a rapid motility of hTHTR2-containing vesicles (Figs. 5 and 6). Comparison of the cumulative velocity histogram for hTHTR2-YFP (Fig. 6A, iv), with that measured for hTHTR1-GFP under similar conditions (17), suggested a more rapid motility of hTHTR2-trafficking vesicles that those containing hTHTR1 (average velocity of 2.3 ± 0.1 versus 1.71 ± 0.13 μm s⁻¹ and hTHTR2 versus hTHTR1 at 37°C, respectively). Further experiments involving transporter co-expression are required to identify whether this implicates independent transport vesicles for individual thiamine transporters or simply reflects more
rapid intracellular trafficking events in cells overexpressing hTHTR2. Given the roles of thiamine pyrophosphate-dependent enzymes in the pathways of energy production, one might speculate a link between enhanced ATP synthetic capacity and activity of ATP-dependent vesicular motors in cells actively accumulating thiamine.

In animal cells, the two major families of microtubule-based motor proteins that convey trafficking vesicles through the cytoplasm are the kinesins (plus end-directed motors) and dyneins (minus end-directed motors). These motor proteins often co-localize on the same structures so that the net direction of cargo transport depends on the antagonistic balance between plus and minus end-directed activities resulting in a frequently observed bi-directional, saltatory motion (40), as observed here with hTHTR2-GFP (Fig. 6). Because microtubules are arranged longitudinally in polarized MDCK cells with their minus ends oriented toward the apical cell surface (24, 25), it was logical to investigate the role of minus end-directed motors (e.g. cytoplasmic dynein) in transporting hTHTR2 toward the apical membrane (41–43). Directed trafficking of vesicles via conventional cytoplasmic dynein requires dynactin, a multisubunit protein complex that directly binds dynein endowing the motor with the ability to convey specific cargo long distances across the microtubule network (reviewed in Ref. 44). One of the subunits of this dynactin complex, dynamitin (p50, DCTN2), plays a crucial role in holding the dynactin complex together (45), as free dynamitin causes p150Glued and p24/22 to be displaced from the dynactin structure. Therefore, the role of dynactin, and by inference dynein-based motility, in cellular processes has commonly been investigated via cytoplasmic dynamitin overexpression (46, 47). By using this approach, we show that inhibition of dynein function leads to a dysregulation of cell surface targeting of hTHTR2 (Fig. 7). However, even though this result implies a direct role for dynein-directed movements in the apical targeting of hTHTR2, we cannot unequivocally rule out a broader effect on the disruption of the microtubular cytoskeleton (48, 49). Therefore, most conservatively, this result supplements the several parallel lines of evidence for microtubule-dependent delivery of hTHTR2 to the cell surface as follows: first, run lengths and velocities are consistent with values reported for microtubule-based vesicular motion in vivo (50), and with the speeds of microtubule motors assayed in vitro (51); second, pharmacological inhibition of motility by nocodazole and colchicine, but not γ-lumicolchicine, a nontubulin-binding analog (52); third, direct resolution of microtubule-associated movement using TIRF microscopy.
(Fig. 6b); and finally, the dysregulation of apical targeting of hTHTR2 in steady-state analyses following dynamin overexpression (Fig. 7). Most interestingly, the apical targeting of rhodopsin has been shown to depend on the binding of Tctex-1, a 14-kDa dynein light chain, to the cytoplasmic COOH-terminal tail of the receptor, a region in which the apical targeting motif is also embedded (42, 53). Because the cytoplasmic tail of hTHTR2 is nonessential for apical targeting (Fig. 2), one must infer either a different region of hTHTR2 interacts with dynein or the interaction is mediated indirectly by another protein, such that apical targeting results from selective sorting of hTHTR2 into a vesicle otherwise destined for apical delivery. If the latter suggestion proves to be true, then more subtle aspects of protein topology/conformation that determine residency in discrete vesicles may prove just as important as targeting signals that act as direct binding partners with the cellular polarization machinery. Perhaps the emerging lack of similarities in sequence and size of identified apical targeting motifs may support such a conformational sorting mechanism (54).

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