Ann L. Jackman
Christopher P. Leamon *Editors*

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Preface

In 1999, a book entitled *Antifolate Drugs in Cancer Therapy* (Humana/Springer) focused on existing and emerging cancer drugs that inhibited folate-dependent enzymes. Several chapters in that volume provided evidence suggesting that the effectiveness and tolerability of antifolate therapy could be further increased by (a) understanding and exploiting some of the molecular determinants of drug sensitivity or (b) by reducing exposure of normal proliferating tissues to these agents. Now, a decade later, we can address the latter subject by reviewing the biological properties of a contemporary class of "targeted agents" that functionally exploit a tumor-associated folate transport protein called the folate receptor (FR).

The FR is a glycosylphosphatidyinositol-linked protein that captures its ligands from the extracellular milieu and transports them inside the cell via a nondestructive, recycling endosomal pathway. FRs have restricted expression in normal tissues, and they are not generally exposed to the bloodstream; however, elevated expression occurs in many human malignancies, especially when associated with aggressively growing cancers. These factors help define "FR targeting" as a viable tumor-targeting strategy. Agents that target the FR range in size from small molecule antifolate drugs and folate-drug conjugates to monoclonal antibodies and nanoparticles. In some cases, the agent need only bind to the FR to elicit a biochemical effect (e.g., diagnostic imaging or immunotherapy); in other cases, such as for high affinity antifolates and folate conjugates of small molecule therapeutics, internalization by the FR/endosomal apparatus and subsequent cytosolic delivery is required for biological activity against intracellular targets.

The discoveries highlighted in this book parallel the emergence of innovative "molecular targeted" small molecules and monoclonal antibodies, i.e., agents that target proteins within highly activated signal transduction pathways that control proliferation. However, many of the tumor-targeted strategies described within cross the boundaries between what is considered to be "molecular-targeted" vs. conventional systemic therapy. Obviously, for these novel agents to be effective, tumors must express a functional form of the FR. But in contrast to the targets of signaling inhibitors, tumor growth is not necessarily dependent on FR expression; rather, this cell surface receptor imparts key therapeutic specificity. Thus, while the

pharmacologic targets of FR-guided drugs and folate-drug conjugates are frequently those of conventional therapy, the selectivity realized through restricted tissue expression of the FR biomarker reduces the adverse effects against untargeted normal tissues. Regardless, both the cellular and molecular targeting approaches share the goal in shifting the paradigm from that of generalized chemotherapy to that of personalized medicine.

Beyond cancer research, FRs are also receiving attention from researchers of inflammatory disorders. Recent discoveries have shown that proinflammatory, activated human monocytes and macrophages express a functional FR isoform. Preclinical and clinical proof has already emerged showing how this marker can be used to identify sites of inflammation (e.g., arthritis) using folate-targeted radiodiagnostic imaging agents, and efforts for therapeutic exploitation are already underway (see Chaps. 9, 10). Clearly, it is only a matter of time before novel FR-targeted anti-inflammatory therapies reach clinical practice.

From a historical and complementary viewpoint, advances in our understanding of other folate transport proteins, such as the reduced folate carrier and the proton-coupled folate transporter, are also reviewed in this book (Chap. 1); however, the main theme of this volume is the FR, with much of the content focused on its basic biology and regulation (Chaps. 2, 3) as well as its exploitation for targeted therapy and diagnostic imaging (Chaps. 4–8). The contributors to this volume are all highly regarded in their fields, and we are very grateful to them for devoting so much time and effort into their excellent contributions. Both of us have benefited tremendously from reviewing their chapters, and we wish for their continued success.

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Chapter 1
Biological Role, Properties, and Therapeutic Applications of the Reduced Folate Carrier (RFC-SLC19A1) and the Proton-Coupled Folate Transporter (PCFT-SLC46A1)

Larry H. Matherly, Ndeye Diop-Bove, and I. David Goldman

Abstract The mechanisms by which folates are transported across cell membranes have been an area of research interest for nearly five decades. Major transport systems include the facilitative carriers, the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT), and the high affinity folate receptors (FRs) α and β which transport folates by endocytosis. RFC is the major transport system in mammalian cells and tissues for folate cofactors and clinically relevant antifolate drugs including methotrexate, raltitrexed, pemetrexed, and pralatrexate. PCFT was identified in 2006 as the mechanism by which folates are transported across the apical brush border of the proximal small intestine. Whereas both PCFT and RFC are widely expressed in tumors, PCFT differs from RFC in its acidic pH optimum which favors transport at the low pH commonly found in the hypoxic microenvironment of solid tumors. Reflecting tumor-specific patterns of expression and/or function, recent studies have focused on the identification of folate-targeted therapeutics with selective transport by PCFT and FRs over RFC. The goal is to circumvent RFC and the potentially toxic consequences of drug transport by RFC in normal tissues. RFC in tumor cells can also influence the pharmacologic activity of PCFT and FR-selective agents by transporting physiological folates which compete for polyglutamylation and binding to intracellular targets. This review focuses on the facilitative pathways of (anti)folate transport, including RFC (SLC19A1) and PCFT (SLC46A1) in relation to their molecular properties, and their physiological and pharmacological roles.

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Abbreviations

AICAR	5-Amino-4-imidazolecarboxamide ribonucleotide
AICARTase	5-Amino-4-imidazolecarboxamide ribonucleotide formyltransferase
ALL	Acute lymphoblastic leukemia
BCRP	Breast-cancer resistant protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
5-FormylTHF	5-Formyltetrahydrofolate
FR	Folate receptor
GARFTase	Glycinamide ribonucleotide formyltransferase
GlpT	Glycerol phosphate/inorganic phosphate antiporter
HFM	Hereditary folate malabsorption
LacY	Lactose/proton symporter
5-MethylTHF	5-Methyltetrahydrofolate
MFS	Major facilitator superfamily
MRP	Multidrug resistance-associated protein
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
OAT	Organic anion transporters
PCFT	Proton-coupled folate transporter
RFC	Reduced folate carrier
RTX	Raltitrexed
SCAM	Substituted cysteine accessibility methods
THF	Tetrahydrofolate
TMD	Transmembrane domain
UTR	Untranslated region

1.1 Introduction

The mechanisms by which folates are transported across cell membranes have been an area of research interest for nearly five decades. Folate cofactors as vitamins are available only from exogenous sources. Reflecting this, there has been a long-standing interest in the mechanism by which these compounds are absorbed in the small intestine (Halsted 1979; Selhub and Rosenberg 1981; Said 2004). Studies on transport of antifolates date from mid- to late 1960s when it was recognized that membrane transport of methotrexate (MTX) is carrier-mediated and is an important determinant of MTX chemotherapeutic activity, and that tumor cells commonly develop resistance to MTX due to an acquired defect in cellular uptake (Sirotnak et al. 1968; Goldman et al. 1968; Hakala 1965).

The first of the folate transporters to be understood at the kinetic and thermodynamic levels was the reduced folate carrier (RFC) (Matherly et al. 2007). Initially characterized in detail in the late 1960s (Goldman et al. 1968), it was nearly 30 years later that this transporter was cloned (Dixon et al. 1994) and its regulation and structure–function understood at the molecular level (Matherly et al. 2007; Matherly and Hou 2008; Zhao et al. 2009a). RFC is a major mode of transport of all the classical antifolate drugs used in the treatment of cancer (Matherly et al. 2007).

The second transport mechanism to be described was an endocytic process mediated by high affinity folate receptors (FRs). While the presence of "folate-binding proteins" was recognized in many tissues and secretions dating back to the 1960s, it was some years later when their role in folate delivery to cells was recognized and characterized (Antony 1992, 1996). This eventually led to the cloning of two endocytic proteins termed folate receptor α (FRα) and folate receptor β (FRβ) in the late 1980s (Elnakat and Ratnam 2004). The initial focus of FR research from a pharmacological perspective involved the role of these receptors in the delivery of MTX into tumor cells. However, this avenue of research proved to be unproductive because of the comparatively poor substrate activity of MTX for FRs, the ubiquitous presence of RFC in tissues and tumors, and the high rates of MTX transport by RFC relative to rates of FR-mediated endocytosis (Sierra et al. 1995; Spinella et al. 1995). However, what evolved over time was the concept of utilizing FRs highly expressed in certain tumor types to deliver a variety of structurally unrelated agents linked to folic acid for therapeutic and diagnostic purposes (Leamon 2008; Hilgenbrink and Low 2005; Salazar and Ratnam 2007) and, more recently, as a vehicle for the selective delivery of cytotoxic antifolates with very low affinities for RFC (Gibbs et al. 2005; Deng et al. 2008a, 2009; Theti et al. 2003; Wang et al. 2010) – a major theme of this book.

Most recently, a third folate transport system was discovered – the proton-coupled folate transporter (PCFT) (Qiu et al. 2006). PCFT is the mechanism by which folates are transported across the apical brush border of the proximal small intestine and operates optimally in an acid environment, a feature that distinguishes it from RFC. Indeed, the properties of PCFT are fully consistent with those previously reported for intestinal folate absorption and for transport of folates and antifolates at the low pH commonly found within the hypoxic microenvironments of human solid tumors (Helmlinger et al. 1997; Raghunand et al. 1999). Based on the latter, novel antifolates are being developed with specificity for PCFT over RFC (Wang et al. 2010; Kugel Desmoulin et al. 2010; Matherly and Gangjee 2011), so as to selectively target solid tumors while minimizing toxicity to normal tissues that express RFC and are exposed to a neutral pH. PCFT is also critical to the transport of folates and antifolates across the blood–choroid plexus barrier into the cerebrospinal fluid (CSF) and may contribute to folate/antifolate export from acidified endosomes during FR-mediated endocytosis (Zhao et al. 2009a, b; Zhao and Goldman 2007).

While the role of membrane transport in the antitumor activities of antifolate drugs has been extensively reviewed (Matherly et al. 2007; Assaraf 2007; Goldman and Matherly 1985; Chattopadhyay et al. 2007; Zhao and Goldman 2003; Goldman et al. 2010), the recent development of novel antifolates, designed for selective transport via FRs or PCFT over RFC (Gibbs et al. 2005; Deng et al. 2008a, 2009; Theti et al. 2003;

Wang et al. 2010; Kugel Desmoulin et al. 2010; Matherly and Gangjee 2011), makes it particularly timely to revisit the features of the parallel transport routes mediated by the endocytotic FRs vs. these facilitative carriers that exist in most tumor cells. In addition to its role in transporting many antifolates, facilitative transport by RFC is also important for physiological folates, thereby influencing pharmacologic activity of FR- or PCFT-targeted antifolates by regulating intracellular folate pools which, in turn, modulate formation of their antifolate polyglutamate derivatives and interactions with target enzymes. Likewise, for antifolate substrates of FRs or PCFT that also preserve RFC substrate activity, RFC provides a route of drug uptake into normal cells with potentially toxic consequences, as noted above. This chapter will focus on facilitative pathways of (anti)folate transport, including RFC (SLC19A1) cal and pharmacological roles. The biology of the FRs will be considered by Kamen (2011) in Chapter 2.

1.2 Role of Membrane Transport in Folate Homeostasis

Folates are a family of B9 vitamins that differ in oxidation of the pteridine ring, the nature of the one-carbon substituent at the N_5 and N_{10} positions, and the extent of γ glutamate conjugation. The major folate in the diet and in the blood of mammals is 5-methyl tetrahydrofolate (5-methylTHF). Within cells, this folate is, in part, oxidized to dihydrofolate during the synthesis of thymidylate and then fully reduced to tetrahydrofolate (THF) with the subsequent formation of a variety of THF cofactors. The biological importance of reduced folates derives from their roles in one-carbon transfers leading to thymidylate, purine nucleotides, serine, and methionine, and in supporting biological methylation reactions from *S*-adenosylmethionine encompassing both small molecules (e.g., phosphatidylethanolamine) and macromolecules (e.g., DNA, histones) (Stokstad 1990; Chiang et al. 1996). Glutamate conjugation, catalyzed by folylpoly-γ-glutamyl synthetase, confers enhanced cellular retention, as folate polyglutamates are poor substrates for folate transporters, and increased rates of one-carbon transfer over monoglutamyl folates, since these derivatives are preferred substrates for folate-requiring enzymes (Shane 1989; Schirch and Strong 1989). Mammals cannot synthesize folates de novo. Hence, to achieve intracellular folate levels sufficient to meet one-carbon biosynthetic needs requires adequate folate intestinal absorption followed by uptake into systemic cells and transport across epithelial barriers into tissue compartments such as the central nervous system (CNS).

Major transport systems for folate uptake include the facilitative carriers, RFC and PCFT, that are widely expressed (Qiu et al. 2006; Zhao et al. 2009a; Whetstine et al. 2002a) but exhibit disparate pH optima (Matherly et al. 2007; Zhao et al. 2009a; Zhao and Goldman 2007; Wang et al. 2004). Other uptake systems include FRα and FRβ, which mediate uptake of folates by endocytosis (Elnakat and Ratnam 2004; Salazar and Ratnam 2007), and the organic anion transporters (OATs, OATPs) that are expressed in epithelial tissues (e.g., kidney, intestine) and transport a broad spectrum of organic ions (e.g., probenecid, bromosulfophthalein) in addition to folates (Rizwan and Burckhardt 2007; Shibayama et al. 2006; Matherly and

Goldman 2003; Masuda 2003). ABC-cassette transporters such as multidrug resistance-associated proteins (MRPs) 1–5 and 8, and ABCG2 (breast cancer resistant protein [BCRP]) also transport folate substrates (Assaraf 2007; Kruh and Belinsky 2003; Kruh et al. 2007), thus exerting opposing effects on the concentrative uptake of these compounds. The impact of these efflux pumps on net transport becomes clear in the presence of energy inhibitors or competitive substrates which result in marked increases in transmembrane gradients for (anti)folate substrates (Hakala 1965; Goldman 1969).

Dietary folates are absorbed in the duodenum and proximal jejunum mediated by PCFT within an acid microenvironment (pH 5.8–6.0) at the cell surface (Zhao et al. 2009a). The critical role that PCFT plays in this process was established by the severe systemic folate deficiency that occurs in patients with hereditary folate malabsorption (HFM) who are null for this transporter (Zhao et al. 2007, 2009a; Qiu et al. 2006; Lasry et al. 2008; Min et al. 2008; Shin et al. 2010, 2011). Although RFC is expressed at the apical brush-border membrane along the entire intestine (Wang et al. 2001), RFC does not likely contribute to folate absorption under physiological conditions, even in intestinal segments in which the pH is more favorable to its function such as the distal small intestine. RFC may, however, contribute to folate absorption when pharmacological doses of folate are administered orally to subjects with HFM (Zhao et al. 2009a). Transport of folates across the basolateral membrane of the jejunum appears to be mediated in part by MRP3 (Kitamura et al. 2008). Hence, in this case, PCFT and MRP3 act in concert to achieve vectorial transport (absorption) across the intestinal epithelium. Folates absorbed in the intestine are delivered to the liver by the hepatic portal vein where both RFC and PCFT are expressed at the sinusoidal membrane (Wang et al. 2001; Horne 1990; Horne and Reed 1992); the pH at this interface would determine the extent to which each transporter contributes to folate uptake into hepatocytes. Folate secretion into the bile at the canalicular membrane is mediated by MRP2 such that in MRP2-null animals, there is a marked defect in the elimination of MTX via the bile (Masuda et al. 1997).

Folates are filtered at the glomerulus and then reabsorbed in the proximal renal tubule. FRα, along with PCFT, is expressed at the apical brush-border membrane and RFC at the basolateral membrane of the proximal renal tubule (Zhao et al. 2009a). In addition, a variety of OATs with much lower specificities for folates may contribute to folate reabsorption in the proximal tubule. These include OATP1 at the apical brush-border membrane and OAT1 and OAT3 at the basolateral membrane (Rizwan and Burckhardt 2007; Masuda 2003; Russel et al. 2002).

Folates are the only, or one of the very few, substrates that are concentrated in the CSF (Geller et al. 2002). To account for this requires active folate transport across the choroid plexus. FRα, RFC, and PCFT are all expressed at the choroid plexus. FRα is expressed primarily at the apical brush-border membrane and to a much lesser extent at the basolateral membrane (Kennedy et al. 2003; Weitman et al. 1992a, b; Selhub and Franklin 1984; Patrick et al. 1997). RFC is expressed at the apical membrane (Wang et al. 2001) and PCFT is expressed at the basolateral membrane (Zhao et al. 2009b). It is now clear that both PCFT and FRα are required for the delivery of folates into the CSF. In HFM, folate is usually undetectable in the CSF and remains quite low even when the folate blood level is normalized

(Geller et al. 2002; Mahadeo et al. 2010a). This abnormality is detected shortly after birth in early infancy. A recent report established that the FRα-null phenotype in humans is also associated with very low CSF folate levels and a clinical syndrome of cerebral folate deficiency (Steinfeld et al. 2009). The mechanism by which PCFT contributes to folate transport into the CSF is unclear. PCFT may contribute to export of folates from endosomes during FRα-mediated endocytosis (Zhao et al. 2009b). Sodium/proton exchangers present at the basolateral membrane of choroid plexus ependymal cells may produce an acidic local microenvironment that provides the driving force for PCFT-mediated transport at this site (Segal 2000).

In Sects. 1.3 and 1.4, we consider the structure, function, and regulation of RFC and PCFT as a prelude to understanding their roles as determinants of antifolate drug response and resistance in cancer. In Sect. 1.5, we examine the roles of these physiologically important facilitative transporters in antifolate chemotherapy, in general, and in relation to applications of folate-based therapeutics with tumor targeting via FRs and PCFT.

1.3 Reduced Folate Carrier

1.3.1 RFC Functional and Structural Characteristics

Properties of RFC have been characterized in a wide assortment of (mostly tumor) cell culture models (both rodent and human). Transport by RFC is temperature dependent, sodium independent, and is characterized by a neutral pH optimum (Matherly et al. 2007).

RFC substrates are structurally diverse with modifications of the ring systems including aromaticity and/or substituents, along with the presence or absence of heteroatoms, the length and composition of the bridge linker between the rings, and replacement of the terminal L-glutamate (Jansen 1999; Westerhof et al. 1995) (Fig. 1.1). The major circulating folate form, 5-methylTHF, is an excellent RFC

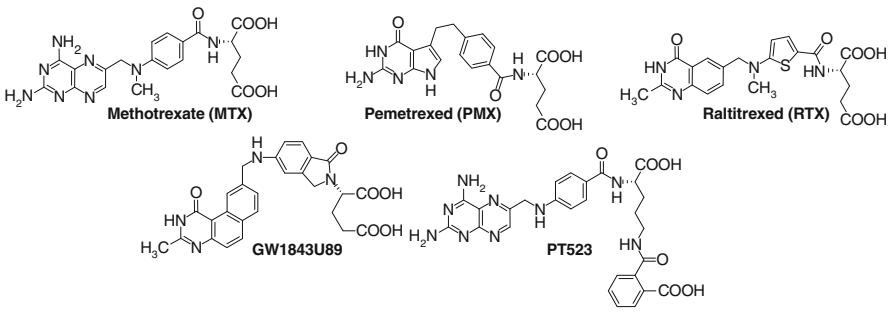

Fig. 1.1 Antifolate structures

substrate as is the active (6S)5-formyl tetrahydrofolate (5-formylTHF) isomer in pharmacologic formulations of folate (i.e., (6R,*S*)5-formylTHF or leucovorin). Transport by RFC is not stereospecific for 5-methylTHF (White et al. 1978), in contrast to 5-formylTHF for which the (6S) stereoisomer is preferred over the (6R) form (Sirotnak et al. 1979). Classical antifolates such as MTX, pemetrexed, and raltitrexed (RTX) (Fig. 1.1) are all RFC substrates (Matherly et al. 2007). These reduced folate and antifolate substrates show saturability at low micromolar concentrations. By contrast, the synthetic form, folic acid, has been generally reported as a poor RFC substrate ($K_t > 200$ μM), representing a distinguishing feature between RFC and FRs or PCFT that have high affinity for folic acid. The benzoquinazoline antifolate GW1843U89 (Smith et al. 1999) and the hemiphthaloylornithine antifolate PT523 (Rosowsky 1999) (Fig. 1.1) are the best RFC substrates known with binding affinities (K_t and K_i) in the submicromolar range and a complete lack of substrate activity with PCFT (Deng et al. 2009; Zhao and Goldman 2007). Pralatrexate (10-propargyl-10-deazaaminopterin) (Sirotnak et al. 1987) was recently approved for treatment of relapsed or refractory peripheral T-cell lymphoma (Thompson 2009), based on potent antitumor effects, reflecting efficient transport by RFC, high affinity for folylpolyglutamate synthetase, resulting in rapid and extensive metabolism to its polyglutamate derivatives.

The most consistent structural feature of RFC substrates relates to their anionic character. Folates are negatively charged at physiologic pH, resulting from ionized α and γ carboxyl groups. Some modifications of the glutamate moiety (e.g., 2-amino-4-phosphonobutanoic acid, L-homocysteic acid, ornithine) are not conducive to RFC binding and transport (Westerhof et al. 1995). Likewise, ICI198583-γ-D-glutamate is a poor transport substrate for RFC, in contrast to the L-isomer (Westerhof et al. 1995). Conversely, modifications of the glutamate-γ-carboxyl (e.g., valine, 2-aminosuberate) are surprisingly well tolerated and both ZD9331 and PT523 are excellent RFC substrates (Jansen 1999; Westerhof et al. 1995). For diaminofuro[2,3-*d*]pyrimidine antifolates with substituted α or γ carboxyl groups, analogs with a single α but no γ carboxyl group bind avidly to RFC, whereas analogs with a single γ but no α carboxyl, or without both α and γ carboxyl groups, do not bind appreciably to RFC (Deng et al. 2008b). Collectively, these results imply that only the α carboxyl group of folate substrates is essential for substrate binding and transport by RFC.

Although RFC generates only small transmembrane chemical gradients, when considered in light of the dianionic character of folates and the membrane potential, RFC produces substantial electrochemical–potential differences across cell membranes. Cellular uptake of folates by RFC is not directly linked to hydrolysis of ATP, nor is it sodium or proton dependent (Henderson and Zevely 1983; Goldman 1971). Rather, the driving force for concentrative uptake of folates appears to involve large gradients for organic phosphates across cell membranes which inhibit folate export via RFC, resulting in uphill folate transport into cells (Goldman 1971). Consistent with this model are findings that phosphorylated derivatives of thiamine are good RFC substrates. Their presence

in cells inhibits MTX export and their efflux is enhanced in cells with elevated RFC levels (Zhao et al. 2001, 2002).

RFC is a member of the major facilitator superfamily (MFS) of proteins comprising of more than 2,000 sequenced members including transporters of amino acids, sugars, vitamins, nucleosides, and organic phosphates, along with neurotransmitters (Matherly et al. 2007; Saier et al. 1999). By computer hydropathy analysis based on the predicted amino acid sequence from cloned RFC cDNAs from various species (Matherly et al. 2007; Matherly and Hou 2008), the carrier conforms to a structure typical of MFS proteins including two bundles of six transmembrane domains (TMDs) connected by a large loop domain between TMDs 6 and 7 and internally oriented N- and C-termini (Fig. 1.2). Much of this topology has been experimentally confirmed by hemagglutinin epitope insertion and cysteine-scanning mutagenesis and accessibility studies (Ferguson and Flintoff 1999; Flintoff et al. 2003; Cao and Matherly 2004; Liu and Matherly 2002). Glycosylation of the single N-glycosylation consensus site at asparagine 58 in human RFC establishes the TMDs 2–3 connecting loop as extracellular (Liu and Matherly 2002). On SDS gels, human RFC appears as a broadly migrating high molecular weight (~85 kDa) species, which shifts to 65 kDa upon treatment with N-glycosidase F (Wong et al. 1998, 1999). Mutation of Asn58 to Gln, abolishing N-glycosylation at this position, has only a nominal effect on surface targeting or membrane transport of human RFC (Wong et al. 1998). There is 64–66% conservation of amino acid sequence between human and rodent RFCs, with somewhat higher homology in TMDs 1–5, 7, and 8, lower homology for TMDs 6 and 9–12, and several of the connecting loops (Matherly and Hou 2008). Both N- and C-termini exhibit low homology. The RFC C-terminus in primates is 50–86 residues longer than that of other species.

RFC structure and function have been studied extensively using state-of-the-art molecular and biochemical techniques for polytopic membrane proteins (Matherly and Hou 2008). Deletions of the N- and C-termini of RFC from hamsters and humans had only minor impact on membrane targeting and transport activity (Sadlish et al. 2002a; Sharina et al. 2002). Deletions of 49 or 60 amino acids of the connecting loop between TMDs 6 and 7 of human RFC abolished activity, whereas replacement of the deleted segments with the nonhomologous loop from the MFS protein SLC19A2 restored transport (Liu et al. 2003). Human RFC was reconstituted in cells from coexpressed TMDs 1–6 and 7–12 RFC half-molecules which co-fold and traffic to the cell surface to restore transport with characteristic properties ranging from kinetics to capacity for *trans*-stimulation (Witt et al. 2004). Thus, neither the N- or C-termini, nor the TMDs 6–7 loop domain appears to directly participate in substrate binding and translocation of folate substrates. Rather, the primary role of the connecting loop between TMDs 6 and 7 is to provide the requisite spacing between two 6 TMD segments.

By exhaustive cysteine-scanning mutagenesis and substituted cysteine accessibility methods (SCAM) of a cysteine-less human RFC, TMDs 4, 5, 7, 8, 10, and 11 were identified as forming the membrane translocation pathway for anionic folates (Hou et al. 2005, 2006). Of 282 Cys substitutions, only ten were inactivating

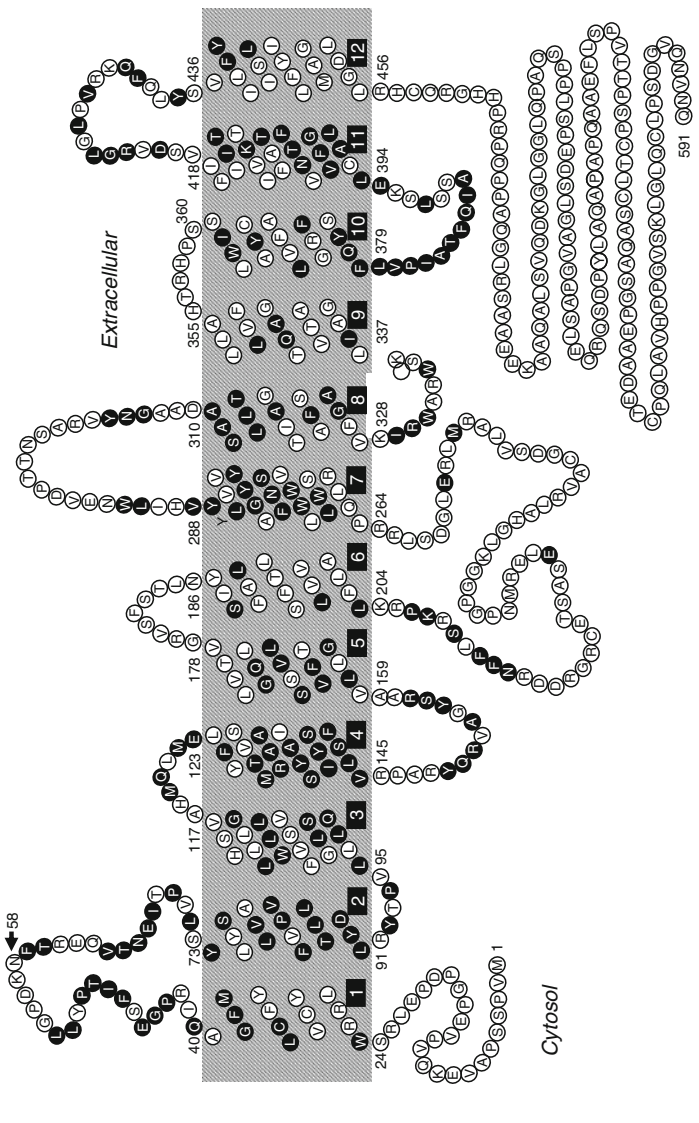

Fig. 1.2 Topology model for human RFC monomer showing conserved residues. A topology model for human RFC, depicting the 12 TMDs, internally oriented N- and C-termini, the N-glycosylation site at Asn-58 and a cytosolic loop connecting TMDs 6 and 7. Amino acids conserved between RFCs from different species including *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Gallus gallus* (chicken), *Danio rerio* (zebrafish), *Bos taurus* (cow), *Rattus norvegicus* (Norway rat), *Cricetulus griseus* (Chinese hamster), *Mus musculus* (mouse), and *Xenopus laevis* (African clawed frog) (Matherly and Hou 2008) are depicted as *black circles*

including a stretch in TMD 4 (Arg133, Ile134, Ala135, Tyr136, Ser138), Tyr281 in TMD 7, Ser313 in TMD 8, and Arg373 in TMD 10, suggesting structural or functional importance (Hou et al. 2005, 2006). Arg133, Arg373, and Ser313 were previously identified as possibly mechanistically important from mutant studies (Liu and Matherly 2001; Sadlish et al. 2002b; Zhao et al. 1999; Sharina et al. 2001). While the γ carboxyl group of folate substrates was not essential for substrate binding to RFC, by N-hydroxysuccinimide [^{3}H]MTX radioaffinity labeling of human RFC, Lys411 was nonetheless found to bind this region (Deng et al. 2008b). From biochemical data for RFC, and solved structures for the bacterial MFS proteins, lactose/proton symporter (LacY) (Abramson et al. 2003) and glycerol-3-phosphate/inorganic phosphate antiporters (GlpTs) (Huang et al. 2003), a three-dimensional homology model for the 591 amino acid human RFC was generated including a membrane translocation pathway comprised of TMDs 1, 2, 4, 5, 7, 8, 10, and 11, and functionally important roles for Ser281, Ser313, and Arg373 (Hou et al. 2006).

1.3.2 RFC Gene Structure and Regulation of RFC Expression and Function

RFCs from humans and rodents are subject to elaborate regulation involving both transcriptional and posttranscriptional mechanisms (Matherly et al. 2007). The human RFC gene maps to chromosome 21q22.2 (Moscow et al. 1995). The gene includes five major coding exons with conserved intron–exon boundaries and up to six alternative noncoding regions and promoters (designated A1/A2, A, B, C, D, and E) (Matherly et al. 2007; Whetstine et al. 2002a; Flatley et al. 2004). A, B, C, D, and E represent noncoding exons, whereas the A1/A2 noncoding sequence is fused to the first coding exon. Promoter activity was confirmed for the 5′ regions proximal to five of the noncoding regions (A1/A2, A, B, C, and D) and for four of these, both tissue-specific (e.g., Ap2, C/EBp, Ikaros) and ubiquitously expressed (e.g., SP, USF) transcription factors and *cis* elements were identified (Matherly et al. 2007; Flatley et al. 2004; Whetstine and Matherly 2001; Whetstine et al. 2002b; Liu et al. 2004; Payton et al. 2005a, b). Thus, net RFC levels achieved in tissues are likely the combined result of levels and post-transcriptional modifications of these factors that determine the transcriptional activities of the multiple RFC promoters. This may be impacted by promoter polymorphisms (see below) and CpG methylation (Worm et al. 2001), as well as by chromatin remodeling.

The upstream noncoding exons for the human RFC gene are alternately spliced to generate heterogenous transcripts comprising of (up to 15) unique untranslated regions (UTRs) linked to a common RFC coding sequence (Matherly et al. 2007; Whetstine et al. 2002a). RFC 5′ UTR diversity results in differences in 5′ CAP-dependent translation and transcript stabilities (Matherly et al. 2007; Payton et al. 2007).

For two 5′ UTRs (A1/A2 and A), upstream AUGs exist in-frame with the RFC coding sequence and result in N-terminally modified RFC proteins with 62 and 22 additional N-terminal amino acids, respectively (Flatley et al. 2004; Payton et al. 2007). However, the biological significance of these N-terminally modified RFC proteins is not well established. While human RFC transcripts and transport are reported to decrease in breast cancer and T-cell acute lymphoblastic leukemia (ALL) cell lines with folate deprivation (Ifergan et al. 2008), it is not clear whether this effect is transcriptional or posttranscriptional.

The human RFC gene is polymorphic and includes high frequency polymorphisms involving nucleotide substitutions, deletions, and insertions in the RFC coding region (G80A, results in R27H in TMD 1), the A1/A2 promoter and noncoding region, and promoter A (Matherly et al. 2007; Flatley et al. 2004; Whetstine et al. 2001, 2002b). While the functional impact and broader health significance of these polymorphisms remain uncertain or even controversial, the 61 bp repeat polymorphism in promoter A is associated with increased promoter activity in reporter assays (Whetstine et al. 2002b). As noted above, transcript variants for human RFC were identified including a CATG insertion at position 191 that generates a frame shift and early translational stop at position 1176 in an MTX-resistant ALL cell line and primary ALL specimens (Wong et al. 1999; Whetstine et al. 2001). Additional human RFC transcript splice variants were reported, involving a 625 bp deletion from exon 7 (positions 1569–2193) and a 988 bp deletion (positions 1294–2281) including all of TMD 12 (Wong et al. 1995; Zhang et al. 1998a; Drori et al. 2000). The former encoded a variant RFC (Wong et al. 1995) that was competent for transport whereas the latter encoded an inactive protein that nonetheless appeared to modulate wild-type RFC activity (Drori et al. 2000).

Although posttranslational regulatory mechanisms involving RFC have been implied including RFC phosphorylation (Kumar et al. 1997), this has not been confirmed. Studies have shown that 5-amino-4-imidazolecarboxamide ribonucleoside, a precursor of 5-amino-4-imidazole carboxamide ribonucleotide (ZMP), potentiates uptake of MTX and 5-formyl THF by CCRF-CEM ALL cells, presumably by RFC (McGuire et al. 2006). The mechanism is unclear.

1.3.3 Human RFC is a Homo-oligomer

While considerations of RFC structure and mechanism have generally focused on RFC monomeric structures, human RFC was recently identified as a homo-oligomer (Hou and Matherly 2009). Thus, (a) crosslinking RFC with a homobifunctional crosslinker resulted in higher order complexes with molecular masses approximating those of dimers, trimers, and tetramers. (b) When coexpressed in RFC-null cells, RFC proteins with different epitope tags (Myc and hemagglutinin) were coimmunoprecipitated with epitope-specific antibodies. (c) In coexpression experiments between wild-type and inactive mutant RFC, a dominant-negative phenotype was

demonstrated involving substantially decreased cell surface RFC of both wild-type and mutant carrier due to profoundly impaired cellular trafficking.

Most recently, the operational significance of human RFC oligomerization and the "minimal functional unit" for transport were studied by negative-dominance experiments in which multimeric transporters composed of different ratios of active and inactive RFC monomers were coexpressed and by expressing covalent RFC dimers composed of active and inactive RFC monomers (Hou et al. 2010). The results strongly support the notion that each RFC monomer comprises a single translocation pathway for anionic folate substrates and functions independently of other monomers. Hence, in spite of an oligomeric structure, human RFC functions as a monomer.

Additional studies are clearly warranted to establish the broader mechanistic and/or regulatory features of RFC oligomerization, including the possibility that RFC oligomerization may have therapeutic implications. Oligomerization can regulate RFC trafficking from the endoplasmic reticulum to the cell surface (Hou et al. 2010) and thus may contribute to antifolate resistance in tumors expressing wild-type and mutant RFCs. Oligomerization may also have regulatory significance as a means of acutely responding to levels of extracellular folates via effects on intracellular trafficking. Whereas no unique biological roles for the Arg27His substitution resulting from the G80A polymorphism in human RFC (Matherly et al. 2007) or N-terminally modified human RFC proteins (Flatley et al. 2004; Payton et al. 2007) have been established (see above), the possibility that these modifications may impact RFC function via effects on carrier oligomerization is not unreasonable. Likewise, no obvious biological significance has been ascribed to naturally occurring human RFC transcript variants (Wong et al. 1995; Zhang et al. 1998a; Drori et al. 2000), although their encoded proteins can be envisaged to act as dominant-negative inhibitors via oligomerization with wild-type RFC, resulting in decreased levels of surface wild-type RFC protein.

1.4 Proton-Coupled Folate Transporter

1.4.1 Identification of the Molecular Entity Responsible for Low-pH Transport in Mammalian Cells

RFC-mediated transport is a process with optimal activity at neutral pH (Matherly et al. 2007). Yet, an unexplained folate transport activity optimal at low pH had been recognized for decades. This was a characteristic of folate absorption in the small intestine and was noted for folate and antifolate transport into a variety of human (Selhub and Rosenberg 1981; Zhao et al. 2009a; Vincent et al. 1985; Mason et al. 1990; Mason and Rosenberg 1994; Horne et al. 1993; Zhao et al. 2004a), rat (Rajgopal et al. 2001; Said et al. 1997), and hamster (Assaraf et al. 1998) cell lines. Indeed, a modest low-pH transport

activity was observed in murine L1210 leukemia cells that had long been an invaluable model for studying the transport properties of RFC (Sierra et al. 1997; Henderson and Strauss 1990). For the lack of a better explanation, the low-pH transport activity was attributed to functionally distinct alternatively spliced or translated RFC forms (Kumar et al. 1998; Chiao et al. 1997). However, no such species was identified that could account for this activity. With time, evidence accumulated indicating that the low-pH folate transport activity must be RFC independent. Most convincing were studies demonstrating that this activity was fully preserved even in the complete absence of genomic RFC (Zhao et al. 2004a), in cell lines in which there were profound loss-of-function mutations of RFC (Chattopadhyay et al. 2006; Wang et al. 2005) or when the RFC gene was silenced (Zhao et al. 2005). Ultimately, this conundrum was resolved with the cloning of PCFT, designated as SLC46A1 in the solute carrier group of integral membrane transport proteins. PCFT was identified using a data mining cloning strategy in which genes with very low homology to RFC across species were identified and then screened by assessing their expression in two HeLa cell lines, both of which lacked genomic RFC, only one of which expressed the low-pH transport activity (Qiu et al. 2006). The human PCFT gene is located on chromosome 17q11.2 and consists of five exons and encodes 459 amino acids (Fig. 1.3). The human protein shares 91% similarity and 87% identity to both the mouse and rat proteins.

1.4.2 A Comparison of the pH Dependence of PCFT-Mediated and RFC-Mediated Transport

Figure 1.4 illustrates the pH profiles of tritiated MTX influx in HeLa cells that lack endogenous transporters and were stably transfected with either RFC or PCFT to achieve levels of expression comparable to those in wild-type HeLa cells. The pH profiles of these transporters are quite distinct. There is little RFC activity below pH 6.5, although a shoulder of residual activity is consistently observed at low pH (Wang et al. 2004). There is little PCFT activity above pH 7.0 when MTX is the transport substrate. The decline in RFC activity as the pH is *reduced* is due almost entirely to a decrease in influx V_{max}; there is a minimal change in influx K_m over a pH range of 7.4–5.5 (Wang et al. 2004). On the other hand, the decline in PCFT transport activity as the pH is *increased* is due to both an increase in influx K_m and decreased influx V_{max} (Qiu et al. 2006). Notably, changes in PCFT-mediated transport with pH depend on the transport substrate. For instance, while these changes are marked for MTX and folic acid, they are more modest for PCFT-mediated pemetrexed transport so that sufficient delivery of this drug is achieved at neutral pH to maintain its activity even in the absence of RFC (Zhao et al. 2008). Similar findings of pH-dependent binding and substrate specificity were recently reported for novel pyrrolo[2,3-*d*]pyrimidine antifolate substrates for human PCFT (Wang et al. 2010; Kugel Desmoulin et al. 2010).

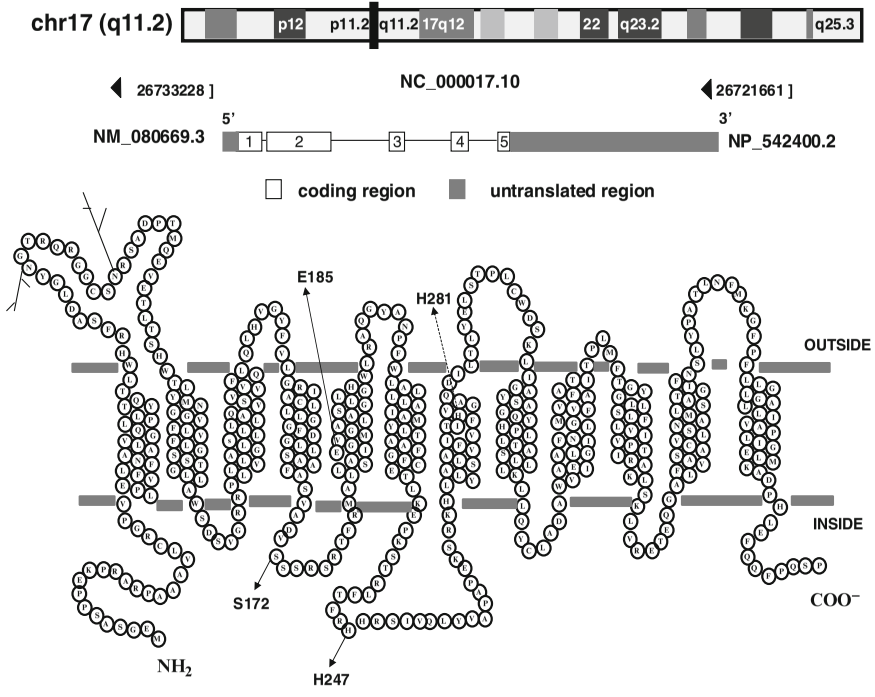

Fig. 1.3 Human PCFT genomic organization, predicted secondary structure, and functionally important residues. Human PCFT is located on chromosome 17q11.2 (UCSC genome browser, *top panel*) and consists of five exons. Exons 1–5 are shown in the *middle panel*. NM_080669.3 and NP_542400.3 are NCBI accession numbers for the mRNA and the protein sequence for human PCFT, respectively. NC_000017.10 represents the genomic sequence. A two-dimensional predicted topology model of human PCFT is shown at the *bottom* of the figure. Twelve TMDs are shown with the N- and C-termini located intracellularly. There are two confirmed N-linked glycosylation sites on the first extracellular loop at asparagines 58 and 68 (Unal et al. 2008). *Arrows* indicate residues that play an important role in function as described in the text