Substituted Cysteine Accessibility Reveals a Novel Transmembrane Domain 2–3 Reentrant Loop and Functional Role for Transmembrane Domain 2 in the Human Proton-coupled Folate Transporter*

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Background: The proton-coupled folate transporter (PCFT) targets cytotoxic antifolates to tumors.

Results: Membrane-impermeable 2-aminoethyl methanethiosulfonate-biotin reacted with cysteine insertions in PCFT transmembrane domain 2 (TMD2) and the TMD 2–3 loop. Residues in TMD2 interact with PCFT substrates, and a reentrant loop connects TMDs 2 and 3. Characterization of PCFT structure is essential to understanding the transport mechanism, including the critical determinants of substrate binding.

The proton-coupled folate transporter (PCFT) is a folate-proton symporter highly expressed in solid tumors that can selectively target cytotoxic antifolates to tumors under acidic microenvironment conditions. Predicted topology models for PCFT suggest that the loop domain between transmembrane domains (TMDs) 2 and 3 resides in the cytosol. Mutations involving Asp-109 or Arg-113 in the TMD2-3 loop result in loss of activity. By structural homology to other solute carriers, TMD2 may form part of the PCFT substrate binding domain. In this study, we mutated the seven cysteine (Cys) residues of human PCFT to serine, creating Cys-less PCFT. Thirty-three single-Cys mutants spanning TMD2 and the TMD2-3 loop in a Cys-less PCFT background were transfected into PCFT-null HeLa cells. All 33 mutants were detected by Western blotting, and 28 were active for [3H]methotrexate uptake at pH 5.5. For the active residues, we performed pulldown assays with membrane-impermeable 2-aminoethyl methanethiosulfonate-biotin and streptavidin beads to determine their aqueous-accessibilities. Multiple residues in TMD2 and the TMD2-3 loop domain reacted with 2-aminoethyl methanethiosulfonate-biotin, establishing aqueous accessibilities. Pemetrexed pretreatment inhibited biotinylation of TMD2 mutants G93C and F94C, and biotinylation of these residues inhibited methotrexate transport activity. Our results suggest that the TMD 2–3 loop domain is aqueous-accessible and forms a novel reentrant loop structure. Residues in TMD2 form an aqueous transmembrane pathway for folate substrates, and Gly-93 and Phe-94 may contribute to a substrate binding domain. Characterization of PCFT structure is essential to understanding the transport mechanism including the critical determinants of substrate binding.

Folates are water-soluble B9 vitamins that are required for cellular growth and development (1). Intracellular folates participate in one-carbon transfer reactions that contribute to the synthesis of serine, methionine, thymidylate, and purine nucleotides. In mammals, folates cannot be synthesized de novo and must be obtained from the diet (1).

The proton-coupled folate transporter (PCFT;3 SLC46A1; NP_54200) is a proton/folate symporter in the major facilitator superfamily (MFS) of transporters, which includes a wide array of symporters and antiporters (2–5). Human PCFT (hPCFT) is expressed abundantly in the proximal jejenum and duodenum and functions as the principal mechanism for the intestinal absorption of dietary folates (3, 6). Loss of hPCFT is associated with hereditary folate malabsorption syndrome, a rare autosomal recessive condition characterized by systemic folate deficiency and dramatically decreased folate levels in the central nervous system (3, 7–17). Whereas hPCFT is also expressed in other normal tissues, the high levels of hPCFT in many solid tumors are of particular interest given their potential for tumor-targeting with novel hPCFT-selective cytotoxic antifolates (18, 19).

Protein structural information for MFS transporters is essential for understanding their mechanisms of membrane transport. For mammalian MFS proteins, detailed structural information is uncommon, reflecting difficulties in isolating sufficient amounts of purified proteins and in crystallizing integral membrane proteins for x-ray diffraction studies. However, x-ray structures have been reported for bacterial MFS proteins, including the lactose/proton symporter (LaY) and the glycerol 2-phosphate/inorganic phosphate antiporter (GlpT) (20–22). Despite their different mechanisms and limited sequence

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†‡§ The abbreviations used are: PCFT, proton-coupled folate transporter; hPCFT, human PCFT; CL, cysteine-less; GlpT, glycerol 2-phosphate/inorganic phosphate transporter; LaY, lactose/proton symporter; MFS, major facilitator superfamily; MTSEA, 2-aminoethyl methanethiosulfonate; MTX, methotrexate; PMX, Pemetrexed; SCAM, substituted cysteine accessibility method; TMD, transmembrane domain; WT, wild-type.
homologies, the structures of LacY and GlpT are similar and show a symmetry in which two bundles of six hydrophobic helices are joined by a hydrophilic linker and surround a central cavity formed by helices 1, 2, 4, and 5 of the N-terminal segment and helices 7, 8, 10, and 11 of the C-terminal segment (23). Helices 3, 6, 9, and 12 do not directly contribute to the central transmembrane cavity and substrate binding domain and are embedded in the lipid membrane (23).

Similarly, hPCFT is predicted to have 12 transmembrane domains (TMDs) with cytosolic N and C termini (Fig. 1) (24, 25). This has been experimentally validated by indirect immunofluorescence staining of N- and C-terminal hemagglutinin (HA)-tagged hPCFT and by substituted cysteine (Cys) accessibility methods (SCAM) involving Cys insertions into the TMD loop domains and reaction with 2-aminothyl methanethiosulfonate (MTSEA)-biotin (25). The extracellular location of the loop connecting TMDs 1 and 2 was further evidenced by the fact that two predicted N-linked glycosylation sites in that loop (Asn-58 and Asn-68) are glycosylated in human PCFT (24). Other aspects of hPCFT structure have been validated, including reactivity of membrane-impermeable methanethiosulfonate reagents with Phe-157, Gly-158, and Leu-161 in TMD4 and with Ile-188 in TMD5. Because methanethiosulfonate reactivity was protected by the hPCFT antifolate substrate Pem etrexed (PMX), these residues likely lie within or near the aqueous accessible substrate binding site (26, 27). However, the contributions of other TMDs to the three-dimensional hPCFT structure have not been determined.

A number of specific amino acid residues were implicated as functionally important in hPCFT including Glu-185 (TMD5; involved in proton coupling), His-281 (TMD7; involved in substrate binding), and Arg-376 (TMD10; impacts proton and substrate binding) (Fig. 1) (7, 28, 29). His-247, localized to the loop domain between TMDs 6 and 7, is predicted to interact with Ser-172 at the cytoplasmic opening to control substrate access to the aqueous binding site (29).

The conserved loop domain linking TMD2 and TMD3 (residues 109–114) is of particular interest. This stretch is predicted to have a cytoplasmic orientation (Fig. 1) and includes Asp-109 and Arg-113, both of which cannot be replaced by other amino acids regardless of charge or polarity (9, 11, 17). It was predicted that the loop region connecting TMD2 and TMD3 including Asp-109 and Arg-113 forms a β-turn structure involving the DSVG tetrapeptide and may be functionally important (11, 30). By homology modeling based on the GlpT template, Arg-113 was predicted to protrude into a hydrophobic cavity composed of TMDs 1, 3, 4, and 6 (11). However, experimental confirmation was not provided.

In this report we use SCAM with a Cys-less (CL) hPCFT that exhibits functional characteristics virtually identical to wild-type (WT) hPCFT and MTSEA-biotin, combined with systematic site-directed mutagenesis, to directly explore the structural and functional significance of the TMD2-3 loop domain and TMD2 in hPCFT. Our results establish that the TMD2-3 domain forms a novel reentrant loop structure (31) and suggest that amino acids at positions 93 and 94 in TMD2 may comprise part of a critical substrate binding domain with aqueous accessible sites. Our results are unprecedented and provide important new insights into key structural and functional determinants of hPCFT uptake.

**EXPERIMENTAL PROCEDURES**

**Reagents**—[3,5,7-3H]Methotrexate (MTX) (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled MTX was provided by the Drug Development Branch, NCI, National Institutes of Health (Bethesda, MD). Folic acid was purchased from Sigma. PMX (Alimta) was purchased from LC Laboratories (Woburn, MA). Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine serum (FBS), which was purchased from Hyclone Technologies (Logan, UT).

**Generation of Cys-less hPCFT and Single-substitution Mutants**—CLhPCFT, including a HA epitope at the C terminus (hereafter, CL-PCFT) in pCDNA3, was prepared by PCR from the C-terminal HA-tagged WT hPCFT (hereafter, WT-PCFT) construct by mutating individual Cys residues (Cys-21, -66, -151, -229, -298, -328, and -397) to serine using the QuikChange™ Multi Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) as described by the manufacturer. Single amino acid substitutions were generated using either WT- or CL-hPCFT as a template and the QuikChange™ site-directed mutagenesis kit (Agilent). Primers were developed using the QuikChange™ Primer Design program (Agilent) and are available upon request. PCR conditions were 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min for 16 cycles. All mutations were confirmed by DNA sequencing by Genewiz, Inc. (South Plainfield, NJ).

**Cell Culture**—The hPCFT-null R1-11 HeLa cell line was a gift of Dr. I. David Goldman (Bronx, NY) (32). Cells were grown in RPMI 1640 media supplemented with 10% FBS, 1% penicillin/ streptomycin, and 2 mM L-glutamine. For transfections with hPCFT constructs, R1-11 cells were seeded in 60-mm culture dishes at a density of 0.8 million cells per plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. After 24 h, the cells were transfected with hPCFT expression constructs (above) in pCDNA3 (1 μg/plate) using Lipofectamine 2000 (Invitrogen) (10 μl/plate) in OptiMEM as described by the manufacturer (Invitrogen). After 4 h the membrane transfection medium was replaced with DMEM containing 10% FBS.

**Membrane Transport**—Forty-eight hours post-transfection, cellular uptake of [3H]MTX (0.5 μM) was measured over 2 min at 37 °C in 60-mm dishes in MES-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose; pH 5.5) as previously described (33). Cells were washed 3× with Dulbecco's phosphate-buffered saline (PBS), and the cells were solubilized in 0.5 N NaOH. Levels of intracellular radioactivity were calculated as pmol/mg of protein based on measurements of radioactivity and protein concentrations of the alkaline cell homogenates (34). In some experiments cells were pretreated for 15 min at 37 °C with MTSEA-biotin (see below) before the transport assays with [3H]MTX treatments. To measure MTX transport kinetics, cells were treated with concentrations of [3H]MTX between 0.33 and 5 μM with results analyzed using Lineweaver-Burke plots for determinations of K<sub>i</sub> and V<sub>max</sub> values. For determining K<sub>i</sub> values for nonradioactive substrates...
including folic acid and PMX, cells were treated with 0.5 \( \mu M \) [\(^{3}H\)]MTX and inhibitors at concentrations from 0.3 to 1.5 \( \mu M \). Results were analyzed by Dixon plots to calculate \( K_i \) values. To compare the optimal pH for transport by WT- and CL-PCFT, transport assays were performed at pH values from 5.5 and 7.2. MES-buffered saline was used for pHs 5.5, 6.0 and 6.5, whereas HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), and 5 mM glucose) was used for pH values 6.8 and 7.2.

Surface Biotinylation with MTSEA-biotin—Forty-eight hours post-transfection (above), cell surface proteins were surface-biotinylated with MTSEA-biotin (Biotium, Hayward, CA). MTSEA-biotin was freshly dissolved in DMSO at 2 mg/100 \( \mu l \) and then diluted 1:100 in PBS. Cells were washed with PBS twice and treated with MTSEA-biotin solution for 30 min at room temperature. The MTSEA-biotin was aspirated, and cell pellets were resuspended in 0.4 ml lysis buffer (50 mM Tris base, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, pH 7.4) by vortexing then mixed on a rotisserie shaker for 1 h at 4 °C. The samples were centrifuged at 16,000 rpm for 15 min at 4 °C, leaving the detergent-solubilized membrane proteins in the supernatant. An aliquot (25 \( \mu l \)) of this fraction was used as a loading control, and the remaining 375 \( \mu l \) were added to 50 \( \mu l \) of streptavidin-agarose resin (Thermo Scientific, Waltham, MA) (prewashed with 3 \( \times \) lysis buffer). This mixture was mixed overnight at 4 °C in a rotisserie shaker. The following day the mixture was centrifuged at 16,000 \( \times \) g for 1 min, and the supernatant was aspirated. The resin was washed 3 times with lysis buffer followed by a wash with lysis buffer supplemented with 2% SDS. Bound proteins were released from the streptavidin resin by heating to 95 °C for 5 min in 2\( \times \) Laemmli loading buffer (35) that contained dithiothreitol. For some experiments protection from MTSEA-biotin biotinylation by the PCFT substrate, PMX, was tested. For these experiments, plates were treated with 2 ml of PBS containing 250 \( \mu M \) PMX for 15 min immediately before MTSEA-biotin treatment. Results were compared with those for untreated controls, incubated in parallel.

Western Blotting—Procedures for plasma membrane preparation, SDS-polyacrylamide gel electrophoresis, and electrotransfer to polyvinylidene difluoride membranes (Pierce) were identical to previously reported methods (36). Blots were probed with anti-HA antibody (Covance, Princeton, NJ) and IRDye800-conjugated secondary antibody. Anti-β-actin antibody (Sigma) was used to probe for loading controls. Imaging and densitometry were performed with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Statistical Analysis—Unpaired \( t \) tests were conducted using GraphPad 6.0 software.

RESULTS AND DISCUSSION

Alanine-scanning Mutagenesis of TMD2 and the TMD2-3 Loop Region of hPCFT—A previous study used progressive alanine substitution mutagenesis with stretches of alanines to disrupt the predicted TMD2-3 3\( \beta \)-turn in hPCFT and found that transport activity was abolished due to impaired intracellular trafficking to the plasma membrane (30). As an extension of this work, we used a targeted alanine-scanning mutagenesis strategy with individual alanine substitutions across this stretch (including flanking regions) with WT-PCFT as a template and extended this to include the entire TMD2 for a total of 33 mutants spanning positions 85–118 (Fig. 1). Mutant hPCFT constructs were transfected into hPCFT-null R1-11 HeLa cells.

FIGURE 1. Membrane topology of hPCFT. TMD2 is highlighted in blue, and the loop region connecting TMDs 2 and 3 is highlighted in green. The seven cysteine residues of hPCFT are highlighted in red. IL, intracellular loop; EL, extracellular loop.
Membrane transport of [3H]MTX was measured at pH 5.5, the pH optimum for PCFT, and HA-tagged hPCFT proteins in crude plasma membranes were assayed by Western blotting with HA-specific antibody. We found that all 33 hPCFT alanine mutants were expressed in crude membranes (Fig. 2B) and that 30 of these were active for transport (25–115% of WT), including all of the mutants in TMD2 (Fig. 2A). Conversely, for residues located in the TMD2-3 loop domain, Ala replacements at positions 109, 112, and 113 abolished uptake ([3H]MTX uptake values were not significantly different from those with R1-11 cells (p > 0.05, noted by asterisks)). WT-PCFT is indicated by the gray bar. TMD2 mutants are shown in black, and the TMD2-3 loop mutants are shown in white. Panels B and C, Western blots are shown for membrane proteins (10 μg) from HA-tagged TMD2 (B) and TMD2-3 loop (C) alanine mutants isolated 48 h after transient transfections of R1-11 cells. Results are shown for membrane proteins probed with HA-specific antibody with equal loading confirmed with β-actin. Results are representative of at least two blots from independent experiments.

These results with Asp-109 and Arg-113 are consistent with previous reports that any amino acid replacement (including even conservative substitutions) at these positions was inactive (9, 11, 17, 30). For Asp-109, mutation disrupts the DSVG tetrad (109–112) and β-turn in the TMD2-3 loop domain. Similarly, mutation of Gly-112 to Pro significantly reduced hPCFT expression on Western blots (Fig. 3B) and caused a complete loss of transport activity (Fig. 3A), further implying that disruption of the β-turn structure results in protein misfolding and protein degradation. By contrast, with Arg-114, we found that mutation to increasingly non-conservative residues, including Lys, His, and Asp, preserved expression on Western blots (Fig. 3B) while progressively decreasing transport without abolishing it altogether (Fig. 3A).

Cys-scanning Mutagenesis and Cys Accessibilities to Biotinylation with MTSEA-biotin for TMD2 and the TMD2-3 Loop Region of hPCFT—Our goal was to further determine the structural and functional significance of TMD2 and the TMD2-3 loop domain in hPCFT using Cys-scanning mutagenesis and SCAM. hPCFT includes 7 Cys residues (Fig. 1), two of which form a disulfide linkage (25). Because Cys residues in WT-PCFT reacted with thiol-reactive MTSEA-biotin in pilot studies (not shown), we generated a “Cys-less”
hPCFT construct (with a C-terminal HA epitope) by mutating the seven Cys residues to serine. When transiently expressed in hPCFT-null R1-11 HeLa cells, CL-PCFT retained 77.5% (±2.8%) of the transport activity of WT-PCFT. The addition of 10 μM PMX completely blocked MTX transport levels for both WT- and CL-PCFT (Fig. 4B). CL-PCFT displayed a similar decrease in the level of membrane hPCFT protein on Western blots (70 ± 10%) of the WT-PCFT level (Fig. 4A). CL-PCFT and WT-PCFT showed nearly identical transport characteristics including \(K_t\) and \(V_{\text{max}}\) values for \([3H]\text{MTX}\) and \(K_i\) values for folic acid and PMX (Table 1). Furthermore, for both CL-PCFT and WT-PCFT, pH-dependent transport of \([3H]\text{MTX}\) was essentially identical with decreasing activity from the high level measured at pH 5.5 and very low levels above pH 7 (Fig. 4C). Collectively, these results establish that WT- and CL-PCFT are functionally equivalent.

To study the roles of the TMD2 and TMD2-3 loop regions in hPCFT structure and transport function, we used site-directed mutagenesis with CL-PCFT as a template to introduce single Cys residues from positions 85 to 118 (Fig. 1). The 33 individual Cys constructs were transiently transfected into R1-11 cells followed by assays of \([3H]\text{MTX}\) uptake at pH 5.5 and of hPCFT protein levels on Western blots (Fig. 5, A–C). Cys substitutions at a number of positions were less tolerated than were the Ala substitutions (compare Figs. 2A and 5A). Twenty-eight of the 33 Cys mutants were considered “active” based on statistically significant increases in \([3H]\text{MTX}\) uptake (>2-fold; \(p < 0.05\)) over the very low residual level in R1-11 cells. These include Cys mutants of several residues (Asn-90, Leu-104, Gly-105, Pro-115) with modest levels of transport. Analogous to their Ala mutant counterparts, Cys replacements at Asp-109, Glu-110, and Pro-115 were tolerated.
Gly112, and Arg-113 were completely inert (Fig. 5A). Whereas Ala replacements of Trp-107 and Arg-114 preserved substantial transport activity (Fig. 2A), transport was abolished for Cys replacements at these positions (Fig. 5A). For many of the Cys mutants, transport activity paralleled levels of hPCFT protein on Western blots (Fig. 5, B and C), suggesting that the substantial losses of transport for the inactive hPCFT Cys mutants were likely the result of mutant protein misfolding, resulting in impaired intracellular trafficking and protein degradation. However, for a few of the Cys mutants in the TMD2-3 loop domain (i.e. Cys-105 and Cys-108), transport was disproportionately low relative to the levels of PCFT protein (Fig. 5, A and C), suggesting functionally inactive protein.

For the 28 active Cys mutants spanning TMD2 and the TMD2-3 loop region, we used MTSEA-biotin to establish their aqueous accessibilities in forming an aqueous transmembrane pathway for anionic (anti)folate substrates. MTSEA-biotin is a membrane-impermeable, bifunctional reagent that includes thiol-reactive MTS and biotin moieties, thus permitting isola-

### TABLE 1

**Kinetic analysis of WT- and CL-PCFT**

WT-PCFT and CL-PCFT were transiently transfected into R1-11 cells, and PCFT transport was measured 48 h later by [3H]MTX uptake assay at pH 5.5 over 2 min. To determine $K_I$ and $V_{max}$ values, cells were treated with [3H]MTX with concentrations between 0.33 and 5 μM with results analyzed by Lineweaver-Burke plots. To determine $V_{max}$ values, cells were incubated with 0.5 μM [3H]MTX with folic acid or PMX as competitors from 0.3 to 1.5 μM, and results were analyzed by Dixon plots. Data are presented as the mean values ± S.E. from three independent experiments.

| Constant | Comounds | WT-PCFT | CL-PCFT |
|----------|----------|---------|---------|
| $K_I$ (μM) | MTX | 0.44 ± 0.06 | 0.51 ± 0.03 |
| $V_{max}$ (pmol/mg/min) | MTX | 276.8 ± 59.7 | 236.2 ± 27.5 |
| $K_I$ (μM) | Folic acid | 1.51 ± 0.18 | 1.49 ± 0.45 |
| $K_I$ (μM) | PMX | 0.13 ± 0.04 | 0.13 ± 0.05 |

**FIGURE 5. Characterization of hPCFT single-Cys mutants.** Panel A, TMD2 and TMD2-3 loop single-Cys hPCFT mutants were transiently transfected into R1-11 cells, and PCFT activity was measured after 48 h by [3H]MTX uptake assay at pH 5.5 over 2 min. Results are expressed as relative to those for CL-PCFT and are reported as mean values ± S.E. (error bars) from triclicate experiments. Transport levels for W107C, D109C, G112C, R113C, and R114C mutants (noted with asterisks) were not significantly increased over the low level in R1-11 cells ($p > 0.05$), whereas transport for the other 28 samples was significantly increased over the transport level in R1-11 cells ($p < 0.05$). CL-PCFT is indicated by the gray bar. The TMD2 mutants are shown in black, and the TMD2-3 loop mutants are shown in white. Panels B and C, Western blot analysis was performed on membrane proteins (10 μg) prepared from HA-tagged TMD2 (B) and the TMD2-3 loop (C) Cys mutants 48 h post-transfection. Results are shown for hPCFT proteins probed with HA-specific antibody, with equal loading confirmed with β-actin. Results are representative of at least two blots from independent experiments.
tion of biotinylated hPCFT protein by precipitation with streptavidin beads for analysis on Western blots. R1-11 cells were transiently transfected with the active Cys hPCFT mutants, then treated with MTSEA-biotin followed by pull-down with streptavidin beads. The G207C mutant located in the extracellular loop 3 domain (Fig. 1) was used as a positive control for the pulldown assays. Biotinylated proteins were eluted and analyzed by Western blotting using an anti-HA antibody (Pull down). An aliquot (25 μl) of the crude membrane fraction before MTSEA-biotin treatment was also analyzed by Western blotting as a control (Membrane). Panels C and D, transiently transfected R1-11 cells were treated with or without 250 μM PMX before treatment with MTSEA-biotin, streptavidin precipitation, and Western blotting as described for panels A and B. Panel E, TMD2 and TMD2-3 loop single-Cys mutants were transiently transfected into R1-11 cells. After 48 h, cells were incubated with or without MTSEA-biotin followed by assay of PCFT transport with [3H]MTX at pH 5.5 over 2 min. Results are expressed relative to the non-treated sample and are reported as the mean values ± S.E. for two (positions 104–111) or three (R1-11, CL, and positions 207, 158, 93, and 94) independent experiments. For positions 158, 93, and 94, losses of transport activity by MTSEA-biotin treatment compared with untreated samples were statistically significant (p < 0.05; noted by asterisk). In the transport experiments, CL-PCFT and G207C were negative controls, whereas MTSEA-biotin-reactive G158C was used as a positive control (all shown in gray) (26). (D. Goldman, personal communication.) Results for the TMD2 mutants are shown in black, and TMD2-3 loop mutants are shown in white.

FIGURE 6. Biotinylation of single-Cys mutants in TMD2 and the TMD2-3 loop with MTSEA-biotin. Panels A and B, TMD2 (A) and TMD2-3 loop (B) single-Cys mutants were transiently transfected into R1-11 cells, and 48 h later cells were treated with MTSEA-biotin. Cells were harvested, and membranes prepared, solubilized, and incubated with streptavidin beads to pull down biotinylated proteins. The G207C mutant located in the extracellular loop 3 domain (Fig. 1) was used as a positive control for the pulldown assays. Biotinylated proteins were eluted and analyzed by Western blotting using an anti-HA antibody (Pull down). An aliquot (25 μl) of the crude membrane fraction before MTSEA-biotin treatment was also analyzed by Western blotting as a control (Membrane). Panels C and D, transiently transfected R1-11 cells were treated with or without 250 μM PMX before treatment with MTSEA-biotin, streptavidin precipitation, and Western blotting as described for panels A and B. Panel E, TMD2 and TMD2-3 loop single-Cys mutants were transiently transfected into R1-11 cells. After 48 h, cells were incubated with or without MTSEA-biotin followed by assay of PCFT transport with [3H]MTX at pH 5.5 over 2 min. Results are expressed relative to the non-treated sample and are reported as the mean values ± S.E. for two (positions 104–111) or three (R1-11, CL, and positions 207, 158, 93, and 94) independent experiments. For positions 158, 93, and 94, losses of transport activity by MTSEA-biotin treatment compared with untreated samples were statistically significant (p < 0.05; noted by asterisk). In the transport experiments, CL-PCFT and G207C were negative controls, whereas MTSEA-biotin-reactive G158C was used as a positive control (all shown in gray) (26). (D. Goldman, personal communication.) Results for the TMD2 mutants are shown in black, and TMD2-3 loop mutants are shown in white.

hydrophilic MTSEA-biotin reagent (Fig. 6A, upper panel). However, other positions in TMD2 also reacted with MTSEA-biotin, especially positions 93 and 94, predicted to lie in the mid-TMD2 region, although flanking positions were poorly or completely unreactive. For the G93C mutant, reaction with MTSEA-biotin is particularly notable given its modest levels in the total membrane fraction (Fig. 6A, lower panel). For the TMD2-3 loop (positions 107–114) and flanking regions (104–106, 115–118), predicted to have a cytosolic orientation, we found that the G105C, A106C, S108C, S110C, and V111C hPCFT mutants were uniquely reactive with MTSEA-biotin in comparison with other mutants (e.g. L104C and P115C) (Fig. 6B). Thus, positions 93 and 94 in TMD2 and positions 105, 106, 108, 110, and 111 in the
TMD2-3 loop region seem to be aqueous accessible despite their predicted membrane topologies.

Impact of hPCFT Substrate on Biotinylation and Transport Inhibition by Biotinylation—To examine whether the MTSEA-biotin-reactive positions in the TMD2 and the TMD2-3 loop regions (positions 93, 94, 105, 106, 108, 110, and 111) contribute to an aqueous binding domain for hydrophilic (anti)folate substrates, we preincubated transiently transfected R1-11 cells expressing the G93C, F94C, L104C, G105C, A106C, S108C, S110C, and V111C mutants with the PCFT substrate PMX (250 μM) before treatment with MTSEA-biotin. Controls were incubated in parallel without PMX. Biotinylated proteins were precipitated with streptavidin beads and analyzed on Western blots. PMX substantially decreased MTSEA-biotin reactivity with Cys residues at positions 93 and 94 (Fig. 6C). However, the biotinylated TMD2-3 loop residues were unaffected by PMX treatment (Fig. 6D). To determine whether biotinylation of these residues also inhibited transport activity, we incubated transiently transfected R1-11 cells expressing G93C, F94C, L104C, G105C, A106C, S108C, S110C, and V111C hPCFT mutants with [3H]MTX (0.5 μM) for 15 min at 37 °C with or without a 15 min MTSEA-biotin pretreatment. In this series of experiments, CL-PCFT and G207C were negative controls, whereas MTSEA-biotin-reactive G158C was used as a positive control (26).

We found that of these residues, only for the G93C and F94C mutants was transport significantly inhibited by biotinylation (Fig. 6E, 63.5 and 18.2%, respectively; p < 0.05). Collectively, these results are consistent with the notion of a direct interaction between positions 93 and 94 and substrate, although an indirect effect mediated by conformational changes resulting from more distal substrate binding cannot be excluded.

Conclusion—In the present study we studied the structural and mechanistic roles of TMD2 and the TMD2-3 loop region in hPCFT. Our results establish aqueous accessibilities of residues located in TMD2 and the TMD2-3 loop despite their predicted membrane topologies. For the TMD2-3 loop, these results are best interpreted as a reentrant loop structure, similar to those reported for other MFS transporters (e.g. EAAT1) (31, 37). These results support those of Subramanian et al. (30), which establish that disruption of the TMD2-3 loop including the DSVG tetrad β-loop results in hPCFT protein misfolding and loss of surface expression and transport activity. In previous studies it was reported that Asp-109 and Arg-113 in this stretch cannot be replaced with even the most conservative substitutions (Glu and Lys, respectively) to restore transport (9, 38). Our results show that this now extends to the G112P mutant as well, although substitutions (Lys, His, Asp) at Arg-114 were tolerated. Finally, our results suggest that positions 93 and 94 in TMD2 may directly participate in forming the hPCFT substrate binding pocket/membrane translocation pathway. Determining the structural determinants of hPCFT membrane transport is requisite to understanding the molecular mechanisms of transport, including critical determinants of (anti)folate binding.

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