Functional roles of aspartate residues of the human proton-coupled folate transporter (PCFT; SLC46A1); D156, mutated in hereditary folate malabsorption, is critical for protein stability; D109 is irreplaceable

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Abbreviations: PCFT, proton-coupled folate transport (SLC46A1); RFC, the reduced folate carrier (SLC19A1); FRα, folate receptor-α; HFM, hereditary folate malabsorption; TMD, transmembrane domain; 5-formylTHF, 5-formyltetrahydrofolate; MW, molecular weight.

Key words: HFM, hereditary folate malabsorption, PCFT, proton-coupled folate transporter, HCP1, PCFT/HCP1, heme carrier protein1, folates, proton-coupled transport, intestinal folate transport

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Abstract

The proton-coupled folate transporter (PCFT; SLC46A1) mediates folate transport into enterocytes in the proximal small intestine; *pcft* loss-of-function mutations are the basis for hereditary folate malabsorption (HFM). The current study explores the role of Asp residues in PCFT function. A novel, homozygous, loss-of-function mutation, D156Y, was identified in a child of Pakistani origin with HFM. Of the six other conserved Asp residues, only one, D109, is shown to be required for function. D156Y along with a variety of other substitutions at this site, (Trp, Phe, Val, Asn, or Lys) lacked function due to instability of the PCFT protein. Substantial function was preserved with Glu, Gly and, to a lesser extent, with Ser, Thr, and Ala substitutions. This correlated with PCFT biotinylated at the cell surface. In contrast, all D109 mutants, including D109E, lacked function irrespective of pH (4.5, 5.5, 7.4) or substrate concentration (0.5-100 µM) despite surface expression comparable to wild-type PCFT. Hence, D156 plays a critical role in PCFT protein stability and D109, located in the 1st intracellular loop between the 2nd and 3rd TMDs, is absolutely required for PCFT function.
Introduction

The folate B9 vitamins are essential cofactors for one-carbon metabolic reactions required for de novo synthesis of nucleotides, methionine and for methylation reactions. These hydrophilic molecules require specific membrane transport processes to reach the metabolic machinery within cells. The major transporter that delivers folates to systemic tissues at their ambient neutral pH is the reduced folate carrier (RFC) \(^1,2\). High-affinity binding proteins mediate transport of folates into cells by an endocytic process \(^3,4\). Folate absorption at the acid microenvironment of the proximal small intestinal brush-border membrane was recently shown to be mediated by a proton-coupled folate transporter (PCFT; SLC46A1; NP_54200) \(^5\). PCFT’s critical role in this process, along with transport across the blood:choroid plexus:cerebrospinal fluid (CSF) barrier, was established by the demonstration of loss-of-function mutations in the pcft gene in subjects with the autosomal recessive disorder, hereditary folate malabsorption (HFM, Online Mendelian Inheritance in Man – OMIM No. 229050) \(^5,6\). Since then additional subjects with HFM and loss-of-function PCFT mutations have been reported by this and other groups \(^7-12\). Mechanisms of folate transport and homeostasis were recently reviewed \(^1\).

PCFT utilizes a proton gradient to drive the uphill transport of folates into cells; transport is optimal at low pH (~5.5), electrogenic, and is accompanied by cellular acidification. As the pH is increased, there is a progressive decrease in the influx \(V_{\text{max}}\) and increase in the influx \(K_m\). However, transport persists even at neural pH in the absence of a pH gradient driven, at least in part, by the voltage gradient across the cell membrane \(^5,13-16\).
Information on specific residues that play an important role in function is emerging. H281 in the 7th transmembrane domain is a determinant of folate binding through its impact on proton-binding. H247 in the 3rd large intracellular loop appears to form a hydrogen bond with S172 in the 2nd intracellular loop, thereby regulating access to the translocation pathway. E185 in 5th TMD was found to be essential for proton-coupling. Most recently, the twelve transmembrane domain PCFT secondary structure was confirmed by the substituted cysteine accessibility method and a sulfhydryl bond between cysteines in two extracellular loops defined. Studies on PCFT mutations associated with HFM also provide insights into residues critical for carrier function. The R113 residue was shown to be mutated in two subjects with HFM and its absence results in a profound loss of function irrespective of the substituted residue.

The current study encompasses an evaluation of the role of Asp residues in PCFT function. Only two of the seven conserved Asp residues were found to be important. D156, mutated in a patient with HFM, was shown to play a critical role in protein stability; D109 is absolutely required for function.
Patient, Materials and Methods

Patient:

This female patient was born in Pakistan to consanguineous parents. Her mother declined taking supplementary folic acid during her pregnancy and, at the time of delivery, had a Hb of 9.8 g/dL. At 2 ½ months of age, the infant developed megaloblastic anemia and was initially treated with blood transfusions, oral folic acid and injections of vitamins. Anemia persisted and, at the age of 8 months, after she had moved to Canada, the patient developed diarrhea and lethargy. Her Hb was 6.6 g/dL, MCV 83fL and serum folate 7.2 nmol/L (normal: 11.8-59.4nmol/L); RFC folate was low, <117 nmol/L (normal: 582-2701nmol/L). Her vitamin B12 level was normal. Bone marrow biopsy was hypercellular with both megaloblastic and dysplastic changes. Oral folic acid was continued but she had frequent episodes of vomiting and/or diarrhea. At 11 months of age, the patient developed seizures. A head CT demonstrated hyperdense cortical/subcortical areas in both the posterior parietal and occipital lobes. By 12 months of age, her weight and length had reached a plateau, dropping from the 25th and 50th percentiles, respectively, to the 3rd percentile. Head circumference was at the 3rd percentile. At 13 months of age the serum and RBC folate levels were normal on continued oral folic acid supplementation but a CSF folate level obtained for the first time was < 5nmol/L (normal: 2-3 times the normal serum folate concentration or ≥10-45 ng/mL). Serum immunoglobulins were normal. The seizures resolved with anticonvulsant therapy and supplementation with 1.6 mg/kg/day of 5-formylTHF orally.
Two months later, the CSF folate level increased to 21 nmol/L but the CSF:serum folate ratio remained <0.5. An MRI of the head at 15 months of age showed normal grey-white matter differentiation with no signal abnormality on the standard sequences. At the age of 18 months, the patient was above the 95th percentile for weight, had normal developmental milestones and a normal neurological assessment except for some speech delay. At present, the patient’s folate supplementation is 5 mg/d of parenteral 5-formylTHF.

**Identification of a PCFT mutation in a patient with HFM:**

This study was approved by the Albert Einstein College of Medicine’s Clinical Committee of Investigation in accordance with the Declaration of Helsinki. After written informed consent was obtained, peripheral blood was collected from the patient and family members. Genomic DNA was isolated at the Albert Einstein College of Medicine DNA Isolation and Cell Expansion Core. Each of the five PCFT exons, with their splice acceptors and donor sites, were amplified by PCR using primers reported previously. PCR products were analyzed on an agarose gel, purified by a gel purification kit (GE Healthcare, Buckinghamshire, HP7 9NA, UK), and sequenced at the Albert Einstein College of Medicine Cancer Center Genomics Shared Resource.

**Chemicals:**

Methotrexate (MTX-disodium salt, (3’, 5’, 7-[3H](N), cat. No. MT701) was obtained from Moravek Biochemicals Inc (Brea, CA), and purified by liquid chromatography before use as previously described. Unlabeled MTX was purchased from Sigma-Aldrich (St. Louis, MO); EZ-Link Sulfo-NHS-LC-Biotin (Sulfosuccin-imidyl-6-
[biotinamido] hexanoate, cat. No. 21335) from Pierce Biotechnology (Rockford, IL),
streptavidin-agarose beads (cat. No. 20349) from Fischer Scientific (Pittsburgh, PA),
and protease inhibitor cocktail (cat. No. 1183670001) from Roche Applied Science
(Mannheim, Germany). MG132 (N-CBZ-Leu-Leu-Leu-AL) was purchased from Sigma-Aldrich (St. Louis, MO) and bafilomycin A1 from LC laboratories (Boston, MA)

**Construction of mutant plasmids by site-directed mutagenesis:**

Mutations were introduced into PCFT cDNA with the Quick change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers for each mutation are listed in Table 1. Mutations in the plasmid constructs were verified by DNA sequencing analysis. A PCFT pcDNA3.1(+) expression vector, which encodes HA-tagged human PCFT at the C-terminus, was used as the template for all site-directed mutants 21.

**Cell lines, cell culture conditions and transient transfection:**

HeLa-R1-11 cells are a stable subclone of the HeLa R1 cell line that lacks both RFC and PCFT expression, the former due to a genetic deletion 22, the latter due to methylation of the promoter and loss of gene copies 23. R1-11 cells have been maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. For transient transfection, R1-11 cells (3.5 X 10⁵/vial) were seeded into 17-mm liquid scintillation vials and grown for 48 hours. The mutant cDNA constructs (0.8 μg of each plasmid), were transiently transfected into the cells with lipofectamine 2000 (Invitrogen, Carlsbad,CA). The cells were processed for transport or Western blot analysis 2 days later.
Membrane transport analyses (functional characterization):

A technique for rapid measurement of $[^3]$HMTX influx was employed. R1-11 transfectants were washed twice with 2ml of HBS buffer (20mM HEPES, 140mM NaCl, 5mM KCl, 2mM MgCl$_2$, and 5mM dextrose at pH 7.4) and incubated in the same buffer in a water bath (37°C) for 20 min. The buffer was then aspirated following which transport buffer, 500µl of HBS or MBS (20mM MES, 140 mM NaCl, 5mM KCl, 2mM MgCl$_2$, and 5mM dextrose) containing $[^3]$HMTX was added. $[^3]$HMTX uptake was halted after 1 min by the addition of 10 volumes of ice-cold HBS buffer at pH 7.4, an interval over which uptake was unidirectional. Cells were then washed 3 times with ice-cold HBS buffer, following which 500µl of 0.2M NaOH was added and cells digested by incubation for 30-45 min at 65°C. A portion of the hydrolysate was then transferred to a vial, fluor added and intracellular radioactivity assessed on a liquid scintillation spectrometer. The protein content of another portion of hydrolysate was determined using the Pierce kit. Influx is expressed as pmoles of $[^3]$HMTX per mg of protein per min and data is reported as mean ± S.E.M. from at least three independent experiments performed on different days.

Cell surface biotinylation assay and Western blot analysis:

These assays have been described previously. Briefly, two days after transfection, cells were washed twice with PBS (2 ml) at pH 8.0 and treated with 1mg/ml EZ-Link Sulfo-NHS-LC-Biotin in PBS (pH 8.0) at room temperature for 30 min. Cells were washed twice and treated with hypotonic buffer (0.5mM Na$_2$HPO$_4$ and 0.1mM EDTA at pH 7.0) containing protease inhibitors and kept on ice for 30 min. Cells were
then scraped from the plates and centrifuged at 14,000 rpm for 15 min at 4°C. The membrane fraction was pelleted and resuspended in 400µl of lysis buffer (0.1% SDS, 1% Triton X-100, 1mM EDTA, 150mM NaCl, 20mM Tris (pH 7.4)) containing protease inhibitors for 30 to 120 min at 4°C (rotating in a cold room). Following centrifugation at 14,000 rpm at 4°C for 15min, 25µl of supernatant was taken from each sample for Western blot analysis (designated as the crude membrane protein fraction). The remaining supernatant was mixed with streptavidin-agarose beads (50µl- that had been pre-washed three times with lysis buffer) overnight at 4°C. The beads were washed four times with the lysis buffer (500µl) containing protease inhibitors. After the final wash, beads-bound protein was stripped by heating for 5 min at 95° in 2X SDS-PAGE loading buffer containing dithiothreitol (DTT) and loaded directly on polyacrylamide gels. The crude membrane fraction was mixed with 2X SDS-PAGE loading buffer(1:1) containing DTT before Western blot analysis. For the crude membrane samples, blots were first probed with a rabbit β-actin antibody (#4967L, Cell signaling Technology, Denvers, MA), then stripped with stripping buffer (100 mM 2-β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7) and reprobed with anti-HA antibody (H6908; Sigma, St. Lois, MO). For biotinylated samples, blots were directly probed with anti-HA antibody.
Results

Identification of a D156Y mutation in a subject with HFM

A novel pcft mutation was identified in the patient described above (c466 G>T) located in exon 2 at position “466” of the cDNA sequence (NM_080669), as indicated in Figure 1A. Both parents were heterozygous for the same mutation. This resulted in a homozygous Asp to Tyr substitution at the D156 residue (NP 54200.2). As shown in Figure 1B, no transport function could be detected in Hela R1-11 cells transfected with the D156Y mutant. On Western blot of the mutated PCFT, HA-tagged at the C-terminus, a protein with a lower molecular weight (MW) than wild-type PCFT was identified in the crude cell membrane fraction; however, no protein was detected at the cell surface (Fig. 1C).

Initial screening of PCFT Asp mutants

PCFT contains seven aspartic acid residues. Six are fully conserved (D72, D109, D156, D170, D286, D331) among species (to xenopus and zebrafish); one is semi-conserved (D54). The location of these residues is illustrated in Figure 2, based upon the current understanding of the PCFT secondary structure 18. D156 is located in the 4th transmembrane domain (TMD). To develop a broader understanding of the role of Asp residues in PCFT function, PCFT mutants with a drastic change to the opposite-charged Lys were evaluated by assessment of [3H]MTX influx at pH 5.5 in cells transiently transfected with these constructs. As indicated in Figure 3A, the only mutants that were not functional were D109K and, as expected, the residue mutated in the subject with HFM, D156K. Hence, D54, D72, D170 and D286 are not required for
function since a marked change at these positions preserved more than 50% of activity. About 25% of activity was observed for the D331K mutant.

To further explore the role and structural requirements of the D109, D156, and D331 residues, each was replaced with Glu, Asn, or Ala. As indicated in Figure 3B, an important role for D331 was excluded by the retention of function despite replacement with a neutral (Ala) or polar (Asn) residue. Full function was retained with the like-charged D331E substitution. No function was retained with any of the substitutions at the D109 residue, including replacement with the like-charged Glu. On the other hand, replacement of the D156 residue with Glu fully preserved function, replacement with Ala preserved ~15% of function and substitution with Asn resulted in a complete loss of activity. Therefore, only D109 and D156 appeared to be critical for PCFT function; further studies focused on these two residues.

**Expression and function of D156 PCFT mutants**

In addition to D156Y, -E, -N, -K, and -A, seven more mutants were generated at this position. As indicated in Figure 4A, substantial function was retained with replacement by Gly (80%) and to an appreciable, though lesser, extent with serine (50%). A low level (~15%) of activity was observed with Ala, Cys and Thr. Substitution with Tyr, Phe and Val resulted in complete loss of activity. Hence, while a negative charge is preferred at this site, nearly full function can be achieved with neutral and polar replacements.

PCFT expression in both the crude cell membrane fraction and at the cell surface was assessed for all twelve D156 mutants. It can be seen that little or no protein could
be detected at the MW of wild-type PCFT in the crude membrane fractions, or at the cell surface after biotinylation, for the mutants that lack function (Fig. 4B and 4C). On the other hand, when some function was preserved, protein was detected both in the crude membrane fraction and at the cell membrane. The mutants with the greatest function tended to have the highest levels of biotinylated protein (D156E, -S, -G). For most of the mutants, except D156N and D156K, a protein of lower MW than wild-type PCFT was detected. This band approximated the MW of deglycosylated PCFT (G-less) in the crude membrane fraction but not at the plasma membrane, indicating that this protein failed to traffic to the surface of the cell.

Further studies were designed to address whether these bands represent deglycosylated forms of PCFT or degraded products of the protein. D156Y was selected as a representative mutant. Instead of the HA tag at the C-terminus, this mutant was HA-tagged at the N-terminus. As previously documented, wild-type and deglycosylated-PCFT activities remain the same regardless of the position of HA tag 21. No mutant protein could be detected on Western blot of the crude membrane fraction (data not shown). Hence, the low MW forms detected with this mutant, and presumably with the other mutants, are not deglycosylated PCFT; rather, these represent the degradation products of the mutant proteins.

Possible roles for ubiquitination and proteasomal degradation in the instability of the mutated D156Y PCFT protein were assessed with MG132 (proteasome inhibitor) and bafilomycin A1 (ubiquitin inhibitor). To optimize concentrations of these inhibitors, growth inhibition was assessed 24 hrs after treatment of R1-11 cells at various inhibitor concentrations; 0.5μM MG132 and 10nM bafilomycin A1 resulted in ~20% inhibition of
cell growth and were utilized at these concentrations. In three separate experiments, treatment of cells transfected with the D156Y PCFT, wild-type PCFT, or the vector with these reagents did not result in any change in $[^3]$H]MTX influx. (data not shown). Further studies, described in the next section, were then undertaken to determine whether the decrease in function of D156G and D156S mutants could be attributed entirely to decreased PCFT expression at the plasma membrane or whether there was, in addition, impaired intrinsic function/properties of the mutated carrier.

**Influx kinetics mediated by D156 mutant PCFTs**

Figure 5 illustrates $[^3]$H]MTX influx kinetics mediated by D156G and D156S PCFT mutants. The influx $K_m$ for D156G was unchanged, but was decreased by a factor of ~4 for D156S, as compared to wild-type PCFT, consistent with a substantial *increase* in the affinity of the latter mutant for its substrate. The $V_{max}$ was decreased for both mutants, but to a greater extent for the Ser than Gly mutant, in comparison to wild-type PCFT, generally consistent with the levels of expression at the cell surface. To more quantitatively compare the levels of protein, biotinylated protein was diluted with loading buffer prior to electrophoresis. A 1:2 dilution (one-part lysate + one-part buffer) of the wild-type PCFT produced a band similar to that of D156G; a 1:3.5 dilution of wild-type PCFT produced a band similar to that of D156S, further supporting the likelihood that $V_{max}$ differences were largely due to differences in the levels of protein at the cell surface. Hence, amino acid substitutions at this site do not appear to impair folate substrate binding nor carrier function.

**Expression and function of D109 PCFT mutants.**
Six PCFT mutants were studied at the D109 position; the results were entirely different from what was observed for the D156 mutants. As indicated in Figure 6A, substitutions of D109 with -E, -N, -K, -A, -G, -S residues resulted in a complete loss of function at pH 5.5 when the [³H]MTX concentration was 0.5 µM; this was the case even with the highly conservative -E mutant. Further, no transport activity was detected for these mutants, in comparison to the mock-transfected cells, at MTX concentrations of 10 µM MTX at pH 5.5 (Fig. 7A) or at 100 µM MTX (Fig. 7B) at pH 5.5 excluding the possibility that the loss of function is due to a decrease (of at least 200-fold) in PCFT affinity for MTX. To determine if the loss of activity might be associated with a decrease in proton binding to the carrier, as observed for other PCFT mutants, the pH of transport buffer was decreased to 4.5; still no activity could be detected for any of the mutants at a MTX level of 0.5 µM (Fig. 7C) or 100 µM (Fig. 7D). The trivial increase in uptake for the -N and -S mutants at 100 µM MTX at pH 5.5 did not reach statistical significance and was not observed at all at pH 4.5. Nor was MTX influx preserved at pH 7.4 excluding defective proton-coupling as a basis for the loss of activity of the D109 mutants (data not shown). Finally, in contrast to the D156 mutants, there was no decrease in protein expression in the crude membrane fraction, nor in the level of surface biotinylation for any of the D109 mutants, thereby excluding alterations in protein stability or trafficking as a basis for the profound loss of function (Fig. 6B).
Discussion

This laboratory recently cloned PCFT and established the physiological role this transporter plays in intestinal folate absorption and transport of folates across the blood:choroid plexus:CSF barrier by the defects in these processes in subjects with HFM \(^5\)\(^-\)\(^7\). In the current paper, the role of Asp residues was examined, one of which (D156Y) was shown to be mutated in a subject with HFM. Despite the fact that five Asp residues are fully conserved beyond D156, only one other residue, D109, was found to be important for function. However, the role of these two residues, and the impact of mutations at these sites, was very different.

The D156 residue, located in the mid-portion of the 4\(^{th}\) TMD, is critical for protein stability. Loss of stability was observed with a variety of polar, neutral or positively charged mutants. However, stability and trafficking to the cell membrane was substantially preserved with the Gly (relatively polar) or to a lesser extent with the Ser (polar) substitutions. It would appear that small size and polarity were associated with retention of stability. Low MW bands were observed with D156 mutants; this was also observed with deglycosylated forms of PCFT that are fully functional \(^{21}\). However, these bands were shown to be degradation products based upon the observation that low MW forms were detected only when PCFT mutants were HA-tagged at the C-terminus. The data suggest that loss of transport function can be attributed largely, if not entirely, to the extent of protein degradation. The influx \(K_m\) for the Gly mutant was unchanged, the decrease in influx \(K_m\) for Ser mutant was consistent with an \textit{increased} rather than decreased affinity for MTX. Differences in maximum velocity observed with these substitutions were due predominantly to differences in the level of PCFT protein at the
cell surface. Hence, mutations at this site do not appear to alter the rate of conformational change during carrier cycling.

It is of interest that Asp residues in the Leishmania FT1 folate transporter, D514 and D529, (located in the 11th TMD and 6th extracellular loop, respectively), are both required for protein stability. Substitution with Val resulted in a complete loss of protein but stability was fully retained with a Glu substitution, similar to what was observed with D156Y. Extensive site-directed mutagenesis of the D156 PCFT suggests that polar substitutions and compact size favor stability. Hence, Gly (D156G) preserved the majority of transport function and polar residues, save for the larger Asn, sustained some function. Thr and Cys residues tend to form hydrogen bonds with carbonyl oxygen atoms in the preceding turn of helices, of particular importance for intrinsic membrane transport proteins. The preservation of activity with the Gly and polar substitutions exclude participation of the D156 residue in a salt bridge.

The properties of the D109 residue were quite different. No substitution, irrespective of charge or polarity preserved function. Indeed, even the most conservative substitution to Glu lacked function. In contrast to the D156 mutants, each D109 mutant protein was expressed and detected at the cell surface at levels comparable to that of wild-type PCFT. Activity could not be detected even at high MTX concentrations excluding the possibility that loss of function was due to an increase in influx $K_m$ to at least two orders of magnitude above the wild-type PCFT influx $K_m$. Likewise, a decrease in the affinity of a proton binding site, that allosterically alters the conformation of the folate binding pocket, as a factor was excluded by the lack of function even when the proton concentration was increased by an order of magnitude.
by decreasing the pH to 4.5. This is in contrast to what was observed for the H281A mutant in which there was marked restoration of function with a similar increase in proton concentration \textsuperscript{17}. Further, the lack of preservation of activity at pH 7.4 excludes a role for this residue in proton coupling. Based upon these finding, the D109 residue is essentially irreplaceable.

D109 is located in the first intracellular loop between the 2\textsuperscript{nd} and 3\textsuperscript{rd} TMDs. Mutations in this loop at R113 (R113S, R113C) were detected in two unrelated families with HFM \textsuperscript{6,8} and, when this residue was mutated to a variety of other amino acids, there was a marked decrease in function, although some activity was preserved with like-charged substitutions \textsuperscript{19}. Hence, it would appear that the first intracellular loop plays an important role in PCFT function. This loop encompasses a D109XXGRR113 sequence required for PCFT trafficking to the apical membrane of polarized Caco2 and MDCK cells \textsuperscript{27}. Mutation of multiple residues to Ala (DSVAAA, AAAAAA) resulted in a trafficking defect in these cell lines. However, the current study demonstrates that in nonpolarized HeLa cells mutation of the D109 residue does not alter protein expression, stability or trafficking to the plasma membrane. It is not clear as to whether a single mutation at this or other sites in this loop would alter trafficking to the apical membrane of polarized cells.

The basis for the critical role the D109 residue plays in PCFT is function is unknown. Multiple alignments of 81 non-homologous protein families indicate that the Asp residue is more highly conserved than any other amino acid due to its short side chain, high charge density, strong polar interactions, and rigidity \textsuperscript{28}. Even Gly, which is small, highly compact and with polar properties, did not substitute for Asp, consistent
with extreme spatial limitations at this site. Hence, in the absence of the Asp residue, substrate either does not bind to the carrier and/or oscillation of the carrier between its inward and outward facing conformations, intrinsic to the alternative-access mechanism, is impaired.\textsuperscript{29, 30}

HFM manifests within the first few months of life and, untreated, may lead to death in early infancy.\textsuperscript{31} However, is not unusual for subjects to live for a year or more before appropriate therapy is instituted, although the chances of neurological involvement and other complications increase as the interval between birth and treatment is increased. There are multi-system clinical manifestations of HFM. Patients always present with anemia, usually megaloblastic, and sometimes accompanied by neutropenia and thrombocytopenia. In some patients the clinical presentation is dominated by an immune deficiency syndrome associated with hypogammaglobulinemia and infections such as \textit{pneumocystis jirovecii}\textsuperscript{9, 6}. Neurological disorders characterized by developmental delays are common; seizures are less frequent but can represent a major clinical challenge particularly when treatment has been delayed. This patient, for whom adequate treatment was substantially delayed, developed seizures at 11 months of age. The clinical picture of HFM has been the subject of recent reviews.\textsuperscript{31, 32}

The neurological consequences of the loss of PCFT function is associated with a failure of folate transport across the choroid plexus where this carrier is highly expressed at the basolateral membrane.\textsuperscript{33} This results in very low levels of folate within the CSF which is normally 2-3 times greater than the blood folate level. Even when the blood folate level is normalized, CSF folate levels remain low as was the case in this
patient. Indeed, the endpoint for treatment of HFM should be a nadir CSF folate level 2-3 times the normal blood folate level which requires achieving supra-normal folate blood levels. Beyond PCFT, two other folate transporters are expressed in the choroid plexus, (i) RFC at the apical membrane in apposition to the CSF 34, and (ii) folate receptor-α (FRα) mainly expressed at the apical membrane but present elsewhere as well in choroid plexus ependymal cells 1. While the role of RFC is not clear, it is clear that FRα is required for folate transport across this epithelium but the impact of loss of this protein differs temporally from what is observed when PCFT function is lost in HFM. Three families have now been reported with loss-of-function FR-α mutations 35, 36. These subjects, with normal folate blood levels, develop neurological signs of folate deficiency beyond the second year of life associated with very low CSF folate levels. The basis for this delay is not clear. The normal folate blood levels may have a protective effect, delaying neurological damage; alternatively, other routes of transport across the choroid plexus may compensate for the absence of FRα during the early years of life.
Acknowledgements

This work was supported by grants from National Institutes of Health, CA-82621, and the Mesothelioma Applied Research Foundation.

Authorship

Contribution: D.S.S performed majority of laboratory experiments and wrote the paper. I.D.G and R.Z equally contributed to guide the designing of the studies and editing the manuscript. L.R provided the patient history and blood specimen. S.H.M performed initial sequencing of the patient's mutation and functional analysis. A.F provided bioinformatics support for this study.

Conflict-of-interest disclosure: The authors declare no competing financial interest.

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Reference List


### TABLE 1. Forward primers utilized for site-directed mutagenesis of Asp mutants.

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<tr>
<th>Primer</th>
<th>Sequence</th>
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<td>D156N</td>
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<td>ACCCGCAACCIGAAGCCACCCTGACGAA</td>
</tr>
<tr>
<td>D170K</td>
<td>GCTTCGGTGCAAAAGTCTCACTGACCAGTC</td>
</tr>
<tr>
<td>D286K</td>
<td>TTTGGGCGCCAGAAAATCTTTAACCGT</td>
</tr>
<tr>
<td>D331E</td>
<td>TACTGCCCTGGCGGAAAGCCTGGGTAGCT</td>
</tr>
<tr>
<td>D331N</td>
<td>TACTGCCCTGGCGGAAAGCCTGGGTAGCT</td>
</tr>
<tr>
<td>D331K</td>
<td>TACTGCCCTGGCGGAAAGCCTGGGTAGCT</td>
</tr>
<tr>
<td>D331A</td>
<td>TACTGCCCTGGCGGAAAGCCTGGGTAGCT</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Identification of a novel D156Y PCFT mutation in a subject with HFM and its expression as well as transport function. A: A chromatogram showing a homozygous mutation in the PCFT gene in a subject with HFM. B: [3H]MTX (0.5µM) influx over 1 min at pH 5.5 and 37°C in Hela-R1-11 cells transiently transfected with wild-type PCFT, D156Y-PCFT, or the vector (mock). C: Western blot analysis of cells transiently transfected with wild-type PCFT, the D156Y mutant, or vector: crude cell membrane fraction (left); biotinylated protein at the cell surface (right). Vertical line has been inserted to indicate a repositioned gel lane.

Figure 2. A topological model for human PCFT. All seven Asp residues are indicated. D54 is semi-conserved; D72, D109, D156, D170, D286, and D331 are fully conserved among species (monkey, horse, rat, mouse, dog, cow, opossum, xenopus and zebra fish). D156 is mutated in the subject with HFM.

Figure 3. Functional assessment of PCFT aspartate mutants. [3H]MTX influx was assessed at pH 5.5 and 37°C over 1 min at a concentration of 0.5µM in Hela-R1-11 transfectants. A: initial screening of Asp mutants. B: Studies focused on D109, D156 and D331 residues replaced with Glu, Asp or Ala. Data are the mean ± SEM from 3 independent experiments.

Figure 4. Expression and function of D156 PCFT mutants. A: [3H]MTX influx was performed at 0.5µM and pH 5.5 over 1 min. Data are the mean ± SEM from 3 independent experiments performed on different days. B and C: Western blot assay of the crude cell membrane fraction (upper panel) and biotinylated protein at the cell
surface (middle panel). β-actin was the loading control (lower panel). These blots are representative of 3 experiments, were performed on 2 gels at the same time.

**Figure 5. MTX influx kinetics mediated by D156 PCFT mutants.** A: \[^{3}H\]MTX influx kinetics mediated by D156G and D156S PCFT as compared to wild-type PCFT. Influx was assessed at pH 5.5 at 37°C over 1 min with \[^{3}H\]MTX concentrations of 0.1, 0.2, 0.5, 1, 2.5, 5 and 10µM. Data are the mean ± SEM from 3 independent experiments. B: Western blot of biotinylated protein obtained from cells transfected with wild-type PCFT and the D156G and D156S mutants. Wild-type PCFT was diluted to more accurately quantify, by comparison, levels of mutant PCFTs. Ten µl of the biotinylated wild-type PCFT protein fraction was added to 10, 20, 25 and 30µl of 2XSDS-loading buffer, described as 1:2, 1:3, 1:3.5 and 1:4 dilutions, respectively. The data is representative of two independent experiments. Vertical line has been inserted to indicate a repositioned gel lane.

**Figure 6. Functional properties of D109 PCFT mutants.** A: \[^{3}H\]MTX influx (0.5µM) over 1 min at pH 5.5. Data are the mean ± SEM from 3 independent experiments. B: Western blot assay of the crude cell membrane fraction (upper panel) and biotinylated protein (middle panel) of D109 PCFT mutants. β-actin was the loading control (lower panel). The graph shown is a representative of two independent experiments. Vertical lines have been inserted to indicate repositioned gel lanes.

**Figure 7. Impact of MTX concentration and pH on the activity of PCFT D109 mutants.** A: \[^{3}H\]MTX influx (10 µM) over 1 min at pH 5.5. B: \[^{3}H\]MTX influx (100 µM) at pH 5.5. C: \[^{3}H\]MTX influx (0.5 µM) at pH 4.5. D: \[^{3}H\]MTX influx (100 µM) at pH 4.5.
Figure 1

A

C

Control

Patient

TCGG CG ACTTC

TCGG CTACTTC

64kDa → 49kDa → 37kDa →

Crude membrane fraction

Cell surface

B

[^3H]MTX influx (pmol/mg protein/min)

WT D156Y MOCK

0 25 50 75 100

Mock

WT D156Y
Figure 4

A

\[\sigma^3_HMTX\text{ influx (pmol/mg protein/min)}\]

WT D156Y D156E D156N D156K D156A D156S D156C D156W D156F D156T D156V D156G

B

Crude membrane fraction

64kDa →
49kDa →
37kDa →

Cell surface

64kDa →
49kDa →
37kDa →

C

Crude membrane fraction

D156C D156W D156F D156T D156V D156G Mack

Cell surface

B-actin
Figure 5

[Graph showing the influx of [3H]MTX in pmol/mg protein/min for different samples:
- WT: $K_m = 2.29 \pm 0.48 \mu M$, $V_{max} = 718 \pm 55$ pmol/mg protein/min
- WT(1:2): $K_m = 2.15 \pm 0.26 \mu M$, $V_{max} = 365 \pm 16$ pmol/mg protein/min
- WT(1:3.5): $K_m = 0.59 \pm 0.08 \mu M$, $V_{max} = 93.1 \pm 3.3$ pmol/mg protein/min
- D156G
- D156S

Dilution:
- WT(1:2)
- WT(1:3)
- WT(1:3.5)
- D156G
- D156S

Protein bands on gel:
- 64kDa → 49kDa → 37kDa

Cell surface]
Figure 6

A

WT
D109E
D109N
D109K
D109A
D109G
D109S
MOCK

[3H]MTX influx (pmol/mg protein/min)

0
25
50
75
100
125

B


64kDa →
49kDa →
37kDa →

Crude membrane fraction

64kDa →
49kDa →
37kDa →

Cell surface

B-actin
**Figure 7**

A. [MTX] = 10 μM
   pH = 5.5

B. [MTX] = 100 μM
   pH = 5.5

C. [MTX] = 0.5 μM
   pH = 4.5

D. [MTX] = 100 μM
   pH = 4.5

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Functional roles of aspartate residues of the human proton-coupled folate transporter (PCFT; SLC46A1); D156, mutated in hereditary folate malabsorption, is critical for protein stability; D109 is irreplaceable

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