Regulation of Folate-mediated One-carbon Metabolism by 10-Formyltetrahydrofolate Dehydrogenase

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10-Formyltetrahydrofolate dehydrogenase (FDH) catalyzes the NADP⁺-dependent conversion of 10-formyltetrahydrofolate to CO₂ and tetrahydrofolate (THF) and is an abundant high affinity folate-binding protein. Although several activities have been ascribed to FDH, its metabolic role in folate-mediated one-carbon metabolism is not well understood. FDH has been proposed to: 1) inhibit purine biosynthesis by depleting 10-formyl-THF pools, 2) maintain cellular folate concentrations by sequestering THF, 3) deplete the supply of folate-activated one-carbon units, and 4) stimulate the generation of THF-activated one-carbon unit synthesis by channeling folate cofactors to other folate-dependent enzymes. The metabolic functions of FDH were investigated in neuroblastoma, which do not contain detectable levels of FDH. Both low and high FDH expression reduced total cellular folate concentrations by 60%, elevated rates of folate catabolism, and depleted cellular S-methyl-THF and S-adenosylmethionine levels. Low FDH expression increased the formyl-THF/THF ratio nearly 10-fold, whereas THF accounted for nearly 50% of total folate in neuroblastoma with high FDH expression. FDH expression did not affect the enrichment of exogenous formate into methionine, serine, or purines and did not suppress de novo purine nucleotide biosynthesis. We conclude that low FDH expression facilitates the incorporation of one-carbon units into the one-carbon pool, whereas high levels of FDH expression deplete the folate-activated one-carbon pool by catalyzing the conversion of 10-formyl-THF to THF. Furthermore, FDH does not increase cellular folate concentrations by sequestering THF in neuroblastoma nor does it inhibit or regulate de novo purine biosynthesis. FDH expression does deplete cellular S-methyl-THF and S-adenosylmethionine levels indicating that FDH impairs the folate-dependent homocysteine remethylation cycle.

Tetrahydrofolate (THF) polyglutamates are cofactors that function as one-carbon donors and ACCEPTORS in a set of reactions known as folate-mediated one-carbon metabolism, which occurs both in the cytoplasm and in mitochondria (see Fig. 1) (1). THF cofactors carry one-carbon units at three oxidation states ranging from formate to methanol (2). The biologically active THF derivatives contain a reduced pteridine and a polyglutamate peptide consisting of five to eight glutamate residues linked by y-peptide bonds (3). Cyttoplasmic folate-mediated one-carbon metabolism is required for the de novo synthesis of purines (supplies the carbon-2 and carbon-8 of the purine ring) and thymidylate (methyl transfer from dUMP to dTMP), and also for remethylation of homocysteine to methionine (2, 3). Methionine can be converted to S-adenosylmethionine (SAM) and serve as a methyl donor for numerous methylation reactions, including the methylation of DNA, RNA, and proteins. Serine is a primary source of folate-activated one-carbon units (4). Cyttoplasmic serine hydroxymethyltransferase (cSHMT) catalyzes the THF-dependent aldol cleavage of serine to methylene-THF and glycine. Alternatively, serine can be converted to glycine and formate in the mitochondria through mitochondrial folate-mediated one-carbon metabolism (5) (Fig. 1). The formate produced in the mitochondria is a major source of one-carbon units for cytoplasmic one-carbon metabolism through its conversion to 10-formyl-THF by the enzyme C1-THF synthase (MTHFD1) (6). Folate deficiency impairs dTMP, methionine, and SAM synthesis, resulting in increased rates of uracil misincorporation into DNA and DNA hypomethylation (5). Therefore, maintenance of cellular folate concentrations is critical for both DNA synthesis and genome stability and expression. Intracellular folate concentrations are influenced by folate availability, uptake, polyglutamylation, turnover, and export (5). Folate monoglutamates are transported into the cell either by folate receptors (FRs) or carrier-mediated proteins (7, 8) and then converted to folate polyglutamates by the enzyme folypolyglutamate synthetase. The addition of the polyglutamate chain is required for cellular retention of folate cofactors (9–12). Reduced folate polyglutamates are sequestered by folate-binding proteins inside the cell that exhibit Kₐ values in the low micromolar to nanomolar range (13). Studies have indicated that the concentration of cellular folate-binding proteins exceeds the concentration of total intracellular folate. In liver, for example, the concentration of folate-binding proteins exceeds the concentration of folate by 5- to 10-fold (5, 13); it has been suggested that the concentration of cellular folate-binding proteins may set the upper limit for intracellular folate accumulation (5).

10-Formyltetrahydrofolate dehydrogenase (FDH) is one of the most abundant folate-binding proteins present in liver (14). FDH catalyzes the NADP⁺-dependent conversion of 10-formyl-THF to THF and CO₂ (1, 15). Mice lacking FDH activity are viable and fertile, indicating that one or more FDH functions are not essential in the laboratory environment (16); FDH may function to regulate the folate-medi-
Folate-mediated one-carbon metabolism

Tetrahydrofolate (THF)-mediated one-carbon metabolism is required for the synthesis of purines, thymidylate, and methionine. The hydroxymethyl group of serine is the major source of one-carbon units, which can be generated in the mitochondria in the form of formate, or in the cytoplasm through the activity of cytoplasmic serine hydroxymethyltransferase. Mitochondrion-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism. MS, methionine synthase; MTHFR, methenyltetrahydrofolate dehydrogenase; cSHMT, cytoplasmic serine hydroxymethyltransferase; mSHMT, mitochondrial serine hydroxymethyltransferase; FDH, formyltetrahydrofolate dehydrogenase; MTHFD1, a trifunctional enzyme encoding 10-formyl-THF synthetase, methylene-THF dehydrogenase, and methenyl-THF cyclohydrolase activities; SAM, 5-adenosylmethionine; SAH, 5-adenosylhomocysteine.

EXPERIMENTAL PROCEDURES

Materials—[65]-[3H]5-Formyl-THF (40 Ci/mmol) was obtained from Moravek Biochemicals, Inc., and [methyl-3H]thymidine was from PerkinElmer Life Sciences. [6R,S]-5-Formyl-THF was from SAPEC, the chemical Division of Cerbios-Pharma SA, Barbeno, Switzerland. Fetal bovine serum, α-minimal essential medium (αMEM), and α-modification lacking sodium bicarbonate, folate, ribosides, ribotides, deoxyribo- and deoxyribotides (defined as αMEM) were obtained from HyClone Laboratories. Folate-depleted fetal bovine serum was prepared by dialyzing serum against ten volumes of phosphate-buffered saline at 4 °C for 24 h with buffer changes every 4 h, then charcoal-treated to remove any remaining folate.

Cell Lines and Culture Medium—The SH-SY5Y human neuroblastoma cell line has been described previously (24). Cells were cultured in αMEM with 10% dialyzed fetal bovine serum for all experiments. FDH expression was induced in cell lines by the addition of 1 μg/ml tetracycline for a minimum of 4 days prior to experimentation.

Generation of Human FDH-expressing Cell Lines—cDNA from a fetal liver library (Clontech) was used as the template to amplify the human FDH open reading frame in two fragments. The first fragment (Fragment A) of the human FDH cDNA (nucleotides 91–1389 from the sequence MN 012190; NCBI) was amplified using the forward primer 5'-CCCTCCTCTGCTACCTGAGAT-3' and the reverse primer 5'-CCCCCCCCATGAAGAGGTGTGTT-3'. The cycling conditions were 94 °C for 45 s, 51 °C for 45 s, and 72 °C for 90 s for a total of 33 cycles. The second fragment (Fragment B) of the human FDH cDNA (nucleotides 1369–2854) was amplified using the forward primer 5'-GGGAGGCAGTGTTTGCCTTCC-3' and the reverse primer 5'-ATGCCTTTTCTAGAATCTGAACTCAGAGGTATT-3'. The cycling conditions were 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 90 s for a total of 33 cycles. Fragments A and B were cloned into the pCR vector using the Blunt End TOPO kit (Invitrogen). The nucleotide sequences of fragments A and B were determined from three different clones and all were identical. This sequence was identical to the sequence of the human FDH available on the NCBI database (GenBankTM Accession no. NM 012190). Fragment A was excised from the pCR plasmid with KpnI and XhoI and subcloned into the pKO vector (generating pKO-A). Fragment B was excised from the pCR-B vector with KpnI and XhoI, the fragment gel purified and digested with SapI (which is present in the 1374–1389 nt overlap region of both Fragments A and B). The vector pKO-A was...
digested with KpnI and Sapl, then ligated with Fragment B (containing Sapl and KpnI sites), generating the entire human FDH cDNA in the pKO vector. The FDH cDNA was excised from the pKO vector with NotI and EcoRV and subcloned into the pCDNA4/TO/myc-His C vector (Invitrogen) generating pcDNA4-FDH.

Stable SH-SY5Y cell lines expressing the human FDH cDNA from the pcDNA6/TR vector (Invitrogen) were generated. SH-SY5Y cells were electroporated with 20 μg of plasmid DNA (pcDNA4-FDH) at 0.22 kV and 950 microfarads (Bio-Rad Gene-Pulsar), then cultured with αMEM for 48 h. The medium was replaced with αMEM containing blasticidin (10 μg/ml) and Zeocin (100 μg/ml, Invitrogen) to select for stable integrants. Individual colonies resistant to Zeocin treatment were selected and expanded using αMEM containing blasticidin and Zeocin, and clonal lines expressing human FDH protein were screened by Western blot analyses.

**Determination of pABG Levels in Culture Medium**—Cells were seeded (5.6 × 10⁴) into 100-mm Primaria culture dishes (Falcon) containing 5 ml of αMEM. Following 5-h incubation, the medium was replaced with defined αMEM containing 25 mM [65]-[5-3H]formyl-THF. Aliquots of medium were taken at various time points, clarified by centrifugation, and stored at -80 °C. Folate degradation products were analyzed in these samples by reversed-phase HPLC as described elsewhere (25). Samples were spiked with unlabeled pABG, [6R,5S]-5-formyl-THF, and [6S]-5-methyl-THF to determine the identity of the radiolabeled fractions. With this method, pABG elutes at 6 min, 5-formyl-THF elutes at 20 min, and 5-methyl-THF elutes at 37 min. For each analysis, 1.0-ml fractions were collected into scintillation tubes and the tritium was quantified using a Beckman LS 6500 liquid scintillation counter.

**Western Blot Analyses of FDH, GAPDH, and FR**—Cells were cultured to 75% confluence in αMEM. Cell pellets were lysed using a buffer containing 10 mM Tris, pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 1% Triton X-100, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged, and protein concentration of the supernatant was determined. Protein extracts (60 μg/lane) were run on an 8% SDS-PAGE gel (for FDH analysis) or a 12% SDS-PAGE gel (for FR and GAPDH analysis), then transferred to a polyvinylidene fluoride microporous membrane (Millipore) using a MiniTransblot apparatus (Bio-Rad). For detection of FDH, the membrane was incubated overnight at 4 °C with primary antiserum (1:10,000 dilution) consisting of purified polyclonal antibodies generated in sheep against a highly conserved peptide sequence of human FDH (amino acid residues 390–410). For detection of GAPDH and FR, the membrane was incubated overnight at 4 °C with polyclonal antibodies (1:1000 dilution) that recognize the human FR or GAPDH (Santa Cruz Biotechnology). The membrane was washed using 0.1% Tween 20 in phosphate-buffered saline, then incubated for 2 h with horseradish peroxidase-conjugated rabbit anti-sheep secondary antibody for the detection of FDH (1:6500 dilution, Pierce), and rabbit anti-goat secondary antibody for the detection of FR and GAPDH (1:7000 dilution, Pierce). Proteins were visualized using the Super Signal West Pico chemiluminescence detection system (Pierce). The protein bands were quantified using Chemilumager 4400 from Alpha Innotech Corp. (San Leandro, CA).

**Cell Growth Assays**—The effect of human FDH cDNA expression on SH-SY5Y cell proliferation was determined using a colorimetric MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay, the yellow MTT reagent is reduced to an insoluble blue formazan product (λmax = 570 nm) by mitochondrial dehydrogenases in viable cells. Cells maintained in αMEM were trypsinized and counted in a hemocytometer. Cells were plated in 96-well V-bottom tissue culture plates at 1 × 10⁴ cells/well in either defined MEM (lacking ribonucleotides, deoxyribonucleotides, serine, and glycine) or defined MEM with 10 mg/L adenosine and 10 mg/L guanosine. Total volume plated was 100 μl of media, and an additional 50 μl of media was added every 24 h; FDH expression was maintained with 1 μg/ml tetracycline. At given time points (0, 24, 48, and 72 h), 15 μl of 5 mg/ml MTT reagent was added to each well. Cells were incubated 4 h at 37 °C in 5% CO2. The insoluble formazan product was pelleted for 10 min at 4000 rpm then resuspended in 100 μl of Me2SO, and A550 was measured on a MRX Microplate Reader (Dynex Technologies). Reported values represent the average and standard deviation of two separate experiments, each with ten replicates. These values were normalized to the lowest A550 value, which was assigned a value of one.

**Determination of Total Folate Content, SAM, SAH, and Polylglutamate Chain Lengths**—Cells were cultured to 70% confluence in αMEM that contained 2 μM folic acid. Total cellular folate, the distribution of folate derivatives, and polyglutamate chain lengths were determined by affinity/reversed-phase HPLC as described elsewhere (26). For SAM and SAH determinations, cells were cultured to 80% confluence in αMEM. 4 h prior to harvest, cells were cultured in αMEM lacking methionine to ensure SAM levels reflected cellular homocysteine remethylation capacity and not exogenous methionine availability. SAM and SAH were extracted from the cells and quantified by HPLC with fluorescence detection relative to standard curves as described elsewhere (27).

**Stable Isotope Tracer Studies**—To quantify the enrichment of [2,3,3-2H3]serine into purines, thymidine, and methionine, cells were plated at 50% confluence in 100-mm plates and cultured in treatment medium, which consisted of defined αMEM lacking deoxyribonucleosides, hypoxanthine, and thymidine but supplemented with 0.05 μM 5-formyl-THF, 10 μM methionine, 0.2 mM glycine, 1 mg/liter pyridoxine, 250 μM formate, 26 mg/liter L-[5,5,5-2H3]leucine, and 250 μM L-[2,3,3-2H3]serine. To quantify the enrichment of [13C]formate into purines, thymidine, and methionine, cells were plated at 50% confluence in 100-mm plates and cultured in treatment medium, which consisted of defined αMEM lacking deoxyribonucleosides, hypoxanthine, and thymidine but supplemented with 250 μM [13C]formate, 0.05 μM 5-formyl-THF, 10 μM methionine, 0.2 mM glycine, 1 mg/liter pyridoxine, 26 mg/liter L-[5,5,5-2H3]leucine. Cells were cultured using treatment medium for a total of 8 days, and the medium was replaced every 2 days. Cells were harvested by washing with ice-cold phosphate-buffered saline, then trypsinized and pelleted by centrifugation. Cellular protein and nuclear DNA were isolated as described previously (27).

Protein pellets were suspended in 6 N HCl (100 μl) in vacuum hydrol-ysis tubes and heated at 100 °C for 20 h. The amino acids were purified by cation exchange chromatography, converted to heptafluorobutyrly n-propyl ester derivatives, and then separated on an HP-5MS column (30 cm × 0.25 mm). Isotopic enrichment was determined in electron capture negative ionization mode by gas chromatography–mass spectrometry using a model 6890 gas chromatograph and model 5973 mass spectrometer (Hewlett-Packard Corp., Palo Alto, CA). Selected ion monitoring was conducted at a mass-to-charge ratio m/z 519–523 for serine, m/z 305–308 for dehydroalanine, m/z 349–353 for leucine, m/z 367–370 for methionine, and m/z 293–295 for glycine.

DNA samples were dried under nitrogen and suspended in formic acid (1 ml) and hydrolyzed at 150 °C for 45 min in vacuum hydrolysis tubes. After drying at 55 °C under nitrogen, the bases were dissolved in 0.2 ml of a 1:1 mixture of N,O-bis[trimethylsilyl]trifluoroacetamide/1% trimethylchlorosilane (Pierce) and acetonitrile and heated at 140 °C for 30 min. The trimethylsilyl-base derivatives were separated on an
HP-5MS column. Isotopic enrichment was determined in positive ionization mode by gas chromatography-mass spectrometry using a model 6890 gas chromatograph and model 5973 mass spectrometer (Hewlett-Packard Corp.). Selected ion monitoring was conducted at a mass-to-charge ratio m/z 255–257 for thymine, m/z 280–283 for adenine, and m/z 368–371 for guanine.

**Formate Suppression Assay**—This assay was developed to quantitate the efficiency of de novo purine nucleotide biosynthesis (as monitored by the incorporation of [14C]formate into purine deoxynucleotides) relative to purine nucleotide biosynthesis through the salvage pathway (as monitored by the incorporation of [3H]hypoxanthine into purine nucleotides). Cells were maintained in minimal essential medium, α modification (HyClone), supplemented with 11% dialyzed fetal bovine serum. The tracer medium consisted of Defined Minimal Essential Medium (HyClone) that lacked glucose, serine, methionine, hypoxanthine, and folate but was supplemented with 200 μM methionine, 20 nM 5-formyl-THF, 2 nM [3H]hypoxanthine, and 20 μM [14C]formate. Cells were split 1:6 and grown in 6-well plates at 37 °C, 5% CO2 in tracer medium until confluent and harvested. The cell pellets were stored at −40 °C. Nuclear DNA was isolated using a DNA Blood Kit from Qiagen, and isotope levels were quantified on a Beckman LS6500 Scintillation Counter in Dual DPM mode. For HPLC separation of nucleosides, cells were split 1:3 into tracer medium in 100-cm² culture plates, grown to confluence, and harvested. DNA was digested to nucleosides prior to separation by HPLC, using procedures described elsewhere (28). Peaks corresponding to nucleosides were identified from standards, collected, and isotope levels quantified.

**RESULTS AND DISCUSSION**

**Cloning of the Human FDH cDNA and Generation of a Tetracycline-inducible Stable Cell Line**—A stable SH-SY5Y cell line that expresses the human FDH cDNA from a tetracycline-inducible promoter (pcDNA4/T/O myc–HisB vector, Invitrogen) was isolated (referred to as SH-SY5YFDH). FDH protein was not detected in nontransfected SH-SY5Y cells; SH-SY5YFDH cells cultured in the absence of tet exhibited very low but detectable levels of FDH protein (1.5-fold over densitometry background) (Fig. 2A). SH-SY5YFDH cells cultured with tet (SH-SY5YFDH + tet) exhibited 13.3-fold greater FDH protein levels than present in SH-SY5YFDH cells culture without tet. These cell lines were used to validate the previously ascribed metabolic roles of FDH in folate-mediated one-carbon metabolism. Specifically, the effects of both low and high level FDH expression on: 1) intracellular folate concentrations, 2) the 10-formyl-THF/THF ratio, 3) SAM levels, and 4) flux within the folate-mediated one-carbon metabolic network were examined.

**Effect of FDH Expression on Cellular Folate Concentrations**—SH-SY5YFDH cells cultured in the absence and presence of tet displayed a 57 and 64% reduction, respectively, in total cellular folate concentrations compared with the parent cell line, SH-SY5Y (Table 1). These results do not support a role for FDH in maintaining cellular folate stores by sequestering or trapping cellular THF. Reductions in cellular folate can result from decreased rates of cellular transport and/or increased rates of turnover (5). Therefore, the effects of FDH expression on both FR expression and folate catabolism were investigated. FR facilitates transport of folic acid into the cell (30), and its expression increases with cellular folate deficiency in cell culture models (31). SH-SY5YFDH cells cultured in the presence or absence of tet displayed increased FR protein levels relative to nontransfected cells as determined by Western blot analyses (Fig. 2B), consistent with FDH expression creating a functional folate deficiency in SH-SY5Y cells. These data also indicate that the FDH-induced decrease in cellular folate concentrations is not the result of impaired FR-mediated folate transport.

The effects of FDH expression on rates of folate catabolism, as evidenced by the generation of the folate degradation product para-aminobenzoylelgutamate (pABG), were determined in culture medium following exposure to [3H]formyl-THF as described previously (25). SH-SY5YFDH cells cultured in the absence and presence of tet generated 20% and 2.5-fold more [3H]pABG, respectively, compared with SH-SY5Y cells (Fig. 3). These results indicate that FDH expression accelerates folate catabolism in these cells, which may contribute to the decreased folate concentrations. However, the modest increase in pABG generation between the SH-SY5Y cells and SH-SY5YFDH cells cultured without tet was not statistically significant, suggesting that increased rates of folate catabolism alone may not account for the observed differences in folate concentrations assuming pABG is the primary product of folate catabolism (Fig. 3). Furthermore, FDH expression had no effect on the average length of intracellular folate polyglutamate chains (Table 1). Increased rates of folate catabolism

### Table 1

| Cell line          | Total folate pmol | % of total folate | Mean polyglutamate lengtha |
|--------------------|------------------|------------------|-----------------------------|
| SH-SY5Y            | 41.7 ± 4.3       | (100)            | 5.2 ± 0.1                   |
| SH-SY5YFDH         | 17.8 ± 1.8       | (43)             | 5.6 ± 0.1                   |
| SH-SY5YFDH + tet   | 14.6 ± 0.3       | (36)             | 5.4 ± 0.2                   |

* a Values indicate number of glutamates.

**Effect of increased human FDH expression on the generation of pABG in culture medium.** SH-SY5Y cells expressing the human FDH cDNA were cultured with defined αMEM containing 250 nM [65]H[^3]formyl-THF. Aliquots of the culture medium were removed at defined time points (0 and 5.5 h) immediately following the addition of [65]H[^3]formyl-THF. Prior to analysis, samples were spiked with unlabeled pABG, 5-formyl-THF, and 5-methyl-THF. Fractions (1 ml) were collected by HPLC, and the tritium for each fraction was quantified by liquid scintillation. Sample A corresponds to SH-SY5Y cells, sample B corresponds to SH-SY5Y cells, and sample C corresponds to SH-SY5Y + tet cells. All values represent duplicate measures, and error bars are ± S.E.

**FIGURE 2.** Western blot analysis of stable cell lines expressing the human FDH cDNA. Cell lysates from SH-SY5Y, SH-SY5YFDH, and SH-SY5YFDH + tet were analyzed by Western blot to determine the expression levels of FDH (A) and FR and GAPDH (B) protein in crude cell extracts (60 μg of protein/lane). Human FR is increased greater than 2-fold in cell lines expressing the FDH cDNA, whereas GAPDH protein levels were unchanged.

**FIGURE 3.** Effect of increased human FDH expression on the generation of pABG in culture medium. SH-SY5Y cells expressing the human FDH cDNA were cultured with defined αMEM containing 250 nM [65]H[^3]formyl-THF. Aliquots of the culture medium were removed at defined time points (0 and 5.5 h) immediately following the addition of [65]H[^3]formyl-THF. Prior to analysis, samples were spiked with unlabeled pABG, 5-formyl-THF, and 5-methyl-THF. Fractions (1 ml) were collected by HPLC, and the tritium for each fraction was quantified by liquid scintillation. Sample A corresponds to SH-SY5Y cells, sample B corresponds to SH-SY5Y cells, and sample C corresponds to SH-SY5Y + tet cells. All values represent duplicate measures, and error bars are ± S.E.
have been demonstrated to increase the average folate polyglutamate chain length, because folate monoglutamates, which have a lower affinity for folate-binding proteins, are more susceptible to degradation (25).

Effected FDH Expression on the Distribution of Folate One-carbon Derivatives and Homocysteine Remethylation—FDH has been proposed to regulate the supply of folate-activated one carbon units by modulating the 10-formyl-THF/THF ratio. Cellular folates are distributed nearly equally in two compartments, the mitochondria and cytoplasm, and these compartments are not in equilibrium (10). 10-Formyl-THF is the primary folate derivative found in the mitochondria of SH-SY5Y cells (24) and should not be available to FDH expressed in the cytoplasm. Surprisingly, the low level of FDH expression in SH-SY5Y/FDH cells increased the cellular formyl-THF/THF ratio by 10-fold compared with SH-SY5Y cells (Table 2). Formyl folates accounted for over 82% of total cellular folates, and the relative levels of both THF and 5-methyl-THF were reduced by ~60% (Table 2). In contrast, high FDH expression decreased the 10-formyl-THF/THF ratio. THF accounted for over 46% of total folate in SH-SY5Y/FDH+tet cells (Table 2).

Prior to these studies, two potentially contradictory metabolic roles had been ascribed to FDH: 1) the removal of excess one-carbon units from the folate-activated one-carbon pool by depleting 10-formyl-THF pools (19), and 2) the generation of folate-activated one-carbons by channeling THF to cSHMT and MTHFD1, two enzymes that catalyze the synthesis of folate-activated one-carbon units (Fig. 1). The results from this study provide evidence that both of these metabolic roles may be operative in cells depending on the expression level of FDH. Low levels of FDH expression increased the 10-formyl-THF/THF ratio, consistent with a role for FDH in facilitating the generation of folate-activated one-carbon units. On the other hand, high levels of FDH expression reduced dramatically the supply of folate activated one-carbon units such that THF became the primary cellular folate derivative. These results are consistent with the known physical properties of FDH. FDH exhibits a higher affinity for THF polyglutamates ($K_d = 15 \text{ nM}$) than 10-formyl-THF polyglutamates ($K_d = 0.9 \text{ \mu M}$). FDH, when expressed at low levels, is expected to bind cellular THF. Under these conditions, total FDH enzymatic activity is expected to be lower compared with its THF-channeling activity to MTHFD1 and cSHMT. Elevated levels of FDH expression provide additional binding sites for 10-formyl-THF, leading to the depletion of 10-formyl-THF pools through the FDH-catalyzed conversion of 10-formyl-THF to THF (the specific activity of FDH exceeds the rate of 10-formyl-THF synthesis). In summary, FDH can either generate or remove folate-activated one-carbon units depending on its level of expression.

Both high and low FDH expression reduced the concentrations of 5-methyl-THF from 37% in nontransfected cells to <8% in FDH-expressing cells (Table 2). Interestingly, other studies have demonstrated that decreases in 5-methyl-THF concentrations impair cellular folate accumulation. The human A222V polymorphic variant of the methyl-10-formyltetrahydrofolate reductase gene encodes a thermolabile enzyme with reduced ability to convert 5,10-methylene-THF to 5-methyl-THF (32). The polymorphic allele impairs the accumulation of 5-methyl-THF in red blood cells in favor of formylated folates (33). Human carriers of the A222V polymorphism also exhibit decreased folate content in red blood cells (33). Therefore, the ability to maintain cellular 5-methyl-THF concentrations may be an important determinant of total cellular folate concentrations.

The effect of FDH-induced depletion of the 5-methyl-THF pool on the homocysteine remethylation cycle was investigated by quantifying cellular SAM and SAH concentrations (Table 3). Because 5-methyl-THF is necessary for folate-dependent homocysteine remethylation, reduction in steady-state 5-methyl-THF concentrations may impair SAM synthesis (Fig. 1). FDH expression at both high and low levels reduced SAM concentrations and the SAM/SAH ratio by 2-fold, indicating that the FDH-induced reduction in 5-methyl-THF and/or total cellular folate concentrations impaired the homocysteine remethylation cycle and reduced the methylation potential (SAM/SAH ratio) of the cell. This inhibitory effect of FDH on homocysteine remethylation is a novel finding that was not anticipated.

Effect of FDH Expression on Purine Biosynthesis and Cell Growth—The one-carbon unit of 10-formyl-THF supplies the C2 and C8 carbon for de novo purine biosynthesis catalyzed by the enzymes 5′-phosphoribosyl-5-aminoimidazole-4-N-succinocarboxamide transformylase (2). Because FDH catalyzes the conversion of 10-formyl-THF to THF, it has been proposed that increased FDH expression may impair cell growth by depleting the supply of one-carbon units (in the form of 10-formyl-THF) for purine biosynthesis. The effect of increased FDH expression on cell proliferation rates was examined in SH-SY5Y. Cell lines expressing the human FDH cDNA were cultured with defined αMEM that lacked nucleosides, nucleotides, serine, and glycine (Fig. 4A). SH-SY5Y cells expressing FDH exhibited lower growth rates relative to nontransfected cells, indicating that increased levels of FDH marginally inhibited cell prolifera-

| Effect of FDH expression on the distribution of folate derivatives in SH-SY5Y cells |
|-----------------------------------------------|
| **Cell line** | **THF** | **5-Methyl-THF** | **Formylated THF (5,10-methylene-THF, 5-formyl-THF, 10-formyl-THF, and 5-formimino-THF)** | **Formyl-THF/THF ratio** |
|----------------|---------|----------------|-------------------------------------------------|--------------------------|
| SH-SY5Y        | 34.8 ± 4.3 | 36.9 ± 0.7 | 28.3 ± 4.6 | 0.8          |
| SH-SY5Y/FDH    | 9.7 ± 1.1  | 7.8 ± 3.7  | 82.4 ± 3.2 | 8.5          |
| SH-SY5Y/FDH+tet | 46.2 ± 8.3 | 5.4 ± 2.1  | 48.5 ± 10.2 | 1.0          |

*p p values, one-way analysis of variance.

Effect of FDH expression on SAM and SAH levels in SH-SY5Y cells

| Effect of FDH expression on SAM and SAH levels in SH-SY5Y cells |
|-----------------------------------------------|
| **Cell line** | **SAM/SAH** | **SAM** | **SAH** |
|----------------|-------------|--------|--------|
| SH-SY5Y        | 0.07 ± 0.05 | 1.2 ± 0.1 | 16.9 ± 0.6 |
| SH-SY5Y/FDH    | 0.03 ± 0.02 | 0.6 ± 0.05 | 18.0 ± 0.9 |
| SH-SY5Y/FDH+tet | 0.04 ± 0.01 | 0.7 ± 0.2  | 17.5 ± 2.4 |

*p values, one-way analysis of variance.
tion in these cells (Fig. 4A). The experiment was repeated using aMEM (containing purine nucleosides, serine, and glycine). The presence of purine nucleosides in the culture medium did not rescue proliferation rates in these cell lines; SH-SY5Y cells expressing FDH exhibited lower growth rates compared with nontransfected cells (Fig. 4B). Therefore, expression of FDH in this SH-SY5Y cell line affected cell growth, but the observed FDH-induced growth inhibition did not result from the inhibition of de novo purine biosynthesis.

The effect of FDH expression on folate-dependent de novo purine biosynthesis was determined directly using a formate-suppression assay, which assesses the ability of the purine salvage pathway to suppress the incorporation of formate into purines through the de novo pathway. In this experiment, cells were grown in the presence of [14C]formate and [3H]hypoxanthine. [14C]Formate is incorporated into purine nucleotides through de novo purine nucleotide biosynthesis, which is folate-dependent, whereas [3H]hypoxanthine is incorporated into purine nucleotides through the salvage pathway (29). FDH expression had no effect on the 14C/[3H] ratio in nuclear DNA or purine deoxynucleosides, demonstrating that, although FDH can deplete 10-formyl-THF pools, this activity does not impair the incorporation of formate into purines (de novo purine biosynthesis) relative to salvage pathway synthesis in SH-SY5Y cells (29). MTHFS may bind and channel 10-formyl-THF to the de novo purine biosynthesis pathway, thus protecting this pathway from FDH-mediated depletion of 10-formyl-THF pools.

**TABLE 4**

| Cell line                  | 14C/[3H] ratio | DNA dpm | da dpm | dG dpm |
|---------------------------|----------------|---------|--------|--------|
| SH-SY5Y                   | 1.8 ± 0.1      | 3.8 ± 0.7| 1.1 ± 0.1|
| SH-SY5YFDH                | 2.0 ± 0.2      | 4.0 ± 0.4| 1.2 ± 0.2|
| SH-SY5YFDH + tet          | 1.9 ± 0.06     | 3.8 ± 0.4| 1.2 ± 0.07|

*a da, deoxyadenosine; dG, deoxyguanosine.

**Effect of FDH Expression on the Flux of Formate between the Thymidylate and Methionine Biosynthetic Pathways**—To determine if SHMT or other folate-dependent reactions were compensating for the FDH-induced depletions in 10-formyl-THF levels, the incorporation of exogenously supplied [13C]formate into methionine and serine (present in cellular protein), and purines (present in nuclear DNA), was determined in SH-SY5Y cells as a function of FDH expression (Fig. 5 and Table 5). The one-carbon precursor pools used for synthesis of purines, serine, and methionine can be derived either from exogenous labeled formate or from endogenously synthesized unlabeled serine metabolites (Fig. 5). Surprisingly, FDH expression had little effect on [13C]formate enrichments into methionine or serine (Table 5A). Furthermore, FDH expression had little impact on the enrichment of the 10-formyl-THF precursor pool used for purine synthesis (Table 5B). These results indicate that FDH expression does not influence the enrichment of [13C]formate into the folate-activated one-carbon pool (Fig. 5 and Table 5B).

To establish directly the contribution of cSHMT-derived one-carbon units into the thymidine and methionine pools in the presence and absence of FDH expression, metabolic isotope tracer experiments were also performed using cells labeled with [1-3H]serine. Methylene-THF that is supplied by cSHMT and incorporated directly into methionine or thymidine is expected to retain the two deuterium atoms (CD2) that are present on the hydroxymethyl group of serine (Fig. 5). Alternatively, if [1-3H]serine enters the mitochondria, the hydroxymethyl group will be released from the mitochondrion as formate, and formate will contain a single deuterium atom (CD1). Previous isotope tracer studies have shown that up to 90% of one-carbon units used for cytoplasmic folate metabolism in mammalian cells are derived from formate generated by mitochondrial serine metabolism (27). During the derivatization of serine for GC analysis, most of the serine is converted to dehydroalanine, with loss of the proton at the C2 position (27). Consequently, the m/z distribution of dehydroalanine isotopes gives the isotopic distributions at the C3 position of serine. Table 6 shows that 73–77% of the isotopically labeled cellular serine pool that provided serine for protein synthesis retained both deuteriums on the C3 carbon (as assessed by dehydroalanine labeling). Serine that contained one deuterium in the C3 position (~30%) was the result of serine that was resynthesized from glycine using the CD2-THF pool (Fig. 5). If the methionine and dTMP one-carbon units were only derived from cSHMT, the mass +2 species of these metabolites should be ~70% of the labeled species. The data in Table 6 indicate that the proportions are much lower, demonstrating that most of the one-carbon units used for cytoplasmic methionine and dTMP synthesis were derived from formate that originated from mitochondrial serine metabolism. Previously, we have shown that in MCF-7...
Tetrahydrofolate metabolism through its conversion to 5,10-methylene-THF by the enzyme SHMT. Single carbons generated from L-[2,3,3-2H3]serine in mitochondria contain one deuterium atom. Mitochondrial-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism.

**TABLE 5**

**Effect of FDH expression on formate metabolism in SH-SY5Y cells**

| Cell line | Met | dT | Met/dT | DHA |
|-----------|-----|----|--------|-----|
| SH-SY5Y   | 0.31| 0.56| 0.53   | 73  |
| SH-SY5YFDH| 0.29| 0.54| 0.60   | 76  |

A) Isotopic enrichments of serine and methionine in protein

| Cell line | dA | dG |
|-----------|----|----|
| SH-SY5Y   | 0.36| 0.36|
| SH-SY5YFDH| 0.33| 0.34|
| SH-SY5YFDH+tet | 0.37| 0.35|

B) Isotopic enrichment of cytosolic 10-formyl-THF pool used for purine synthesis

methionine. Low levels of FDH expression (as observed in SH-SY5YFDH cells cultured without tet) did not affect the incorporation of CD2 into either methionine or dTMP, whereas cells with high levels of FDH expression (SH-SY5YFDH+tet cells) exhibited a 60% increase in CD2 in methionine and dTMP compared with the nontransfected cell line. These results demonstrate that FDH expression increased the flux of cSHMT derived one-carbons into methionine and dTMP, although the overall enrichment of cSHMT derived one-carbons into methionine was similar to that observed in nontransfected cells as evidenced by the higher percentage of CD2 in dTMP compared with the nontransfected cell line. FDH values represent the amount of serine used for protein synthesis, serving as an internal control. Enrichment of [2,3,3-2H3]serine in thymidine (dT) was determined by analysis of nuclear DNA. All values are expressed as the percent of L-[2,3,3-2H3]serine-derived carbon that contains two deuterium atoms in the target compound (the ratio of carbons containing two deuterium atoms in the target compound divided by the total number of carbons that contain one or two deuterium atoms × 100). Two independent experiments were performed with duplicate measurements for each sample, and similar values were obtained for both experiments. The results from one experiment are shown.
and dTMP did not exceed 8%. Furthermore, the data indicate that if FDH depresses rates of formate incorporation into methionine and dTMP, the cSHMT enzyme can only compensate by making very minor contributions to the one-carbon network by providing one-carbon units for methionine and dTMP synthesis.

CONCLUSIONS

These studies examined several purported metabolic functions of FDH in a cell culture model. As predicted from its in vitro activity, FDH was demonstrated to affect the 10-formyl-THF pools in cells, but FDH-induced alterations in 10-formyl-THF levels did not impair de novo purine biosynthesis or confer a purine requirement to these cells as has been speculated. We also demonstrate that increased FDH expression did not result in increased cellular folate accumulation in neuroblastoma, and therefore its folate tight-binding properties do not enable this enzyme to serve as a reservoir for cellular folate. The results of this study have identified a new and unanticipated metabolic role for FDH; both 5-methyl-THF and SAM levels were sensitive to FDH expression level.

The effect of FDH on cellular methylation reactions warrants additional study. Neither the metabolic role of FDH within the folate-dependent one-carbon network, nor its effects on total cellular folate content is easily predicted from its catalytic, biochemical, or biophysical properties. Furthermore, its physiological function and impact on one-carbon metabolism and cellular folate content may vary in different cell types and be dependent on the expression of other tissue-specific folate-dependent enzymes and binding proteins.

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