Published in final edited form as:
Expert Rev Mol Med. ; 11: e4. doi:10.1017/S1462399409000969.

Membrane Transporters and Folate Homeostasis; Intestinal Absorption, Transport into Systemic Compartments and Tissues

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Abstract

Folates, the generic term for the family of B vitamins, are derived entirely from dietary sources, and are key one-carbon donors required for *de novo* nucleotide and methionine synthesis. These highly hydrophilic molecules utilize genetically distinct and functionally diverse transport systems to enter cells: the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT), and the folate receptors. Each plays a unique role in mediating folate transport across epithelia and into systemic tissues. With the recent discovery of the mechanism of intestinal folate absorption, and the clarification of the genetic basis for the autosomal recessive disorder, hereditary folate malabsorption, involving loss-of-function mutations in PCFT protein, it is now possible to piece together how these folate transporters contribute, both individually and collectively, to folate homeostasis in humans. This review focuses on the physiological roles of these major folate transporters with a brief consideration of their impact on the pharmacological activities of antifolates.

1. Introduction

Folates are a family of B9 vitamins that play a critical role in key biosynthetic processes in mammalian cells. These one-carbon donors are required for purine nucleotide and thymidylate synthesis and, hence, are essential for the *de novo* production of RNA and DNA. Folates are also required for vitamin B12-dependent synthesis of methionine, from which S-adenosylmethionine is formed, required for methylation of DNA, histones, lipids and neurotransmitters (1). Mammalian cells, unlike bacteria, do not have the metabolic machinery to synthesize folates. Hence, folate requirements must be met entirely from dietary sources. Traditionally, folates were derived from foods such as liver and dark green leafy vegetables. The recent commercial fortification of cereals, grains, and bread with folic acid now represents an important source of folates. This has resulted in a rise in tissue and blood folate levels (2). Because of the hydrophilic nature of the charged folate molecule, there is minimal passive diffusion across cell membranes. Rather, highly specific transporters are required to mediate intestinal folate absorption and the transport of folates into systemic tissues. Yet, despite the importance of these vitamins and their membrane transport, aspects of folate physiology, particularly relating to their transport, were uncharacterized and/or misunderstood until recently.

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There is considerable information, acquired over many decades, regarding the properties of two folate transporters. (i) The reduced folate carrier (RFC), a member of the superfamily of solute carriers (SLC19A1), is an anionic exchanger and a major route of delivery of folates to systemic tissues at physiological pH (3,4). (ii) Two folate receptors (FRs) (FRα and FRβ), embedded in the cell membrane by a glycosylphosphoinositol (GPI) anchor, transport folates via receptor-mediated endocytosis at neutral to mildly acidic pH (5,6). Recently, a third folate transporter was identified, the proton-coupled folate transporter (PCFT). PCFT also belongs to the superfamily of solute carriers (SLC46A1) and functions optimally at low pH (7,8). PCFT accounts for the low-pH folate transport activity in many normal and malignant tissues, most notably the small intestine. The critical role that PCFT plays in intestinal folate absorption was established by the demonstration of loss-of-function mutations in this protein in patients with hereditary folate malabsorption (HFM) (7,9,10).

Structural analogs of folate compounds have been used for treating cancer and autoimmune diseases (11,12). These include methotrexate (MTX) and, more recently, pemetrexed, (12,13). These agents utilize the same transporters as physiological folates to enter tumor cells. Transport is an important determinant of antifolate activities and impaired transport is an important mechanism of resistance (12).

This review considers how these three transport systems contribute both individually and collectively to folate homeostasis in man and to delivery of antifolates in the treatment of cancer.

2.0 The tetrahydrofolate cofactors: interconversions and utilization in folate-dependent biochemical reactions

The folate molecule consists of a pteridine moiety with A and B rings linked at carbon 6 by a methylene bridge to p–aminobenzoylglutamic acid (Figure 1). “Folate” is a generic term for members of the B9 family of vitamins and will be used as such in this review. The parent structure is folic acid, a pharmacologic agent not found in nature. Because of its stability, folic acid has been used as a folate source in cell culture medium and in vitamins; it is reduced at the B ring first to dihydrofolate (DHF) and then to tetrahydrofolate (THF) within cells (Figure 1). Folic acid is also used to discriminate among folate transport routes for which it has markedly different affinities. THF is a good substrate for the enzyme, folylpolyglutamate synthetase (FPGS), which progressively adds glutamate molecules at the gamma carboxyl residue through an amide bond to form glutamate-peptide chains of varying length (Figure 1). THF polyglutamates associate with methyl, formyl, methylene, or methenyl one-carbon moieties at the N5 and/or N10 positions that serve as one-carbon donors in biosynthetic reactions (1). The folate polyglutamate derivatives are, with few exceptions, the preferred substrates for one-carbon transfer reactions over their non-polyglutamate (hereafter, designated “monoglutamate”) precursors (14).

The predominant natural folate in the diet and blood of humans and rodents is 5-methylTHF (Figure 1). Utilization of this form in cells begins with the transfer of a methyl group to homocysteine, in a vitamin B12-dependent reaction mediated by methionine synthase, to form methionine (Figure 2). The unsubstituted THF released then reacts with formate (via 10-formylTHF synthetase) to produce 10-formylTHF which donates two carbons to the synthesis of the purine ring in reactions mediated first by β-glycinamideribonucleotide (GAR) transformylase and then by 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) transformylase. 10-FormylTHF also undergoes dehydration to 5,10-methenylTHF which in turn is reduced to 5,10-methyleneTHF. 5,10-MethyleneTHF is also formed from THF by serinehydroxymethyltransferase which interconverts serine to glycine. 5,10-MethyleneTHF is reduced in an irreversible reaction by 5,10-methyleneTHF reductase

(MTHFR) to 5-methylTHF. 5,10-MethyleneTHF is required for *de novo* synthesis of thymidylate, catalyzed by thymidylate synthase, in which a one-carbon moiety is transferred to deoxyuridylate along with a reducing equivalent from the pteridine B ring. This results in oxidation of the THF moiety to DHF. Because of its rapidity and irreversibility, this reaction has the potential to deplete cellular THF cofactors which rapidly interconvert to 5,10-methyleneTHF by mass action as this folate is oxidized. However, THF cofactor levels are maintained because of the rapid reduction of DHF to THF, mediated by dihydrofolate reductase (DHFR) (Figure 2). When DHFR is inhibited by MTX, aminopterin, or the more recent PT523, there is rapid interconversion of THF cofactors to DHF, resulting in cessation of one-carbon-dependent processes (12,15,16).

3.0 The folate transporters

3.1 The reduced folate carrier

(RFC,SLC19A1) was the first folate transporter described at the kinetic, thermodynamic and molecular levels (3,17–19) . The gene that encodes human RFC (hRFC) is located on chromosome 21q22.3. The K_m for 5-methylTHF, 5-formylTHF, and MTX influx mediated by RFC is in the 3–7 μM range; the K_m for PT523 is much lower. RFC has a very low affinity for folic acid ($K_i \sim 150–200$ μM). The high affinity of RFC for PT523, its very low affinity for folic acid, and its neutral pH optimum clearly distinguish this carrier from PCFT. MTX is frequently used to characterize RFC-mediated transport because it is not metabolized over short intervals and because of the ease and accuracy of influx determinations and distinguishing between free and tightly bound drug within cells (17).

RFC generates only small transmembrane *chemical* gradients. However, folates are negatively charged due to the two glutamate carboxyl groups that are fully ionized at physiological pH (Figure 1). When this is considered within the context of the membrane potential, RFC actually produces a substantial electrochemical-potential difference for folates across cell membranes (17). The energy source for this uphill process is unique. RFC function is not directly linked to ATP hydrolysis, neither is it Na^+ nor H^+-dependent (20,21). Rather, RFC-mediated transport is highly sensitive to the transmembrane anion gradient, in particular, the organic phosphate gradient (20,21). Organic phosphates are highly concentrated in cells where they are synthesized in ATP-dependent reactions and are largely retained. Their resulting asymmetrical distribution across cell membranes provides the driving force for RFC-mediated uphill transport of folates into cells (21).

The most substantive support for this model comes from studies on thiamine transport and metabolism. There are two other members of the SLC19 family, SLC19A2 and SLC19A3, with ~40% homology to RFC (22–26). However, both are thiamine, not folate, transporters. Neither does RFC transport thiamine. However, after thiamine is transported into cells, it is converted to thiamine pyrophosphate and thiamine monophosphate (27); both are transported by RFC (K_is of ~ 25 μM and 32 μM, respectively) (28,29). When cells are exposed to thiamine, these phosphorylated derivatives accumulate intracellularly such that the higher the level of RFC expression, the lower the level of cellular accumulation of thiamine pyrophosphate due to *enhanced* export mediated by RFC. Likewise, when cells are loaded with MTX, accumulation of the phosphorylated derivatives of thiamine is *enhanced* due to MTX inhibition of their export by RFC (28). These observations can only be explained if MTX and phosphorylated derivatives of thiamine use the same carrier, RFC. These interactions among thiamine, its phosphorylated derivates, and folates are illustrated in Figure 3 (upper panel).

Additional evidence for utilization of RFC by structurally unrelated anions comes from the observation that folate influx is enhanced in RFC-containing membrane vesicles by

phosphate or sulfate in the *trans*-compartment (30). Likewise, depletion of extracellular anions impairs efflux of folates from cells, an activity that is restored when extracellular anions such as thiamine pyrophosphate, AMP, or phosphate are added (20).

Thus, RFC is not influenced by the cellular energy charge, per se. Indeed, inhibition of anaerobic or aerobic metabolism resulting in ATP depletion enhances rather than diminishes the transmembrane folate gradient mediated by RFC (31–33). This is likely due to (i) inhibition of ATP-binding cassette proteins that export folates and oppose the concentrative impact of RFC (34), and (ii) maintenance of the net transmembrane adenine nucleotide gradient since ADP and AMP generated from ATP are more potent inhibitors of RFC-mediated folate transport than ATP (20,21).

RFC is characterized by twelve membrane-spanning transmembrane domains (TMDs) and cytoplasmically oriented N- and C-termini (35–37) (Figure 4). hRFC is N-glycosylated at a N-glycosylation consensus site in the loop connecting TMDs 1 and 2 (at Asn58) (38). A large loop domain that connects TMDs 6 and 7 is poorly conserved between species and can be replaced altogether by a non-homologous segment from the SLC19A2 carrier (39). When separate TMD1–6 and TMD7–12 hRFC half-molecules are co-expressed in hRFC-null cells, they are targeted to the plasma membrane surface and restore transport activity (40). Hence, the role of the TMD6–7 loop domain is primarily to provide appropriate spacing between the TMD1–6 and TMD7–12 segments for optimal transport.

Studies with RFC mutants implicated conserved amino acids in TMDs 1, 2, 3, 4, 8, 10, and 11 as important to transport (4) and recent results with N-hydroxysuccinimide ^{3}H-MTX radioaffinity labeling localized binding of the substrate gamma carboxyl group to Lys411 in TMD11 of hRFC (41). By exhaustive cysteine-scanning mutagenesis of a “cysteine-less” hRFC molecule, amino acids localized in TMDs 4, 5, 7, 8, 10, and 11 were implicated in forming the putative substrate binding pocket (42,43) consistent with crystal structures for the bacterial MFS proteins, LacY and GlpT (44,45).

RFC is ubiquitously expressed with patterns of localization (e.g., intestine, hepatocytes, renal epithelial cells, choroid plexus) that suggest its integral role in specialized tissue functions important for *in vivo* folate homeostasis (46,47). hRFC is intricately regulated by transcriptional controls involving as many as 6 alternate promoters and non-coding regions that generate up to 15 distinct 5’ untranslated regions (UTRs) fused to a common coding sequence (4) (Figure 4). The net level of hRFC transcripts among different tissues likely result from both ubiquitous (e.g., Sp, USF) and tissue-specific (Ap1, C/EBP) transcription factors that, when combined, transactivate or repress transcription in response to tissue-specific stimuli. Other factors likely important in the regulation of this gene include promoter methylation, general promoter architecture, and chromatin structure (4).

The diverse hRFC 5’UTRs are subject to posttranscriptional controls including effects on 5’CAP-dependent translation and transcript stabilities (48). For many 5’UTRs, the effects on steady-state hRFC transcripts and proteins are subtle and can be overshadowed by differences in promoter activities. However, for non-coding exons A1/A2, A, and D, there were profound decreases in steady-state hRFC compared to other 5’UTRs (48). For the A1/A2 and A 5’UTRs, upstream AUGs occur in-frame within hRFC coding sequence and result in N-terminally modified hRFC protein isoforms, with 64 and 22 additional N-terminal amino acids, respectively (48,49). The biological significance of these modified hRFC proteins is unclear.

High frequency polymorphisms have been described in hRFC involving both the coding region (G80A) and promoters A1/A2 and A (49–52). G80A hRFC was associated with an increased risk of fetal abnormalities (53,54) and MTX toxicity in patients with acute

lymphoblastic leukemia (ALL) (55). However, the clinical significance of G80A remains controversial (4,56). The presence of a 61 bp repeat polymorphism in the hRFC-A promoter may protect against neural tube defects (57).

3.2 The proton-coupled folate transporter (PCFT)

is a recently discovered folate carrier (SLC46A1) (7,58,59). There are two other members of this family (SLC46A2 and SLC46A3) but their functions are not known. The gene that encodes human PCFT is located on chromosome 17q11.2. PCFT was initially reported to be a low-affinity, pH-independent, heme transporter (heme influx K_m of 125 μM) that mediates heme-associated iron absorption in the small intestine (60); however, subsequent studies were not confirmatory (7,58). Rather, PCFT is a high-affinity folate transporter with a low pH optimum. Its identification now explains the molecular basis for the long-recognized low pH folate transport activities in normal and malignant mammalian cells (8,61–65).

Folate transport mediated by human, mouse and rat PCFTs is electrogenic, indicating that there is a net translocation of positive charges as each folate molecule is transported (7,59,66,67). Folate-induced currents in *Xenopus* oocytes microinjected with PCFT cRNA increase as the pH is decreased in parallel to increased transport of tritiated folates (7,66). This is opposite to what is observed for RFC where transport activity falls with decreasing pH from pH 7.4 and is negligible below a pH of 6.0–6.5 (68,69). If folates are bivalent anions, as has been assumed to be the case, more than two protons must be co-transported with each folate molecule to account for the positive charge of the PCFT-folate-proton complex. Hence, PCFT functions as a folate-proton symporter: the downhill flow of protons via PCFT is coupled to the uphill flow of folates into cells via PCFT (Figure 3). Early studies with rodent jejunal apical brush-border membrane vesicles provided insight into the energetics of what would later be recognized as PCFT-mediated transport. A transvesicular pH gradient resulted in increased unidirectional folate transport, and substantial transmembrane folate concentration gradients, from the low- to high- pH compartment, consistent with a proton-coupled process (70).

While the substrate specificity for PCFT shares some similarity with RFC, there are important differences including a marked difference in pH optima that impacts affinities for transport substrates. The RFC influx K_ms are comparable at pHs 7.4 and 5.5. The fall in transport with decreasing pH is due instead to a marked decrease in influx V_{max} (69). In contrast, for PCFT the influx K_m for folates is increased and V_{max} is decreased with an increase in pH from 5.5 to 7.4. However, the pattern and magnitude of these changes varies among different transport substrates (7). Most notable is the relatively small change in influx K_m and V_{max} for pemetrexed, compared to other folates, with increasing pH. Because of this, PCFT mediates pemetrexed pharmacological effects at physiological pH (7,67). Hence, like the divalent metal-ion transporter, DMT1, PCFT mediates transport of folates in the absence of a proton gradient (71). RFC and PCFT are both stereospecific for 5-formylTHF (7,72).

The PCFT expression pattern in human tissues provides clues as to its functional roles. In murine and human tissues, there are high levels of PCFT transcripts in small intestine, kidney, liver, placenta, retina, and brain. Within the intestine, the highest PCFT levels are found in the proximal jejunum and duodenum (7,66,73).

Information on PCFT secondary structure is emerging. Hydropathy analyses predict a protein with twelve TMDs and amino- and carboxy- termini oriented to the cytoplasm (Figure 5). Th e latter was confirmed by immunofluorescence studies of hemagglutin-tagged PCFT (66,74). The loop domain between the first and second TMDs must be extracellular since the two putative N-glycosylation consensus sites in this region are glycosylated. N-

glycosylation does not appear to be required for PCFT intracellular trafficking nor function (74).

Both RFC (47) and PCFT (66) expressions are markedly increased in small intestine when mice are fed a folate-deficient diet. However, the underlying regulatory mechanisms for these changes await definition.

3.2.1 The impact of pH on the relative contributions of RFC and PCFT to the transport of folates; focus on intestinal folate absorption—The pH at the transport interface is an important determinant of the relative contributions of RFC and PCFT to overall folate uptake when these transporters are expressed at the same site in the same tissue, as is frequently the case. When the pH is 7.4, PCFT transport function is low to negligible (depending on the folate substrate), and RFC function is maximal. As the pH drops, RFC function decreases and PCFT activity increases. At some point, RFC transport disappears altogether and PCFT becomes the sole transport route (7,66,69). From the physiological perspective, the critical transport interfaces where there is an acid pH include: (i) the microclimate of the duodenum and proximal jejunum apical brush-border membranes where several Na^+/H^+ exchangers generate pHs of 5.8–6.0, and where folates are absorbed from the diet (75–78); and (ii) within acidified endosomes containing FRs (pH ~6.5) from which, following internalization from the cell surface, folates are exported into the cytoplasm (79) (see Section 3.3). It is possible that there are other, as yet unidentified, transport interfaces where ambient pH is low due to localized activities of Na^+/H^+ exchangers.

RFC and PCFT are expressed at the apical brush-border membrane of the proximal jejunum (58,66,80,81); however, the acidic pH clearly favors PCFT over RFC (Figure 6). Indeed, there is a large literature that demonstrates a low-pH optimum at this site for folate transport and a K_m for folic acid at or below 1μM, properties clearly different from RFC. These encompass a broad spectrum of systems: intact intestine in man and rodents; isolated intestinal loops, rings and everted sacs *in vitro*; and isolated intestinal cells or cell lines of intestinal origin (82–84).

Yet, despite this, because RFC was identified at the intestinal apical brush-border membrane and there was no other *known* transporter at this location, absorption was attributed to RFC. Indeed, the functional discrepancies between intestinal transport and RFC-mediated transport were attributed to post-translational modifications of RFC that altered its functional properties, although such modifications were never identified (85,86). Further evidence that the low-pH folate transport activity is due to a carrier genetically distinct from RFC came from studies in which RFC was deleted from the genome, mutated with loss-of-function, or was silenced. In these studies, with a variety of cell lines including those of intestinal origin, transport at pH 7.4 was lost or markedly diminished but the low pH transport activity was preserved (62,87–89). However, it remains unexplained how in one report siRNA to RFC suppressed folate transport activity in rat-derived intestinal epithelia cells at low, but not physiological pH (90).

Ultimately, the critical role that PCFT plays in intestinal folate absorption was unequivocally established with the demonstration that subjects with HFM invariably have loss-of-function mutations in the PCFT gene (see Section 4.0).

3.3 The folate receptors

FRs are very high affinity folate binding proteins, encoded by three distinct genes designated α, β, and γ, located on chromosome 11 (6,91). FRs are homologous proteins (68–79% identical amino acid sequences), characterized by disparate patterns of tissue

expression (see below). FRα and FRβ are both GPI-anchored proteins that mediate folate transport (5,6,91,92).

FRs have high affinity for folic acid (Kd 1–10 nM). FRα and FRβ from both human and murine sources exhibit different specificities for (6S) and (6R) diastereomers of reduced folates such as 5-methylTHF and 5-formylTHF (93,94). The 50-fold lower binding affinity of (6S) 5-methylTHF for FRβ compared to FRα was attributed to Leu49, Phe104, and Gly164 in FRβ since replacement of these residues with the corresponding residues from FRα (Ala, Val, and Glu, respectively) reconstituted the FRα phenotype (95).

Mechanistically, folate internalization by membrane-associated FRs involves a receptor-mediated endocytosis (5,6,91,96,97). The process is initiated when a folate molecule binds to a FR on the cell surface. This is followed by invagination of the plasma membrane at that site and the formation of a vesicle (endosome), which migrates into the cytoplasm where it is acidified to a pH of ~ 6.5, resulting in dissociation of the folate from the FR complex (79). Folate ligand is then exported into the cytoplasm (96,97). This trans-endosomal exporter was thought to be an anion exchanger and require a trans-endosomal pH gradient (96,98,99). Most recently, PCFT was proposed to account for folate export from endosomes (7,59,100) (Figure 3, see below).

The physiologic roles of FRs are unclear in all but a few cases. While FRs α and β can transport folate into cells, this is inefficient compared to transporters such as RFC (101,102). FRα is expressed in epithelial cells of the kidney, choroid plexus, retina, uterus, and placenta (6,103). In proximal renal tubule cells, FRα is expressed at the apical (luminal) surface. In retinal pigment epithelium, FRα is expressed on the basolateral membrane (104,105). FRα is also expressed in tumors, particularly non-mucinous adenocarcinomas of the ovary, uterus and cervix (103). For ovarian carcinomas, FRα expression correlates with histologic grade and stage (106). FRα is negatively regulated by the estrogen receptor (107). Dexamethasone, a glucocorticoid receptor agonist, is a positive regulator of FRα (108).

FRβ is expressed during normal myelopoiesis and is present in placenta, spleen, thymus, and in CD34+ monocytes (109–112). A non-functional form of FRβ was reported in CD34+ human hematopoietic cells (111). FRβ is expressed in leukemic blasts in chronic myelogenous leukemia and in acute myelogenous leukemia (AML) (110,112,113). In AML, FRβ expression is induced by treatment with retinoid receptor agonists including all-*trans*-retinoic acid (110). This is independent of cell differentiation or proliferation status and was not observed in non-myeloid cells.

3.4 Folate transport mediated by ATP-binding cassette transport proteins and members of the SLC21 and SLC22 families of solute carriers

Beyond the transporters that are highly specific for folates, there are other potential folate transport routes. Relevant ATP-binding cassette exporters include the multidrug resistance-associated proteins, MRP1–5 (ABCC1-ABCC5) and the breast cancer resistance protein, BCRP, (ABCG2) (34,114,115). These are low affinity, high capacity transporters (K_ms ~0.2 – 2 mM for folates/antifolates). Members of this family are widely expressed in mammalian cells and suppress the level of free folates or antifolates that accumulate in most cells grown *in vitro* (see Section 3.1) (34). Some of the shorter chain-length polyglutamate folates may be weak substrates for MRPs. However, in general, folate polyglutamates are retained within cells (12,34,116).

From a physiological perspective, MRP2 plays a critical role in the export of folates across the bile canalicular membrane, as demonstrated by impaired biliary secretion of MTX in MRP2 (−/−) mice (117). The role that MRP family members play in the vectorial transport

of folates in other tissues is less clear. Of particular interest is their potential role in the export of folates from jejunal enterocytes. MRP2 is expressed at the apical membrane which would oppose absorption mediated by PCFT (118). Expression of MRP3 at the basolateral membrane of rat jejunum (119) suggests its role in the transport of folates across the jejunal enterocyte by mediating export across the basolateral membrane. MRPs1–4 and BCRP are expressed in human jejunum although relative levels have not been firmly established (120,121). While MRP1 and MRP 4 were localized to the basolateral membrane of some tissues, their localization in human jejunum is not known (34).

The SLC21 family of facilitative carriers transport organic anions and the SLC22 family transport both organic anions and cations (122–124). Both are expressed in tissues that mediate vectorial transport, i.e, intestine, kidney, and liver. Some of these carriers transport folates and antifolates. Of the SLC21 members, OAT-K1 and OAT-K2 are kidney-specific and expressed at the apical brush-border membrane. These transporters have K_ms for MTX and 5-methylTHF of ~2 μM and could play a role in reabsorption of folates at the proximal tubule (125). Of the SLC22 members, OAT1, OAT2, and OAT3 are expressed at the basolateral membrane of renal tubules (126). These transporters generally have low affinities for MTX (10–700 μM), depending on the expression system (127–131). Since RFC is also present at the basolateral membrane, it is unlikely that the SLC22 transporters play a major role in renal reabsorption of folates.

4.0 Genetic deletion of folate transporter genes: the PCFT-null phenotype, hereditary folate malabsorption (HFM), and what can be learned from this disorder about the role of PCFT (and RFC) in folate biology

RFC and the FRs have been genetically deleted in the mouse. RFC heterozygous (+/−) mice have no phenotype. Loss of both RFC alleles (−/−) is embryonic lethal; however, mice can be brought to term and live for 1–2 weeks when pregnant females receive parenteral folic acid. At death these animals have evidence of failure of erythropoiesis in bone marrow, liver, and spleen (132). More recent studies on RFC (−/−) mice from less folate-supplemented pregnant females reveal a variety of congenital malformations (133). Mice heterozygous (+/−) for FRα have no phenotype. Loss of both alleles (−/−) is embryonic lethal; the embryos are arrested at the time of neural tube closure (134). If, however, pregnant females receive parental 5-formylTHF throughout gestation, the pups are normal and can be maintained without any apparent abnormalities through adulthood on a standard chow (R. Finnell, personal communication).

The human PCFT-null genotype/phenotype is known within the context of the rare autosomal recessive disorder, HFM (7,9,10,135). The major defects in HFM are: (i) impaired intestinal folate absorption, resulting in severe systemic folate deficiency; and (ii) impaired transport of folates into the central nervous system associated with very low to undetectable folate in the cerebrospinal fluid (CSF).

Individuals who are PCFT (+/−) are completely normal. Infants with HFM are normal at birth. Neural tube defects are not seen, indicating that there is adequate placental delivery of folates from mother to fetus. Signs of HFM begin several months after birth, presumably when folate stores, provided by the mother during gestation, are depleted. The infants develop anemia, sometimes pancytopenia, reflecting folate deficiency in rapidly proliferating hematopoietic cells. Gastrointestinal involvement manifests as severe diarrhea. Immune deficiency due to hypoimmunoglobulinemia is frequent, resulting in infections usually associated with immune-compromised states (i.e. *Pneumocystis carinii* and cytomegalovirus pneumonia). Patients frequently have neurological signs including seizures,

neuro-developmental defects, and/or mental retardation. Systemic signs of the disease can be completely reversed by low-dose parenteral folate or higher-dose oral folate which normalizes blood folate levels. However, very high blood folate levels are required to achieve CSF folate levels in the normal range (135).

Eight families with HFM have been reported to date with loss-of-function mutations in PCFT (Four additional families not as yet reported; Goldman, unpublished). The mutations are indicated in Figure 5 (7,9,10,136). No mutations were detected in RFC or FRα in patients with HFM. These observations *unequivocally* establish PCFT as the mechanism by which folates are absorbed across the apical brush-border membrane of the proximal jejunum under normal conditions. This is consistent with the low pH (5.8–6.0) at the jejunal brush-border membrane and observations that: (i) when jejunal pH is decreased, as occurs with pancreatic insufficiency, folate absorption is increased (137); and (ii) when the pH is increased, as occurs with ingestion of antacids or after gastrectomy, intestinal folate absorption is decreased (138).

The clinical observation that high-doses of oral folates can restore systemic folate levels in individuals with HFM raises the question of how intestinal folate is absorbed under these conditions (135). Defects due to PCFT mutations that decrease affinities for folates could be overcome by high doses of oral folates. However, in several cases, mutations resulted in a stop codon, a frameshift in exon 1, or a splicing defect, resulting in the absence of PCFT protein. In other cases, point mutations resulted in defects in trafficking/stability and/or a complete loss of intrinsic function (7,9,10,136). Since passive diffusion of folates is assumed to be negligible even in the presence of pharmacological doses of folates, it is likely that absorption is mediated by RFC, albeit inefficiently, under these conditions.

5.0 The folate-one-carbon journey from food to macromolecules

This section traces the pathway of folates ingested and the various folate transporters that mediate folate transport across epithelia and into systemic tissues.

5.1 Hydrolysis of folate polyglutamates

The major natural folate forms in the diet are polyglutamate congeners of 5-methylTHF that are not substrates for the absorptive process (139,140). This is consistent with the recent demonstration that affinities of PCFT for the di- to penta- glutamates of MTX are very low (66). Rather, folate polyglutamate derivatives must be hydrolyzed to their monoglutamate forms by glutamate carboxypeptidase II in human intestine and γ-glutamyl hydrolase in rat intestine (141,142). The γ-glutamate hydrolytic reaction has a low pH optimum like the absorptive process, consistent with the low pH at the surface of mucosal cells in the proximal jejunum.

5.2 Intestinal Absorption

Once folate monoglutamate is generated, it is transported across the apical brush border membrane of the proximal jejunum mediated by PCFT. Because PCFT transport is concentrative, driven by the transmembrane proton gradient, the high folate levels in enterocytes should facilitate folate efflux across the basolateral membrane into the periserosal space and, from there, enter the vascular system. The mechanism of export from enterocytes is not clear; neither RFC nor PCFT is expressed at the basolateral membrane. However, MRPs are expressed at this site, particularly MRP3 (34), and may represent the route of export of folates (Section 3.4).

5.3 Transport to, and disposition in, the liver

After intestinal absorption, folates enter the hepatic portal system and are delivered to the hepatic sinusoids in apposition to the hepatocyte basolateral (sinusoidal) membrane. PCFT is highly expressed in the liver, and localizes to the sinusoidal membrane (Wang and Goldman, unpublished). This is consistent with a dominant low pH folate transport activity in hepatocytes and in sinusoidal membranes derived from hepatocytes (143,144). In the latter, concentrative folate transport is produced with a transvesicular pH gradient. There is minimal transport activity at pH 7.4, consistent with a low level of RFC activity (145,146), although RFC is detected at the hepatocyte membrane (80). It is unclear as to the pH within hepatic sinusoids where hepatic portal vein blood derived from the intestine, pancreas, and spleen mixes with hepatic artery blood. It has been proposed that a Na^+/H^+ antiporter at the sinusoidal membrane may generate a local extracellular region of high acidity (147), as is the case in the proximal jejunum.

Folates that enter the liver have three potential destinations. (i) Folates can be converted to polyglutamate storage forms or (ii) they can be secreted into the bile at the hepatic canalicular membrane, mediated by MRP2 (117), whereby they return to the duodenum and jejunum and are subsequently reabsorbed, thus completing the cycle of enterohepatic circulation. (iii) Folate monoglutamates, formed by hydrolysis of polyglutamate forms stored in hepatocytes, or delivered directly from the hepatic portal vein, enter the hepatic vein, ultimately reaching the systemic circulation whereby they accumulate in, and meet the one-carbon requirements of, peripheral tissues. Folates in blood also encounter epithelial barriers separating compartments containing highly folate-dependent tissues. Notable are neural tissues isolated by the vascular blood-brain-barrier and blood-choroid plexus-CSF-barrier.

5.4 Transport into systemic tissues

Membrane transport of folates into systemic tissues via the arterial system where the pH is 7.4 is mediated by the RFC. While PCFT is often co-expressed with RFC, PCFT function would be minimal and not likely contribute to the delivery of folates systemically unless, as suggested above, there are tissues where Na^+/H^+ exchangers produce acid microenvironments at transport interfaces. PCFT may also play an important role in FRα–mediated transport (see Sections 3.3 and 5.6).

5.5 Filtration and reabsorption in the kidney

Blood folates, not bound to serum proteins, are filtered at the glomerulus but, due to a highly efficient reabsorptive mechanism, little or none is lost in the urine at physiological levels of folate intake (148). The initial step in reabsorption involves tight binding to FRα highly expressed at the luminal brush-border membrane of proximal tubules. The affinities of folates for this site are consistent with their affinities for FRα (149,150) and the urinary clearance of folates is inversely proportional to their affinities for FRα (151). In the FRα (−/−) mouse, (maintained on standard chow – see above, Section 4) there is increased folic acid clearance, and decreased 5-methylTHF uptake (152). Folates accumulate to high levels in the kidney, reflecting the large component bound to FRα. This is followed by slow internalization of receptors, release of folate into the cytoplasm, and export at the basolateral membrane by RFC where the pH is optimal for carrier function (80,148,149). Several SLC21 solute carriers are expressed at the apical brush-border membrane of proximal renal tubule cells (see Section 3.4) and probably contribute to renal folate absorption, particularly at high folate loads, when FRα-mediated absorption is saturated (152). The role of PCFT in renal folate reabsorption is not clear. PCFT mRNA is highly expressed in the kidney but its exact location has not been defined (7,66). In renal brush-border membrane vesicles, the folate flux and net transport is optimal at low pH when the pH of the trans-compartment is

7.4, consistent with a PCFT-mediated process (153,154). The pH in the proximal tubule starts at 7.4 and falls to ~6.8 as filtrate passes down the tubule, bicarbonate is reabsorbed, and chloride is secreted (155). Under these conditions, should PCFT be present, only modest PCFT-mediated transport at the brush-border membrane would be expected.

While a rigorous analysis of renal folate clearance in subjects with HFM has not been reported, some information can be gleaned from published studies. In one patient with HFM, the clearance of folate from blood, after an intravenous bolus, was not different from that of a normal subject over the first 2−3 hours (156). In another case, clearance in a patient with HFM over this interval was similar to what was observed in historical controls (148,157). These observations suggest that PCFT is not required for reabsorption of folates in the proximal tubule. However, since the initial phase of folate clearance from blood is influenced by uptake and retention in systemic tissues and tight binding of filtered folate to FRα, more than a few hours may be necessary to fully decipher the role of PCFT in the reabsorptive process. It is also possible that at least a component of the FR-mediated folate reabsorption in the proximal tubule represents a transcytosis in which vesicles formed at the brush-border membrane retain folate as they transit intact to the basolateral membrane where their contents are discharged to the peritubular space. However, uptake of tritiated folate alone, or folate coupled to colloidal gold particles, into renal tubular epithelial cells is consistent with translocation of vesicles across the brush-border membrane, followed by recycling back to that membrane via dense apical tubules (158,159).

5.6 Transport of folates across the blood-brain-barrier and the choroid plexus-CSF-barrier

There are two potential routes of delivery of substrates to the brain, the blood-brain-barrier at the cerebral vascular endothelium, and the blood-CSF-barrier at the choroid plexus. RFC (80) and PCFT (Zhao et al, unpublished) proteins are localized to the vascular blood-brain-barrier. FRα message is neither expressed in brain nor is protein detected at the blood-brain-barrier (160,161). The high rate of delivery of 5-methylTHF to brain parenchyma is consistent with folate extraction from blood mediated predominantly at the vascular blood-brain-barrier (162). Transport of 5-methylTHF into human brain capillaries is saturable and can be inhibited by low levels of 5-methylTHF or folic acid (162). While this has been interpreted as implicating FRα in this process, despite the absence of the receptor in brain, this is also consistent with a PCFT-mediated process for which folic acid and 5-methylTHF have comparable affinities. However, there is no evidence that the pH at this transport interface is acidic.

The choroid plexus represents the pathway for the active transport of substrates and metabolites into and out of the CSF (163). The concentration of most small molecules in CSF is less than in blood (163). The opposite is the case for folates. The usual CSF:blood folate ratio is 2–3:1 (135), consistent with concentrative folate transport across the choroid plexus. It is clear that PCFT is required to achieve usual blood:CSF folate gradients, since this ratio is reversed in subjects with HFM (135). Evidence that FRα plays a role in this process comes from the observations that: (i) this protein is highly expressed in choroid plexus; and (ii) blocking autoantibodies to FR are found in the blood of patients with cerebral folate deficiency. In this disorder, there are low levels of CSF folate even though folate intestinal absorption and folate blood levels are normal (164–168). These subjects do not have mutations in FRα, RFC, or PCFT (164).

Studies on transport of folates into the choroid plexus *in vivo* and *in vitro* further substantiate a role for this organ in the transport of folates (161,162,169,170). PCFT protein is expressed at the basolateral membrane of ependymal cells at the capillary interface (R. Zhao et al., unpublished); RFC is expressed at the apical membrane at the CSF interface (80). FRα mRNA is expressed in the choroid plexus, and is visualized at the basolateral and,

to a greater extent, apical basolateral membranes (161,171–174). While both interfaces are likely at neutral pH, Na^+/H^+ antiporters expressed at the basolateral membrane (175) could produce an acid microclimate that facilitates PCFT function, as occurs in the proximal small intestine. One possible model that includes FRα and PCFT envisions FRα-mediated endocytosis across the basolateral membrane, generating a folate gradient across ependymal cells, followed by the downhill flow of folate across the apical membrane into the CSF mediated by RFC. While RFC produces small folate gradients directed into cells, it mediates very rapid bidirectional fluxes of folates (176). According to this paradigm, PCFT facilitates FRα-mediated endocytosis by serving as the route for folate export from acidified endosomes into the cytoplasm (Figure 3). As observed for transport across the vascular blood-brain-barrier, 5-methylTHF appears to be the preferred substrate for transport across the choroid plexus (162,170,177).

5.7 Transport of folates across the placenta

FRα, RFC, and PCFT are all highly expressed in the placenta (7,46,109,172,178), although the location of, and relationship among, these transporters has not been established. However, the observation that low levels of folic acid, but not MTX, inhibit maternal to fetal [^{3}H]folic acid transport across guinea pig placenta suggests that FRα plays an important role in the initial phase of folate transport from maternal blood at the apical membrane of the syncytiotrophoblast (179,180). Further evidence of a role for FRα in placental transport involves the inhibitory effects of bafilomycin A, which blocks the V-type proton pump, or FCCP, which abolishes the transmembrane proton gradient, on folic acid transport into tumor cells of trophoblastic origin (JAR). This is presumably due to their inhibitory effects on folate export from endosomes in the endocytic pathway (99). Transport of folic acid in the BeWo trophoblastic cell line is maximal at pH 5.0, reflecting PCFT-mediated transport, and decreases as pH is increased, although a second peak of activity at pH 7.4 is also detected, likely reflecting RFC-mediated transport (181). Vectorial transport of folic acid across BeWo cells, from the apical compartment (maternal side) at pH 5.5, separated by a semi-permeable membrane from the basal compartment (fetal side) at pH 7.4, was far greater than transport from the basal to apical compartments when the pH gradient was reversed (182). This suggests a higher level of PCFT activity at the apical than basal interface.

Because the placenta is fetal in origin, and because PCFT (−/−) subjects with HFM do not have developmental defects associated with folate deficiency, PCFT does not appear to be necessary for placental transport of folate from mother to embryo or fetus despite its high level of placental expression and its potential role in FRα-mediated endocytosis. The histories of families with HFM reveal that PCFT (+/−) mothers can support a normal pregnancy. Unanswered as yet is whether the loss of two PCFT alleles will affect maternal-to-fetus folate delivery. The oldest reported female with HFM is now age 28 and has had normal menarche but has not, as yet, become pregnant (10,183,184).

6.0 Clinical Implications/Applications

Folate transport is a critical determinant of folate sufficiency and the integrity of one-carbon metabolism in man. Hence, expression of folate transporters, as determined by genetic, epigenetic and other regulatory factors, is of considerable importance. The requirements for RFC and FRα in embryonic development, and PCFT in infancy, are clear. Folate deficiency during adult life may be associated with transport variations that contribute to other pathophysiologic states including cancer, cardiovascular disease, and neurological disorders (185–187). Folate deficiency in pregnant women is a major factor in fetal abnormalities (188). Mild transport variations could be compounded by alterations in activities of folate-dependent interconverting and biosynthetic enzymes for which there are known allelic

variations (e.g., C677T in MTHFR) that impact on cellular distributions of individual THF cofactor forms (189). The cumulative effects could be impaired nucleotide biosynthesis, impaired repair of DNA damage, DNA hypomethylation resulting in aberrant oncogene expression, or DNA hypermethylation resulting in silencing of protective genes such as tumor suppressors (185).

An understanding of the molecular basis for HFM now makes possible a definitive diagnosis of this disorder in families. This allows genetic counseling along with prenatal and *in vitro* testing. Individualization of treatment with specific folates may be possible, based upon the functional characteristics and specificity of individual mutant PCFTs in patients with HFM.

RFC-mediated transport is also key to antitumor activities of antifolate chemotherapeutic drugs such as MTX (4,12). Early on, it was recognized that intrinsic and acquired resistance to MTX is frequently due to impaired transport in cell lines (190–192) and is a factor in resistance to MTX in ALL and osteogenic sarcoma (193–195). Enhanced accumulations of MTX and its polyglutamates accompany increased levels of hRFC transcripts and copies of chromosome 21 in hyperdiploid B-precursor ALL (196).

Recently, pemetrexed was approved for the treatment of cancer in the United States, more than fifty-years following the introduction of MTX (13). Pemetrexed has an affinity for RFC comparable to that of MTX. However, pemetrexed differs from MTX in that its affinity for PCFT is sufficiently high, even at physiological pH, that its activity is preserved in tumor cells that are MTX resistant due to loss of RFC function (13,87,197).

7.0 Research in progress and outstanding research questions

The transcriptional and post-transcriptional controls that contribute to tissue-specific expression of RFC have been largely defined (4). While there have been extensive studies of high frequency sequence polymorphisms in the hRFC coding and noncoding regions in relation to a variety of pathological states (4,56), these associations remain controversial. The recent production of a “humanized” RFC mouse in which the mouse RFC gene is functionally replaced by the hRFC gene locus (198) holds promise for extending *in vitro* findings of hRFC regulation and function including polymorphic hRFC gene variants to a clinically relevant *in vivo* model. This will allow correlations with dietary folate status and biological manifestations of folate deficiency.

Structure-function studies of hRFC have provided substantive insights into its membrane topology, N-glycosylation, important domains and amino acids, and three dimensional helix packing associations (4). Recent studies indicate that there are higher order oligomers (e.g., dimers) of hRFC (Z. Hou and L.H. Matherly, unpublished), as has been observed for other transporters (199). Clarification of RFC oligomeric character is especially important to understanding carrier structure and function, including the mechanism of concentrative folate transport. The existence of homo-oligomeric hRFC may also play an important role in antifolate resistance through potential “dominant-negative” interactions between mutant and wild-type hRFCs that result in alterations in function and/or trafficking defects.

Since PCFT was only recently discovered, there is only scant information on the structural properties of this transporter. A predicted secondary structure was described and localization of the N- and C-termini and the first extracellular loop was verified (66,74). Studies are ongoing to identify key amino acid residues that are determinants of substrate binding, proton-coupling, and carrier mobility. Future studies will emphasize helix packing and tertiary structure using approaches such as cysteine-scanning mutagenesis and accessibility methods (35,200). Of course, the finding of higher order hRFC oligomers raises the intriguing possibility that oligomeric PCFT may be important, as well. All these studies of

PCFT structure and function would be enhanced considerably by the availability of a three dimensional structural model of this transporter. The extrapolation of a prokaryotic model based upon the GlpT crystal structure to PCFT is appealing (136) since GlpT is also proton-coupled although, unlike PCFT, this is a proton antiporter (201). Extrapolation of this and other models to PCFT or other transporters will require rigorous experimental verification, as noted above for RFC.

Since folate deficiency is associated with increased risk of colorectal cancer, deciphering the role of RFC and PCFT in the delivery of folates to the large intestine and their regulation at this site, within the context of dietary and environmental factors, will be of considerable importance. These studies need to take into consideration the recent paradigm that folate deficiency results in an increased frequency of oncogenesis, but once cancers are formed, excess folates may accelerate tumor growth (202,203). For RFC, causal associations with loss of carrier function and colorectal cancer were described in mouse models (204,205).

The hypothesis that PCFT is required for FR function, by serving as a route of export from acidified endosomes, broadens the potential biological importance of this carrier. Clarification of this issue could establish a critical role for PCFT in the transport of folates across the blood-choroid plexus-barrier and clarify why transport into this compartment is impaired in HFM. Additional information is also required to clarify the functional role of PCFT in other tissues in which it is highly expressed, particularly those expected to harbor a neutral pH milieu. This relates, in particular, to the extent to which PCFT mediates folate transport from the hepatic portal vein across the sinusoidal membrane to hepatic cells, reabsorption in the proximal renal tubule, transport across the placenta to the fetus, and transport across the choroid plexus and into brain parenchyma at the blood-brain-barrier. These questions will be best addressed by studies on a PCFT-null mouse.

The role that PCFT plays in antifolate delivery to tumor cells requires further exploration. PCFT has a high affinity for pemetrexed and, when transfected into tumor cells, selectivity augments the drug's growth inhibitory activity without a salutary effect on other antifolates such as MTX, PT523 or raltitrexed which have a much lower affinity for PCFT at physiological pH (67,197). It will be important to determine whether PCFT might contribute more to the activity of pemetrexed, or at all to the activities of other antifolates, when transport occurs within solid tumors *in vivo* where cells are hypoxic, the microenvironment is somewhat acidic, and PCFT would be expected to operate more efficiently (206–208). Likewise, because of the high K_m s for antifolate transport at physiological pH, it will be important to determine the role PCFT plays in the delivery of antifolates at the high blood levels achieved in clinical regimens under conditions in which RFC is saturated.

Finally, there continues to be an ongoing interest in the development of new-generation antifolates. Antifolate thymidylate synthase inhibitors were designed with very low affinity for RFC but very high affinity for FRα, so as to minimize toxicity due to uptake via RFC which is ubiquitously expressed, and to maximize uptake via FRs that are more selectively expressed in certain tumors (209–211). An analogous strategy was very recently described for a series of highly potent FR-targeted antifolate GARFT inhibitors (212). It will be of particular interest to determine whether the activities of these novel FR-targeted agents will be limited by their efficacies as substrates for PCFT which may be required for their optimal export from endosomes during the endocytic cycle.

Acknowledgments

The authors wish to thank the reviewers of this manuscript for their very helpful suggestions. This work was supported by grants CA53535 (LHM) and CA82621 (IDG) from the National Institutes of Health and from the Mesothelioma Applied Research Foundation (IDG).

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9.0 Website

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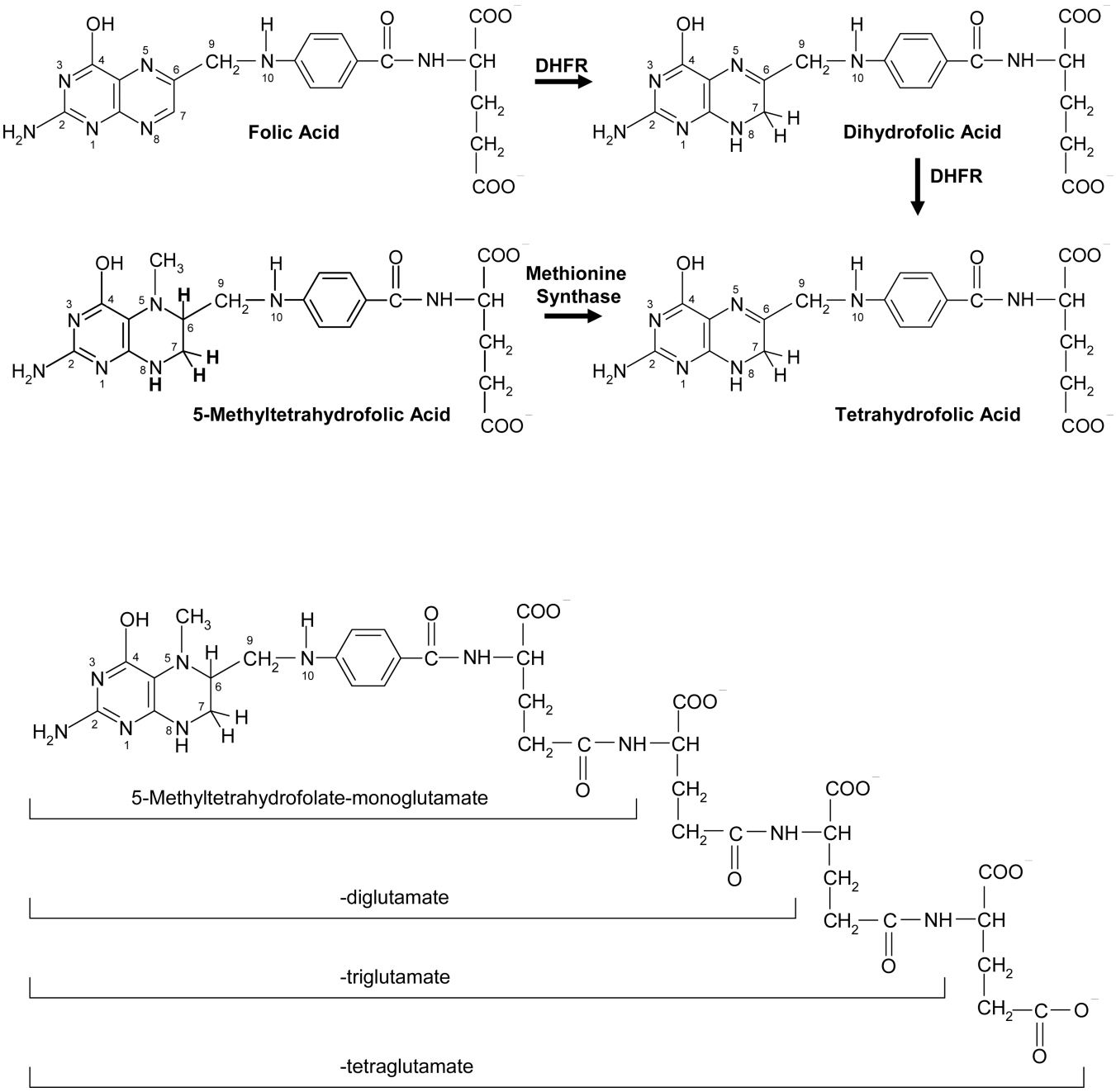

Figure 1.

Upper Panel: Structures of representative folate compounds. Folic acid is reduced to dihydrofolic acid at the positions 7,8 of the B ring which, in turn, is reduced to tetrahydrofolic acid at the positions 5,6 of the B ring in reactions mediated by dihydrofolate reductase (DHFR). Endogenous folic acid is not found in cells. Rather, its presence is related to the consumption of folic acid added to cereals and grains and vitamins. The major oxidized folate in cells is dihydrofolic acid. 5-Methyltetrahydrofolic acid (5-methylTHF) is the major dietary form and the major folate found in blood. It is formed endogenously by the reduction of 5,10-methylene-tetrahydrofolic acid (5,10-methyleneTHF). With the utilization of 5-methylTHF in methionine synthesis, tetrahydrofolate (THF) is generated. These

relationships are depicted in Figure 2. The two carboxyl groups of the glutamate moiety are fully ionized at physiological pH.

Lower Panel: Structure of 5-methylTHF tetraglutamate. This is one of the 5-methylTHF forms found in nature and in mammalian cells. This is a poly-anion and a very poor substrate for folate transporters and multidrug resistance associated proteins.

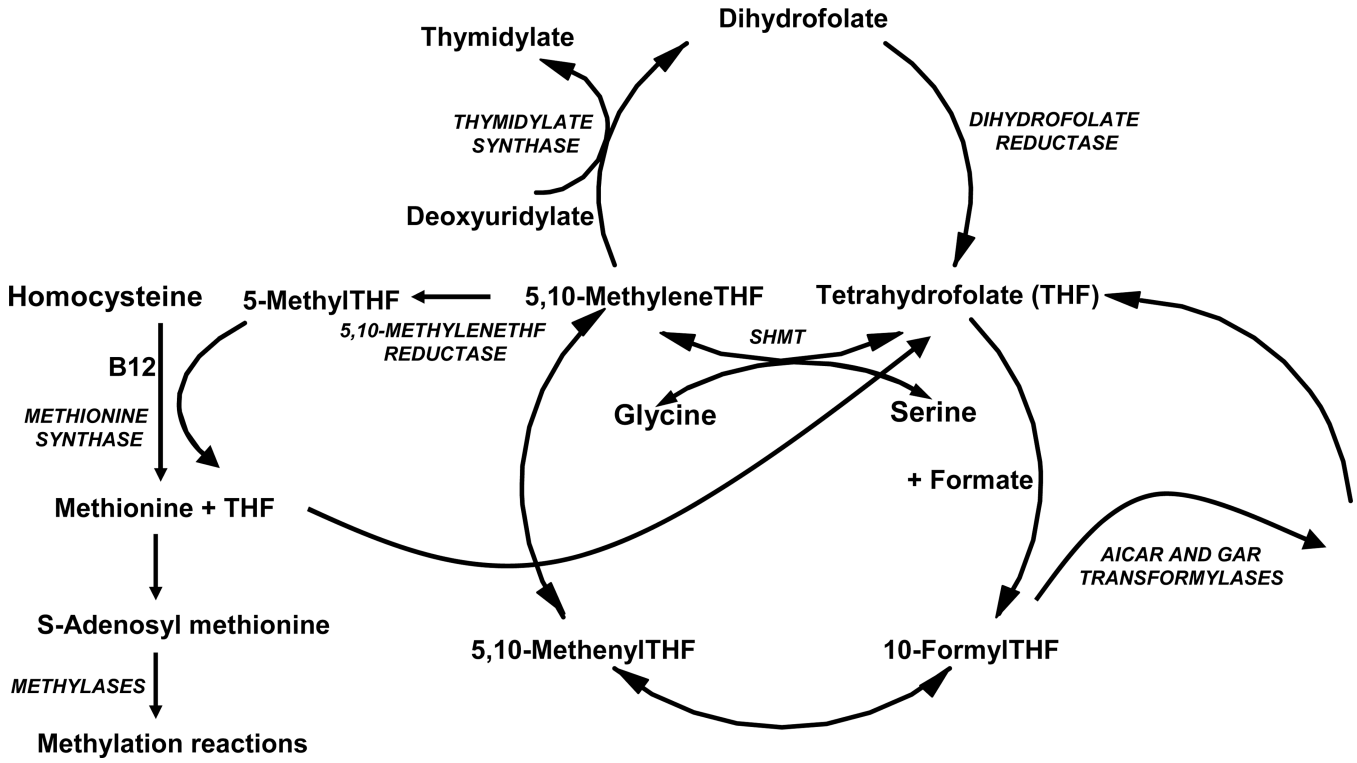

Figure 2.

Folate metabolic pathways. 5-MethylTHF enters the metabolic cycle following transport into cells where, with homocysteine, it is utilized in the synthesis of methionine mediated by methionine synthase and vitamin B12. The THF product then acquires a carbon from formate or serine, at the N5, N10, or shared between the N5 and N10 positions that, through a series of interconversions, results in several other folate forms that provide carbons for purine, thymidylate, and methionine synthesis. Abbreviations: (AICAR, 5-amino-4-imidazolecarboxamide ribonucleotide; GAR, β-glycinamideribonucleotide; SHMT, serinehydroxymethyltransferase.

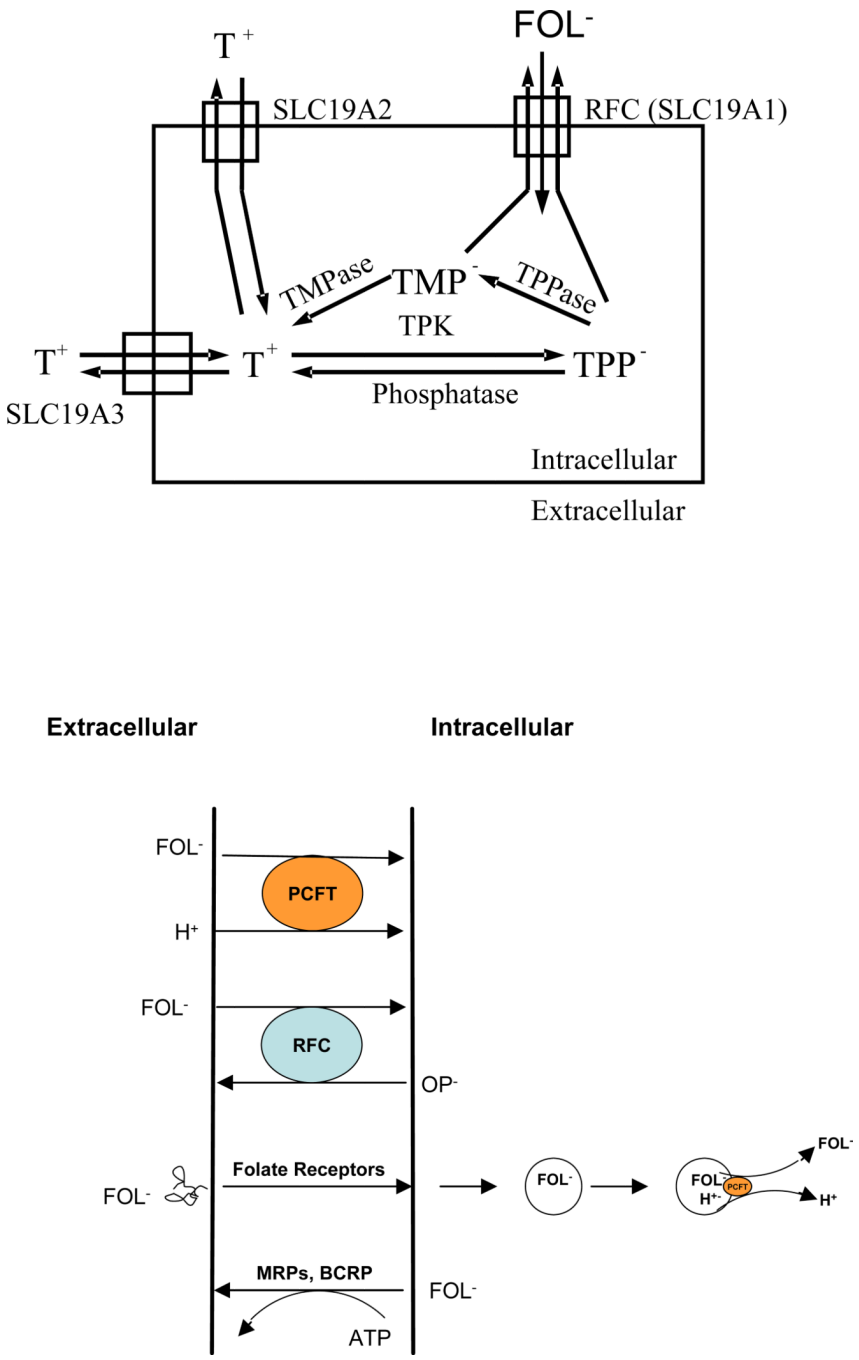

Figure 3.

Upper panel: Interactions among thiamine, its phosphorylated derivatives, folates, and the SLC19 family of transporters. Thiamine (T^+), a cation, is transported into cells via SLC19A2 and SLC19A3. Within cells thiamine is metabolized to thiamine pyrophosphate (TPP^-), an anion, in a reaction mediated by thiamine pyrophosphate kinase (TPK). TPP^- can be hydrolyzed to thiamine monophosphate (TMP^-), also an anion, mediated by thiamine pyrophosphatase (TPPase). Both are substrates for the reduced folate carrier (RFC-SLC19A1) and are exported by that mechanism. Folates enter cells via RFC and are not substrates for the thiamine transporters. Likewise, thiamine is not a substrate for RFC. Lower panel: A schema of the current known folate transporters. The proton-coupled folate

transporter (PCFT) is a folate (FOL^-)-H^+ symporter that functions most efficiently in an acidic extracellular environment. The reduced folate carrier (RFC) is an anion exchanger that utilizes the transmembrane organic phosphate (OP^-) gradient to achieve uphill transport into cells. Folate receptors (FRs) α and β are high- affinity folate binding proteins that transport folates into cells via an endocytic mechanism. Once in the cytoplasm, the vesicle acidifies, folate is release from receptor and is exported from the endosome via PCFT. Folate monoglutamates are exported from cells via the multidrug-associated resistance proteins (MRPs) and the breast cancer resistant protein (BCRP).

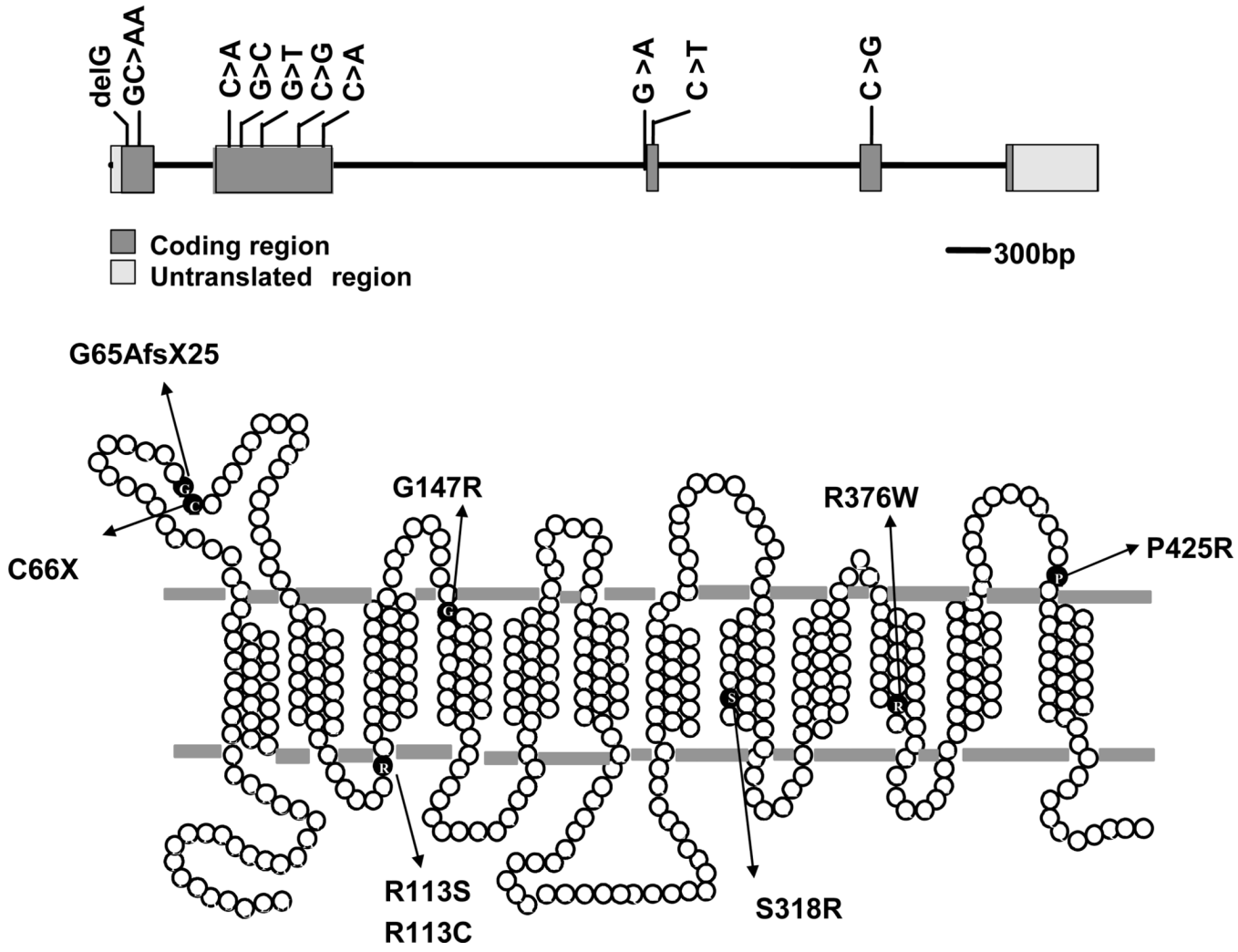

Figure 4.

Upstream RFC gene structure and membrane topology. Upper Panel: A schematic of the upstream region of the human RFC gene including up to 6 alternate non-coding regions, each preceded by a separate promoter, spanning ~35 kb upstream of the major translational start site (shown). The alternate promoters transcribe unique RFC transcripts each with a distinct 5' untranslated region (5'UTRs) fused to a common coding sequence. Alternate splicing for the A1/A2, A, B, and D 5'UTRs have been described. For the A1/A2 and A 5'UTRs, upstream AUGs occur in-frame within the RFC non-coding sequence and result in N-terminally modified hRFC protein isoforms, with 64 and 22 additional N-terminal amino acids, respectively. Lower Panel: A schematic is shown for the membrane topology of the human RFC including 12 transmembrane domains (TMDs), internally oriented N- and C-termini, the large cytosolic loop between TMDs 6 and 7, and the N-glycosylation site at Asparagine 58.

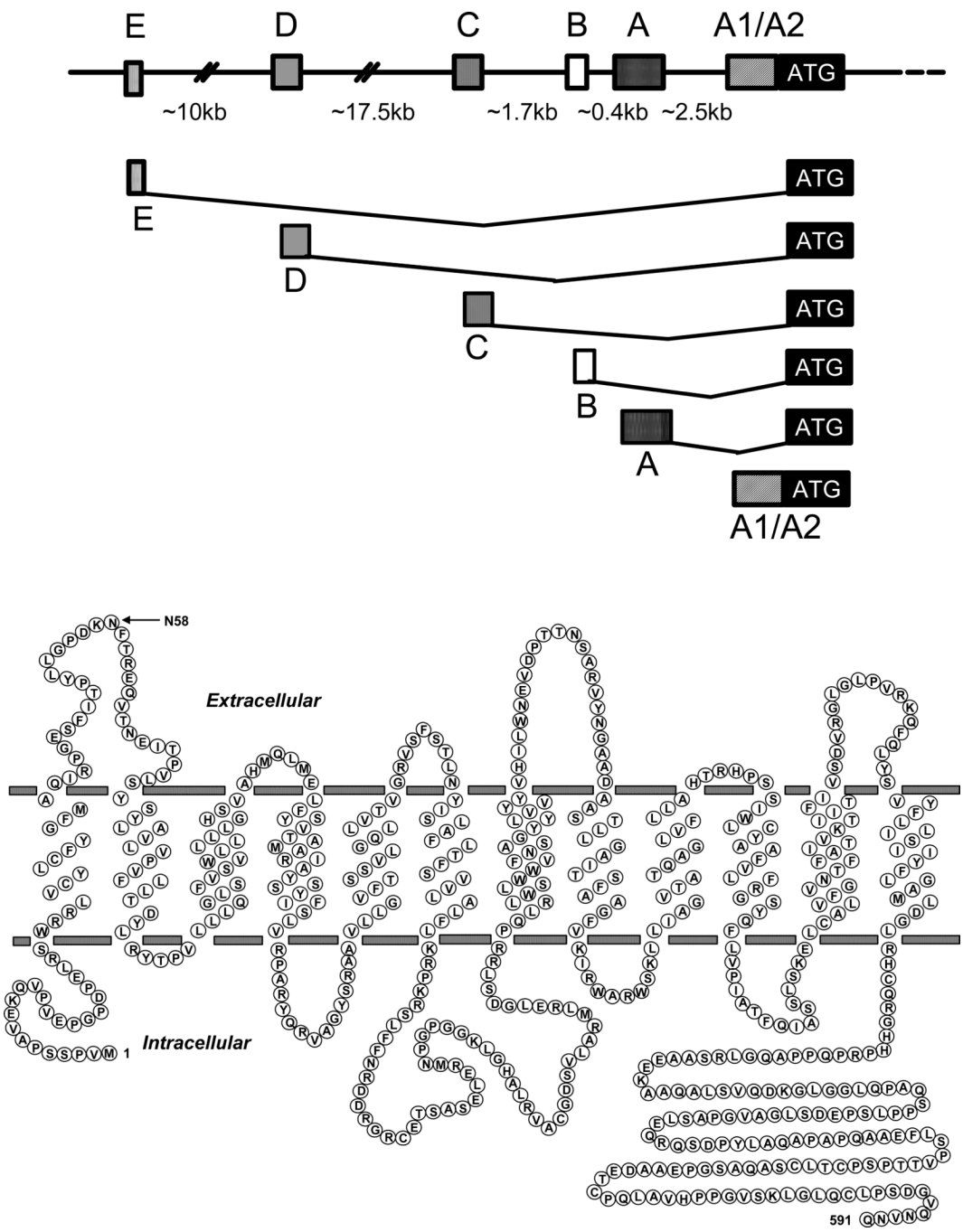

Figure 5.

The genomic organization and predicted secondary structure of PCFT and the spectrum of reported mutations in the protein derived from patients with the autosomal recessive disorder, hereditary folate malabsorption (HFM). Upper Panel: The genomic organization of PCFT; the positions of the various base mutations in the first through fourth exons are indicated. Lower Panel: A schematic of a predicted human PCFT topology showing confirmed N-glycosylation sites between the first and second TMDs with the N- and C-termini oriented to the cytoplasm. The location and identity of the mutated amino acid residues involved with HFM are indicated.

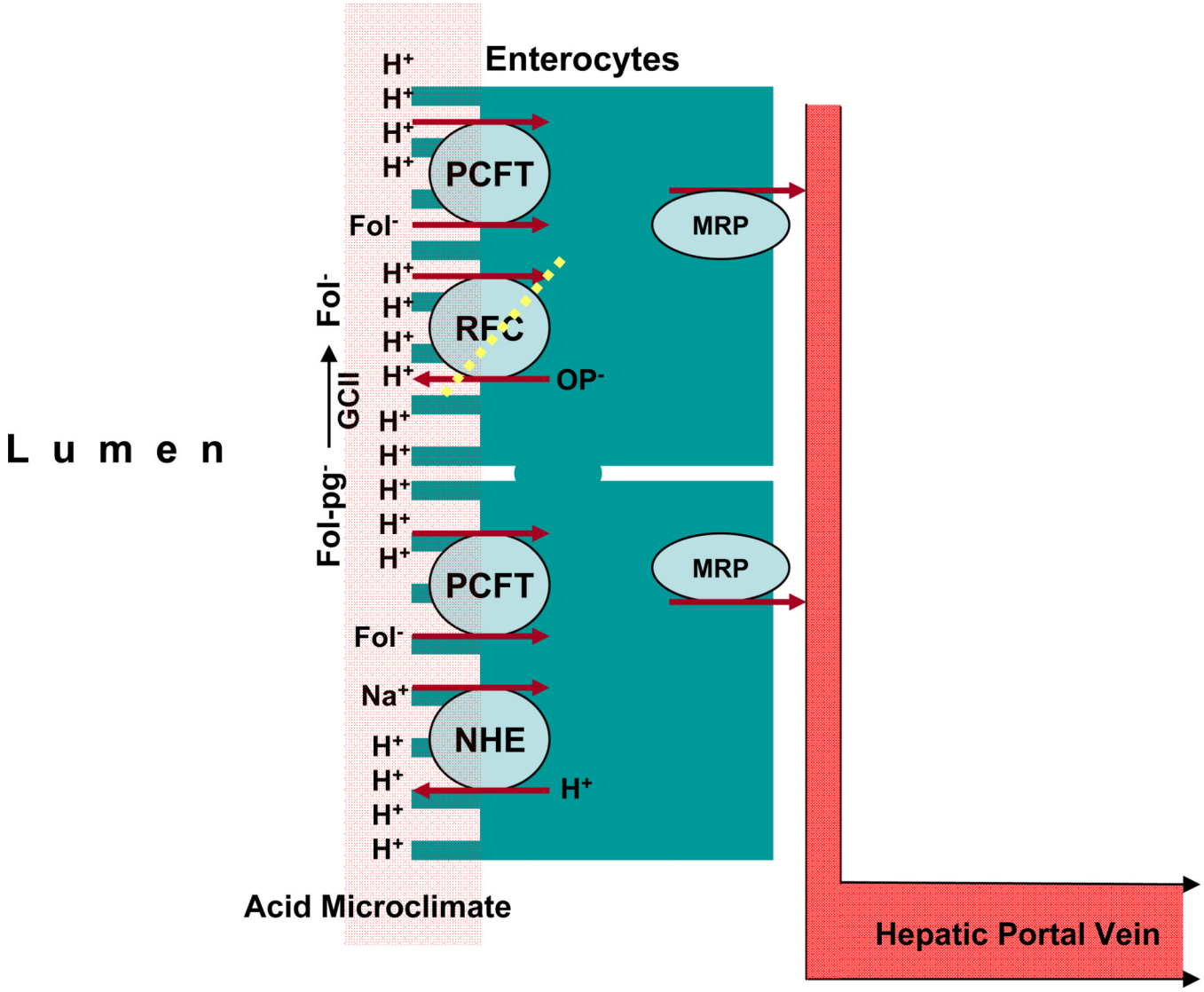

Figure 6.

Schematic of the sequence of events that occur in the proximal jejunum during the process of hydrolysis and absorption of dietary folate polyglutamates. Dietary 5-methylTHF polyglutamates (Fol-pg^{-}) are hydrolyzed to monoglutamate by glutamate carboxypeptidase II (GCII) and then transported into cells via PCFT; both reactions have a low pH optimum and are favored by the low pH within the microenvironment at the jejunal villi (indicated in pink) that is generated by NHE Na^+/H^+ exchangers. Cellular folates exit the basolateral membrane by an, as yet, unconfirmed mechanism likely related to a member(s) of the multidrug-associated protein (MRP) family of ABC cassette exporters.