Rodent intestinal folate transporters (SLC46A1): secondary structure, functional properties, and response to dietary folate restriction

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FOLATES are essential one-carbon donors critical for nucleic acid synthesis and methylation reactions (28). The requirement for this B vitamin is met entirely from the absorption of dietary folate. The further characterization of the role of PCFT in mouse development and folate delivery to intestinal and other tissues and for the investigation of PCFT as a factor in the pathogenesis of cancer in mouse models.

MATERIALS AND METHODS

Reagents. [3H]folic acid, [3H]methotrexate (MTX), and [3H](6S)-5-methyltetrahydrofolate (5-methylTHF) were obtained from Moravek Biochemicals (Brea, CA) and purified as previously described (5). MTX di-, tri-, tetra-, penta-, and hexaglutamates, (6S)-5-methylTHF, and (6R)- and (6S)-5-formyltetrahydrofolate (5-formylTHF) were ob-
tained from Schircks Laboratories (Jona, Switzerland). PT523, a MTX analog, was obtained from Roche (Basel, Switzerland). Folic acid, MTX, and other chemicals were obtained from commercial sources.

**Plasmid construction.** Full-length \textit{MmPCFT} and \textit{RnPCFT} cDNA clones were obtained from Open Biosystems (Huntsville, AL). The open reading frames (ORFs) were PCR amplified with PfUItra DNA polymerase (primers are shown in Table 1) and subsequently cloned into the BglII site of the pS64T vector for in vitro transcription of the capped sense \textit{MmPCFT} and \textit{RnPCFT} cDNA from a SP6 promoter using the mMESSAGE mMACHINE system (Ambion, Austin, TX) and into the BamHI site of pcDNA3.1(+) (+) to generate pcDNA3.1(+)\textit{MmPCFT} and pcDNA3.1(+)\textit{RnPCFT}, respectively. \textit{MmPCFT} and \textit{RnPCFT} ORFs were tagged at either NH2- or COOH-termini with a hemaglutinin (HA) epitope by PCR (primers shown in Table 1) and cloned into pcDNA3.1(+) with the same strategy as above. cDNA inserts were verified by DNA sequencing at the Albert Einstein Center Genomics Shared Resource.

**Cell culture and transfection.** HeLa and HepG2 cells obtained from the American Type Culture Collection (Manassas, VA) were maintained as previously described (30). Plasmid DNA was transfected into these cells using Lipofectamine 2000 (Invitrogen). HepG2 cells stably transfected with either pcDNA3.1(+), pcDNA3.1(+}\textit{MmPCFT}, or pcDNA3.1(+}\textit{RnPCFT} were generated by G418 selection (600 \mu g/ml).

**Experiments in mice.** The impact of dietary folate restriction on \textit{PCFT} mRNA levels was assessed in 3- to 4-mo-old male C57BL/6 specific-pathogen-free mice. Animals were maintained on either folate-containing mouse chow (2 mg/kg folic acid) or a folate acid-free diet over 8 wk, after which they were killed in a CO2 chamber. The proximal small intestine was dissected and rinsed in ice-cold PBS (pH 7.4), and total RNA was isolated with TRIzol reagent (Invitrogen). Data on RFC and folate receptor (FR) were tagged at either NH2- or COOH-termini with a hemaglutinin (HA) epitope by PCR (primers shown in Table 1) and cloned into pcDNA3.1(+) with the same strategy as above. cDNA inserts were verified by DNA sequencing at the Albert Einstein Center Genomics Shared Resource.

**Production of peptide antibody and immunohistochemistry.** For immunohistochemical experiments, C57BL/6 adult mice maintained on the usual mouse chow were killed, and segments of intestines were dissected, rinsed in ice-cold PBS (pH 7.4), and embedded in freezing embedding OCT medium (Andwin Scientific, Addison, IL) for cryosectioning at 5 \mu m thickness. Similar intestine segments were dissected for the isolation of total RNA. Production of peptide antibody and immunohistochemistry was carried out using standard methods.

**Table 1. Primer sequences of \textit{MmPCFT} and \textit{RnPCFT} ORFs as well as \textit{MmPCFT} and \textit{GAPDH} primers used for quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
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</thead>
<tbody>
<tr>
<td>\textit{MmPCFT ORF} Forward</td>
<td>5'-TATAGATCTTCACCATGAGGGGCGCGTGAG3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TATAGATCTTCAGGGGCTTGAAGAAC3'</td>
</tr>
<tr>
<td>Forward (N-HA tag)</td>
<td>5'-TATAGATCTCACCAATACCGTCATTCAAGAGGCGCGTGAG3'</td>
</tr>
<tr>
<td>Reverse (C-HA tag)</td>
<td>5'-TCAAGATCTTAAGCTTTATCTGAAGACAGTGAGGCGCGTGAG3'</td>
</tr>
<tr>
<td>\textit{RnPCFT ORF} Forward</td>
<td>5'-TCAAGATCTCACCGGATGGGCGCCGCGTGAG3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCAAGATCTCACCGGATGGGCGCCGCGTGAG3'</td>
</tr>
<tr>
<td>Forward (N-HA tag)</td>
<td>5'-TCAAGATCTCACCGGATGGGCGCCGCGTGAG3'</td>
</tr>
<tr>
<td>Reverse (C-HA tag)</td>
<td>5'-TCAAGATCTAACGGAAACTG3'</td>
</tr>
</tbody>
</table>

**Quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{MmPCFT} Forward</td>
<td>5'-GAGTGGTTGCTTTCTGCTGAG3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCCGTACCCGCTGAAGATGA3'</td>
</tr>
<tr>
<td>\textit{GAPDH} Forward</td>
<td>5'-GGCAATTGCTTCTCAATGACAA3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCCATGTGCTTGACACGATAT3'</td>
</tr>
</tbody>
</table>

\textit{MmPCFT}, mouse (\textit{Mus musculus}) protein-coupled folate transporter (PCFT); \textit{RnPCFT}, rat (\textit{Rattus norvegicus}) PCFT; ORF, open reading frame; N-HA and C-HA tags, NH2- and COOH-terminal hemaglutinin tags, respectively. Underlined regions indicate BglII restriction sites.
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MgCl₂, and 5 mM dextrose) at pH 5.5 or 6.5 containing either preimmune or anti-MmPCFT serum (1:50 dilution) at 37°C for 2 min.

To examine the effects of sodium on the transport activities of MmPCFT or RnPCFT, [³H]folinic acid (0.5 μM) uptake was assessed in MBS (pH5.5) over 2 min in which 140 mM NaCl was replaced by the same concentration of choline chloride. Uptake was normalized to protein content.

Electrophysiological analyses in Xenopus oocytes. Preparation and microinjection with 50 nl of water or PCFT cRNA (30–50 ng) into defolliculated Xenopus laevis oocytes followed previously described procedures; oocytes were kept at 17°C in horse serum medium [82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2.3 mM CaCl₂, 5 mM HEPES, and 5% horse serum (pH 7.5)], and electrophysiological recordings in a solution of 90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Tris, and 5 mM MES were conducted 3–7 days after eRNA injection (10, 22). For the determination of Kₐ values, currents were measured at different folate concentrations with the membrane potential clamped at –80 mV. Influx kinetic constants were obtained from the normalized currents from different experiments fitted to the following equation: $I = (I_{\text{max}} \times S)/(K_{\text{m}} + S)$, where I is the current induced by a given substrate concentration (S) and $I_{\text{max}}$ is the maximal current generated.

Northern blot analysis. A Northern blot containing polyA⁺ RNA (2 μg/lane) from 12 mouse tissues was obtained from Orgene (Rockville, MD). [³²P]dCTP-labeled cDNA probes were made from a MmPCFT cDNA segment (1094–1440 bp, GenBank Accession No. NM_026740) and hybridized to the membrane as previously described (22). After the membrane had been stripped, β-actin mRNA was probed as the loading control.

Total RNA (10 μg) isolated from different intestinal segments was resolved on a 1% denaturing agarose gel and transferred to a Nytran membrane (Whatman, Florham Park, NJ). Northern blot hybridization was subsequently performed using the [³²P]dCTP-labeled MmPCFT cDNA probe described above. 18S and 28S rRNAs were visualized after ethidium bromide staining as the loading control.

Quantitative RT-PCR. Total RNA isolated from mice fed folate-replete or folate-deficient diets, as previously reported (15) and described above, was reverse transcribed to cDNA with Superscript Reverse Transcriptase II (Invitrogen). Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers specific for MmPCFT (Table 1). GAPDH was simultaneously amplified with specific primers (Table 1) to normalize MmPCFT expression.

RESULTS

Structural analysis of MmPCFT and RnPCFT. MmPCFT and RnPCFT (GenBank Accession Nos. AAH57976 and AAH89868, respectively) were identified in the National Center for Biotechnology Information (NCBI) GenBank database based on high amino acid identity to human PCFT protein. The human, mouse, and rat orthologs encode proteins of 459 amino acids with a predicted molecular weight of ~50 kDa (Fig. 1A). PCFT proteins are highly conserved in mammals. Based on sequence alignment analysis with ClustalW (13), the predicted MmPCFT and RnPCFT proteins share 95% amino acid identity to each other, 87% identity to HsPCFT, and >80% identity to their counterparts in other mammalian species, e.g., the dog (Canis familiaris; XP_548286), rhesus monkey (Macaca mulatta; XP_001106954), and domestic cow (Bos taurus; XM_588367) (Fig. 1A and B). PCFT orthologs also appear to be present in nonmammalian vertebrates, e.g., the chicken (Gallus gallus (GgPCFT); XP415815), African clawed frog (X. laevis (XipcFT); AAH77859), and zebrafish (Danio rerio (DrPCFT); AAH49421), all of which share >50% amino acid identity to mammalian PCFTs (Fig. 1B).

Invertebrate homologs could be identified in the NCBI GenBank database with >30% amino acid identity to vertebrate PCFTs.

MmPCFT and RnPCFT genes are located at Chr11B5 and Chr10q25, respectively, and have the same gene structure as HsPCFT (Chr17q11.2): five exons and four introns with exons highly conserved between these three species (Fig. 1C).

Similar gene structure and exon conservation are also present in other PCFT genes, for instance, the dog (GenelD: 491166), rhesus monkey (GenelD: 708727), domestic cow (GenelD: 511097), and chicken (GenelD: 417569). The zebrafish (GenelD: 393255) gene contains six exons and five introns; exons 2 and 3 of the DrPCFT gene correspond to exon 2 of the mammalian and chicken PCFT genes.

Subcellular localization and topological properties of MmPCFT and RnPCFT proteins. Topology prediction programs [DAS, HMMTOP, PredictProtein, SOSUI, TMHMM, TMpred, TopPred, and Hydrophy Analysis (http://www.expasy.org/tools/#topology and http://www.tcdb.org/analyze.php)] were employed to characterize the PCFT secondary structure. The numbers of transmembrane domains (TMDs) varied with the different algorithms. Most of the programs predicted a polytopic integral membrane protein with 11 or 12 transmembrane segments, although TMpred predicted 10 transmembrane segments. A model with 12 TMDs, as predicted by DAS, HMMTOP, ProteinPrediction, and Hydrophy Analysis programs, is shown in Fig. 1A and indicates the high degree of homology within TMDs among human and rodent transporters.

An immunohistochemistry approach was used to assess the localization of the NH₂- and COOH-termini of rodent PCFT proteins. MmPCFT and RnPCFT were tagged with an HA epitope at either terminus, and the fusion proteins were subsequently immunodetected with an anti-HA antibody in HeLa cells transiently transfected with each construct with or without permeabilization before being immunostained. As shown in Fig. 2A, MmPCFT and RnPCFT HA fusion proteins could only be localized to the plasma membrane of permeabilized cells (top); these epitopes could not be stained in cells that were not permeabilized (bottom). Similar immunolocalization was also observed for wild-type MmPCFT expressed in HeLa cells (Fig. 2A, wild-type MmPCFT), which could only be stained in permeabilized cells with an anti-MmPCFT peptide antibody that specifically recognizes an epitope in the COOH-terminus. These observations are consistent with an intracellular localization of the NH₂- and COOH-termini of MmPCFT and RnPCFT and a predicted topology in which there are an even number of transmembrane segments, likely 12 transmembrane segments, as based on the topological analyses.

When cDNAs encoding wild-type or HA-tagged transporters were transiently transfected into HeLa cells, all the constructs noted above produced [³H]MTX influx activity comparable with that of cells transfected with native MmPCFT and RnPCFT, which was >14 times higher than cells transfected with vector control alone (Fig. 2B, mock) and consistent with their plasma membrane localization and the reported folate transport activity for the human gene (22). This also indicates that the HA epitope at either the NH₂- or COOH-terminus does not alter the transport activity of MmPCFT or RnPCFT.

Anti-MmPCFT serum, which specifically recognizes the COOH-terminus of the murine transporter, did not alter folate acid uptake in HepG2 cells stably transfected with MmPCFT,

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further supporting the intracellular localization of the COOH-terminus of *Mm*PCFT protein (Fig. 2C).

Using anti-*Mm*PCFT serum, *Mm*PCFT protein was detected as broad bands in the Western blot analysis in both transiently transfected HeLa cells and stably transfected HepG2 cells with molecular weights higher than predicted (~50 kDa) (Fig. 2D). This is consistent with the N-linked glycosylation site(s) in the extracellular loop between *TMD 1* and *TMD 2*, which are...
highly conserved among rodent and human PCFTs (Fig. 1A) and have been confirmed in other studies by this laboratory (unpublished observations). In both HepG2 and HeLa transfectants, there was a band at 35 kDa detected in both mock- and MmPCFT-transfected HeLa and HepG2 cells that disappeared in the presence of blocking peptide. The origin of this band was not clear.

**pH dependence of folate transport mediated by MmPCFT and RnPCFT.** The initial uptake of 0.5 μM [3H]folic acid (Fig. 3A), [3H]MTX (Fig. 3B), and [3H](6S)5-methylTHF (Fig. 3C) by MmPCFT and RnPCFT in stably transfected HepG2 cells was highly pH dependent, decreasing as pH was increased from pH 5.5 to 7.4. Whereas there was negligible folic acid and MTX transport at pH 7.0, there was residual activity for (6S)5-methylTHF at this pH. There was ~50% of maximum activity for all these folate substrates at pH 6.0, the pH at the upper small intestinal absorptive surface (17). [3H]Folic acid transport mediated by MmPCFT and RnPCFT in stably transfected HepG2 cells was not sodium dependent; influx of both species was the same in sodium-containing buffer and buffer in which sodium was replaced by choline (Fig. 3D).

**Electrophysiological characterization of MmPCFT and RnPCFT in Xenopus oocytes.** Xenopus oocytes injected with either MmPCFT or RnPCFT cRNA displayed currents induced by folic acid, (6S)5-methylTHF, or MTX in two-electrode voltage-clamp experiments (shown for folic acid in Fig. 3E); no currents were induced by folates in water-injected oocytes. In general, the folate-induced current amplitude was greater with MmPCFT than with RnPCFT. Folic acid-induced current amplitudes were proportional to the applied transmembrane voltage in oocytes expressing either MmPCFT (Fig. 3F) or RnPCFT (data not shown).

**Folate influx kinetics.** Initial rates of [3H]folic acid, [3H]MTX, and [3H](6S)5-methylTHF uptake in HepG2 cells stably trans-
fected with cDNAs encoding *Mm*PCFT and *Rn*PCFT followed Michaelis-Menten kinetics (as illustrated for folic acid in Fig. 4A). Folic acid-induced currents in *Xenopus* oocytes injected with *Mm*PCFT cRNA was saturable with increasing substrate concentrations at both pH 5.5 and 6.5 (Fig. 4B). Table 2 shows data comparing relative influx $K_m$ values for the various folates for mouse and rat PCFTs at pH 5.5 versus 6.5. Both *Mm*PCFT and *Rn*PCFT had comparable high affinities for folic acid at pH 5.5. When pH was increased from 5.5 to 6.5, the $K_m$ value for folic acid, (6S)-5-methylTHF, and MTX was
creased and $V_{\text{max}}$ was decreased. For instance, $K_m$ values for MTX increased from 1.0 $\mu$M to 4.6 $\mu$M and 2.6 $\mu$M for MmPCFT and RnPCFT, respectively, and $V_{\text{max}}$ decreased from 289.6 pmol 2 min$^{-1}$ to 137.9 pmol 2 min$^{-1}$ and 130.2 pmol 2 min$^{-1}$, respectively. There was a lesser change in influx $K_m$ values for folic acid over this pH range, with no change for (6S)-5-methylTHF as assessed by electrophysiological measurements in *Xenopus* oocytes (Table 2). $K_m$ values were somewhat overestimated using tritiated folates due to substrate depletion at low concentrations, which could account for differences in this parameter based on current measurements (see MATERIALS AND METHODS). In addition, these differences might be due to voltage-related changes in $K_m$ that occur during electrophysiological measurements.

**MmPCFT and RnPCFT substrate specificity.** Influx of 0.5 $\mu$M [3H]folic acid was assessed at pH 5.5 in the absence (control) or presence of a 10-fold (5 $\mu$M) higher concentration of nonlabeled folates or antifolates in MmPCFT−, RnPCFT−, and mock-transfected HepG2 cells. As shown in Fig. 4C, there was substantial inhibition of [3H]folic acid uptake by folates and antifolates. The most potent inhibitors were pemetrexed and (6S)-5-formylTHF followed by folic acid and (6S)-5-methylTHF and then MTX and ZD1694 ($P<0.001$). The unnatural (6R) isomer of 5-formylTHF was a weaker inhibitor than the natural (6S) isomer ($P<0.001$, as assessed by one-way ANOVA followed by Tukey’s test). PT523, a 4-amino folic acid (aminopterin) analog with a hemiphthaloyl-L-ornithine side chain, did not inhibit at all.

As shown in Fig. 4D, whereas uptake of 0.5 $\mu$M [3H]MTX at pH 5.5 mediated by MmPCFT and RnPCFT was nearly

![Fig. 5](http://ajpcell.physiology.org/)

**Table 2. Relative influx $K_m$ values for the various folates for MmPCFT and RnPCFT at pH 5.5 versus 6.5**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MmPCFT</th>
<th>RnPCFT</th>
<th>MmPCFT</th>
<th>RnPCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.5</td>
<td>pH 6.5</td>
<td>pH 5.5</td>
<td>pH 6.5</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.1±0.1</td>
<td>3.9±0.4</td>
<td>0.8±0.1</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>(6S)-5-methylTHF</td>
<td>0.8±0.2</td>
<td>1.6±0.8</td>
<td>0.7±0.1</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>MTX</td>
<td>1.0±0.1</td>
<td>4.6±1.8</td>
<td>0.6±0.1</td>
<td>2.6±0.8</td>
</tr>
</tbody>
</table>

Data are means ± SE of $K_m$ values (in $\mu$M). The following substrates were used: folic acid, (6S)-5-methyltetrahydrofolate ((6S)-5-methylTHF), and methotrexate (MTX).
abolished by 50 μM nonlabeled MTX monoglutamate, the same concentration of MTX di-, tri-, tetra-, penta-, or hexaglutamate had no inhibitory effect at all, indicating that MmPCFT and RnPCFT are highly specific for monoglutamyl forms of folates.

**Expression of PCFT mRNA and protein in mouse tissues.** MmPCFT mRNA, with a molecular size of ~2.2 kb, was abundantly expressed in the small intestine, liver, and kidney and to a lesser extent in the brain, testis, skin, and stomach (Fig. 5A). There were low levels in the heart and lung, very low levels in the spleen and thymus, and no detectable MmPCFT mRNA in skeletal muscle. A shorter isoform of MmPCFT mRNA, with a molecular size of ~1.3 kb, was also detected in the brain, kidney, liver, skin, small intestine, and stomach. Focusing on the intestinal tract, MmPCFT mRNA was highly expressed in the duodenum and proximal jejunum, with lower expression in the distal jejunum and even lower levels in the cecum and colon (Fig. 5B). There was just a trace of expression in the ileum and rectum.

MmPCFT protein localization was analyzed by immunohistochemistry using affinity-purified polyclonal anti-MmPCFT antibody. As shown in Fig. 5C, there was substantial expression of MmPCFT protein in the duodenum and proximal jejunal localized exclusively to the apical brush-border membrane. The peptide antigen used to produce the anti-MmPCFT antibody completely blocked staining of MmPCFT, consistent with the high specificity of this antibody for MmPCFT protein.

**Altersations in MmPCFT mRNA levels in the proximal small intestine in response to dietary folate.** A previous study (15) demonstrated that folate deprivation in mice resulted in increased levels of both FR-α and RFC mRNA. Using the RNA from these animals, we assessed the impact of folate deprivation on PCFT mRNA in the proximal small intestine in mice on a normal chow diet versus mice on a folate-deficient diet. As shown in Fig. 6, inset, MmPCFT mRNA levels were increased in each of 10 folate-deficient mice compared with folate-replete mice (n = 7) without an overlap in levels between the two groups. Overall, the MmPCFT mRNA level was increased by a factor of ~13 in folate-deficient mice compared with folate-replete mice (Fig. 6).

**DISCUSSION**

The present study demonstrates that murine and rat PCFTs, like the human ortholog, are high-affinity carriers with a high degree of specificity for folates. Both MmPCFT and RnPCFT are highly specific for monoglutamate MTX but not polyglutamate derivatives of MTX. This explains, at the molecular level, why the transport of dietary folates across the luminal brush-border membranes of proximal small intestinal epithelial cells requires the hydrolysis of folate polyglutamates by γ-glutamyl carboxypeptidase II to monoglutamate forms before transport into enterocytes can occur (7).

Transport of folic acid, (6S)5-methylTHF, and MTX by mouse and rat PCFTs expressed in *Xenopus* oocytes was electrogenic, consistent with a net charge translocation during folate transport. The magnitude of the current was dependent on the extracellular folate concentration and on the extracellular pH. Alterations in protonation of the folate molecule do not occur over the pH range of 5.5 to 7.4 (20); however, at lower pHs, these measurements would be complicated by changes in substrate protonation, which, in turn, changes the concentration of the substrate recognized by the transporter. The change in current amplitude as a function of pH, under conditions in which changes in protonation of the folate molecule do not occur, suggest that pH changes not only alter the magnitude of the driving force for folate transport but may also titrate amino acids within PCFT that result in an alteration in the rate of transport. Studies are underway to identify the amino acids involved in these pH-dependent effects.

MmPCFT has been previously reported to be a heme carrier protein (HCP1) that transports heme with low affinity (~125 μM) and independent of pH (14, 25). Within the context of that report, HCP1 was predicted to consist of nine transmembrane domains with the NH2- and COOH-termini located intracellularly and extracellularly, respectively. However, the present study, along with the previous report from this laboratory, indicates that transport mediated by this carrier is highly specific and pH dependent for folates, with an affinity at least two orders of magnitude higher than that reported for heme. These data, together with topological prediction based on multiple hydropathy analyses, also support intracellular localization of both the NH2- and COOH-termini of MmPCFT and RnPCFT expressed in HeLa cells and an even number of transmembrane segments, most likely 12. However, in view of the limitations of the methodologies employed, confirmation of the localization of the NH2- and COOH-termini and other elements of this transporter will require further verification of the secondary structure.

The functional properties of MmPCFT and RnPCFT are consistent with the folate transport activities that have been...
reported for rat and mouse intestinal segments, brush-border membranes, and intestinal cell lines that have a low pH maximum (16, 29). Both transporters have a high level of folate transport activity at pH 6.0, the pH at the apical surface of the proximal small intestinal epithelium (17). \textit{Mn}PCFT mRNA and protein were highly expressed in the duodenum and upper jejunum with the protein exclusively localized to the brush-border membrane, consistent with its functional role as the rate-limiting first step in the absorption of dietary folates. The functional role of \textit{Mn}PCFT in intestinal folate absorption is further supported by the observation that \textit{Mn}PCFT mRNA levels in the murine proximal small intestine increased 13-fold in mice fed a folate-deficient diet. It is of interest that both RFC and FR-\(\alpha\) mRNA levels also increased in the same tissues from the same mice on a folate-deficient diet, indicating that expression of all these transporters is folate responsive (15). However, it is only PCFT that, in fact, mediates the translocation of folates at the acidic environment of the proximal small intestine at usual dietary folate levels. This conclusion is based on the observation that this gene is mutated in patients with hereditary folate malabsorption. Hence, this folate-responsive regulation of the PCFT gene is particularly important in folate deficiency states. It is possible, however, that some RFC-mediated transport can occur with the administration of pharmacological doses of folates, as in the treatment of hereditary folate malabsorption (6).

In addition to its high expression in the small intestine at the sites of folate absorption, PCFT is also highly expressed in other tissues. Very high expression of \textit{Mn}PCFT mRNA was observed in the liver and kidney, tissues that manifest a high level of folate transport activity at low pH (1, 9). The major fraction of folates in the glomerular filtrate, which is at pH 6.8 (11), are reabsorbed at the proximal tubules. At this pH, PCFT has substantial folate transport activity, suggesting that it may play a direct role in reabsorption of folates at this site. This is supported by the presence of substantial low pH folate transport activity in brush-border membrane vesicles from the rat kidney (1). The contribution of PCFT to folate transport in the liver, where it is also highly expressed, is not clear. Transport of folates into the liver from the arterial system occurs at neutral pH; however, the pH within the hepatic portal sinusoids, where folates are delivered via the portal system from the intestine to hepatocytes, is not known and may be acidic (9). Data for human and rodent PCFTs indicate that there is residual 5-methyl\(\text{THF}\) transport activity at pH 7.0 so that this carrier can operate, albeit less efficiently, at neutral pH.

Beyond transport at the level of the cell membrane, PCFT may have other functions. For instance, this carrier may be incorporated into membrane vesicles that accommodate folate receptors and transport folates by receptor-mediated endocytosis or transcytosis (33). FR-\(\alpha\) is highly expressed at the apical proximal renal tubule membrane and has been suggested to be a major route of folate reabsorption in the kidney (2). Folates bind to FRs that are anchored to the cell membrane via a glycosylphosphoinositol moiety. The resultant complex is internalized in a vesicle that traffics, intact, within the cytoplasm, where it is acidified, resulting in the release of the folate molecule from receptor (18, 19, 34). The mechanism by which folate is then exported from the vesicle has not been clarified.

If PCFT is present in these endocytic vesicles, it could provide the route of folate transport into the cytoplasm using the outward transvesicular proton gradient. Such a mechanism has been proposed earlier, although the identity of a vesicular transporter that could function at low pH is not known (12, 21). This mechanism could also explain, in part, why patients with hereditary folate malabsorption have a defect in folate transport into the central nervous system where the pH is neutral at the transport site but FR-\(\alpha\) is highly expressed at the choroid plexus (32). The detection of substantial levels of PCFT mRNA in the mouse brain is consistent with this hypothesis.

Analysis of the NCBI GenBank genome database shows that PCFT is highly conserved in other mammalian species at the level of both the gene and primary protein structures, e.g., in the dog, rhesus monkey, and domestic cow and, to a lesser extent, in nonmammalian vertebrates such as birds, amphibians, and fish. Together with its functional conservation in humans, mice, and rats, this suggests that PCFT is evolutionarily conserved for intestinal folate absorption in mammalian species and likely plays this role in nonmammalian vertebrates as well. PCFT does not appear to be conserved in invertebrates according to a protein-protein BLAST search of the NCBI GenBank database using vertebrate PCFTs as queries, suggesting that this gene might have originated and evolved in vertebrates.

GRANTS

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