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Hereditary folate malabsorption: A positively charged amino acid at position 113 of the proton-coupled folate transporter (PCFT/SLC46A1) is required for folic acid binding

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ABSTRACT

The proton-coupled folate transporter (PCFT/SLC46A1) mediates intestinal folate uptake at acidic pH. Some loss of folic acid (FA) transport mutations in PCFT from hereditary folate malabsorption (HFM) patients cluster in R113, thereby suggesting a functional role for this residue. Herein, unlike non-conservative substitutions, an R113H mutant displayed 80-fold increase in the FA transport Km while retaining parental Vmax, hence indicating a major fall in folate substrate affinity. Furthermore, consistent with the preservation of 9% of parental transport activity, R113H transfectants displayed a substantial decrease in the FA growth requirement relative to mock transfectants. Homology modeling based on the crystal structures of the *Escherichia coli* transporter homologues EmrD and glycerol-3-phosphate transporter revealed that the R113H rotamer properly protrudes into the cytoplasmic face of the minor cleft normally occupied by R113. These findings constitute the first demonstration that a basic amino acid at position 113 is required for folate substrate binding.

Introduction

A novel route of folate uptake with optimal transport activity at acidic pH has been recently identified and termed proton-coupled folate transporter (PCFT/SLC46A1) [1]. Functioning optimally at low pH (5.5), PCFT recognizes folic acid (FA), reduced folates and methotrexate (MTX) with comparable high affinities (Km = 1–5 μM) [2–5]. PCFT plays a key role in the absorption of folates within the acidic microenvironment of the small intestine. Consistent with the important role that PCFT plays in intestinal folate absorption, the Goldman group recently showed that loss-of-function mutations in the PCFT gene constitute the molecular basis of hereditary folate malabsorption (HFM; OMIM 229050) [1,6]; these findings were recently corroborated in a study from our laboratory with an independent HFM patient [7]. HFM is a rare autosomal recessive disorder caused by impaired intestinal folate absorption with folate deficiency characterized by anemia, hypoimmunoglobulinemia, and recurrent infections. Importantly, upon early diagnosis of HFM, the signs and symptoms can be obviated by high oral doses of folates.

Recently, two loss-of-function mutations in the PCFT gene from HFM patients mapped to a highly conserved R113 residue [6,7], thereby suggesting a functional role for this cationic amino acid [7]. Herein, non-conservative R113 substitutions resulted in the loss of FA influx at pH 5.5. In contrast, a conservative R113H mutant was properly sorted out to the plasma membrane and displayed a dramatic increase in the FA transport Km, while fully retaining the wt transport Vmax. These results suggested a major fall in substrate affinity while preserving carrier mobility. Hence, these findings establish for the first time that a positive charge in residue 113 contributes to folate substrate binding by PCFT.

Materials and methods

Biochemicals and antibodies. FA was purchased from Sigma–Aldrich Chemie BV (Zwijndrecht, the Netherlands). Anti-Myc monoclonal antibodies were a generous gift from Prof. Ami Aronheim (Rappaport Faculty of Medicine, Technion, Haifa, Israel).

Tissue cultures. Cells were grown under monolayer conditions in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin (Biological Industries, Beth-Haemek, Is-

Abbreviations: HFM, hereditary folate malabsorption; PCFT, proton-coupled folate transporter; FA, folic acid; MTX, methotrexate

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rael) in a humidified atmosphere of 5% CO_2. The growth medium of transfectant cells also contained 500 μg/ml G-418 (Calbiochem, San Diego,CA) .

Site-directed mutagenesis. The PCFT mutations R113A, R113D, R113H, R113K, and R113C were introduced into a C-terminally Myc-tagged wt (R113) PCFT construct [7] using Pfu Turbo DNA polymerase and a QuickChange kit according to the instructions of the manufacturer (Stratagene, La Jolla, CA).

Establishment of stable Myc-tagged PCFT transfectants. RFC transport null Chinese hamster ovary (CHO) C5 $MTX^{R0.15}$ cells (2×10^7) [8] were trypsinized and then transfected by electroporation (1000 μF, 234 V) with 10 μg DNA of the following constructs: pCDNA 3.1(−) (Invitrogene) containing C-terminally Myc-tagged wt PCFT, each of the various R113 PCFT mutants as well as the empty vector (mock) in a final volume of 0.4 ml of serum-free RPMI-1640 medium containing 10 mM glucose and 0.1 mM DTT. Cells were then suspended in 10 ml of complete RPMI-1640 medium at 37 °C, allowed to recover for 48 h and grown in medium containing 500 μg/ml G-418.

Assay of [3H]*Folic acid influx and transport kinetics.* See Supplemental Materials and methods.

Folate growth requirement. FA growth requirement was determined as previously described [7] with some modifications as detailed in Fig. 3 legend. EC_{50} is defined as the FA concentration necessary to produce 50% of maximal cell growth.

Bioinformatics methodology. See Supplemental Materials and methods.

Statistical analysis. We used a paired student's *t*-test to examine the significance of the difference between two populations for a certain variable. A difference between the averages of two populations was considered significant if the *P*-value obtained was <0.05.

Results

Subcellular localization of various R113 PCFT mutants in stable transfectants

We first explored the subcellular trafficking of the C-terminally Myc-tagged wt PCFT and the various site-directed R113 substitutions including R113H, R113K, R113C, R113A, R113D, and empty vector (mock) stably transfected into RFC-null CHO cells (Supplemental data, Fig. 1). Immunofluorescence microscopy with the wt R113 as well as the R113H, R113K, and R113C mutants revealed plasma membrane targeting, albeit, some cytoplasmic localization was detectable. In contrast, cytoplasmic retention of the R113A and R113D mutant PCFTs was observed and no plasma membrane localization was seen with these mutants. Mock transfectants and wt PCFT transfectants incubated only with the secondary anti-mouse antibody revealed background fluorescence. Real-time PCR analysis confirmed that the wt- and the various R113 PCFT mutant transfectants expressed comparable transcript levels which were significantly different from the background signal obtained with mock transfectants (Supplemental data, Fig. 2).

Folic acid transport in the wt R113 and the various R113 mutant transfectants

To explore folate transport in the various R113 mutants, initial rates of [3H]FA uptake were determined at pH 5.5 (Fig. 1). The low basal [^{3}H]FA influx in the R113A, R113D, and R113C PCFT transfectants was indistinguishable from the background transport levels obtained with mock transfectants (0.49–0.6 pmol/10^7 cells/min, respectively; Fig. 1A). In contrast, retention of a positive charge in the conservative R113K and R113H mutants resulted in the preservation of 5% and 9%, respectively, of [^{3}H]FA influx, relative to the wt PCFT transfectant, thereby achieving statistical significance when compared to the basal transport rates obtained with mock transfectants. We next determined [^{3}H]FA influx at pH 7.3 (Fig. 1B); the wt PCFT displayed a very low [^{3}H]FA influx of only 0.15 ± 0.06 pmol/10^7 cells/min at neutral pH, hence being 94-fold lower than the FA uptake rate at pH 5.5 (Fig. 1A). When compared to the wt PCFT, the conservative R113K and R113H mutants preserved 45% and 53% of wt PCFT [^{3}H]FA influx at pH 7.3, respectively (Fig. 1B). In contrast, the non-conservative mutants displayed very low transport rates that were indistinguishable from mock transfectants. Hence, based on the preservation of a substantial FA uptake at acidic pH for the R113H mutant, a transport kinetic study was undertaken and data analyzed using both Eadie-Hofstee (Fig. 2) and Lineweaver-Burk plots (Fig. 2, *inset*). Whereas the wt PCFT displayed a low FA transport Km of 0.28 ± 0.05 μM, the mutant R113H exhibited a dramatic increase of 80-fold in the transport Km (i.e. Km = 22.5 ± 7.2 μM; Fig. 2). In contrast, the mutant R113H displayed a FA transport Vmax (12.9 ± 5.1 pmol/10^7 cells/min) which was essentially identical to that obtained with the wt R113 PCFT (Vmax = 11.4 ± 2.6 pmol/10^7 cells/min). These results establish that the mutant R113H PCFT displays a dramatic fall in the transport affinity for FA.

Folic acid growth requirement in the wt R113 and the various R113 mutant transfectants

To further corroborate the preservation of some folate transport function for the R113H and R113K mutants, the various PCFT mutant transfectants were examined for FA growth requirement (Fig. 3). The C-terminally Myc-tagged wt PCFT transfectant displayed a 50% FA growth requirement concentration (EC_{50}) of 18.0 ± 8.1 nM, whereas the R113D, R113A, and R113C transfectants exhibited 12-fold higher EC_{50} values (∼216–245 nM) that were not statistically different from mock-transfected cells. In contrast, stable introduction of the R113H and R113 K PCFT cDNAs into RFC-null CHO cells, significantly decreased the FA EC_{50} values when compared to the mock transfectant. Hence, these results support the preservation of some FA uptake at physiological pH in the R113H mutant and to a lesser extent in the R113K mutant.

Homology modeling of the R113 mutant PCFTs

Using advanced bioinformatics methodology, we have recently shown that the human PCFT shares a similar fold with several *Escherichia coli* transporters including the glycerol-3-phosphate transporter, the multidrug resistance transporter EmrD and the lactose permease [7]. To explore the possible structural basis for the preservation of some FA transport activity in the conservative R113H mutant and to a lesser extent in R113K, while completely losing FA transport activity in the other mutants, this structural platform was further explored here. HHpred is a method for sequence database searching and structure prediction that is much more sensitive in identifying remote homologues than BLAST. HHpred identified three significant PDB matches encompassing the majority of the PCFT amino acid sequence; these included the *E. coli* transporters 1pw4 (glycerol-3-phosphate transporter, GlpT) [9,10], 2gfp (multidrug resistance protein D; EmrD) [11] and 2cfq (lactose permease) [12]. In our previous paper [7] we used the 1pw4A (GlpT) template to construct a PCFT homology-based model, since the HHpred *P*-value was the highest for this homologue (i.e. the probability for a match to be a true positive). Herein, we built a second model based on the 2gfp template (EmrD), which is the second best homologue identified by HHpred. EmrD was chosen here since although the overall *P*-value of the latter is slightly lower than GlpT, its identity with PCFT at the vicinity of the R113 residue (S108-P115) is almost perfect (Fig. 4A), thus allowing for a more accurate reconstruction of this important loop