THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 42, pp. 29386–29396, October 17, 2014
Published in the U.S.A.

Mammalian Mitochondrial and Cytosolic Folylpolyglutamate Synthetase Maintain the Subcellular Compartmentalization of Folates*

Received for publication, July 1, 2014, and in revised form, August 15, 2014 Published, JBC Papers in Press, August 27, 2014, DOI 10.1074/jbc.M114.593244

Scott A. Lawrence[‡], Steven A. Titus[§], Jennifer Ferguson[§], Amy L. Heineman[§], Shirley M. Taylor[§¶], and Richard G. Moran[‡§1]

From the Departments of [‡]Pharmacology and Toxicology and [¶]Microbiology and Immunology, and the [§]Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298

Background: Folylpolyglutamates are in the cytosol and mitochondria and the enzyme that makes these compounds, folylpolyglutamate synthetase (FPGS) is in both compartments.
Results: Folylpolyglutamates cannot traverse mitochondrial membranes in either direction.
Conclusion: Subcellular isoforms of FPGS are required to establish and maintain subcellular folate compartmentalization and function.
Significance: Mitochondrial folates are a separate metabolic pool not in equilibrium with cytosol.

Folylpoly-γ-glutamate synthetase (FPGS) catalyze the addition of multiple glutamates to tetrahydrofolate derivatives. Two mRNAs for the *fpgs* gene direct isoforms of FPGS to the cytosol and to mitochondria in mouse and human tissues. We sought to clarify the functions of these two compartmentalized isoforms. Stable cell lines were created that express cDNAs for the mitochondrial and cytosolic isoforms of human FPGS under control of a doxycycline-inducible promoter in the AUXB1 cell line. AUXB1 are devoid of endogenous FPGS activity due to a premature translational stop at codon 432 in the *fpgs* gene. Loss of folates was not measurable from these doxycycline-induced cells or from parental CHO cells over the course of three CHO cell generations. Likewise, there was no detectable transfer of folate polyglutamates either from the cytosol to mitochondria, or from mitochondria to the cytosol. The cell line expressing cytosolic FPGS required exogenous glycine but not thymidine or purine, whereas cells expressing the mitochondrial isoform required exogenous thymidine and purine but not glycine for optimal growth and survival. We concluded that mitochondrial FPGS is required because folate polyglutamates are not substrates for transport across the mitochondrial membrane in either direction and that polyglutamation not only traps folates in the cytosol, but also in the mitochondrial matrix.

Folates are required for cell growth and survival because of their use as cofactors in one-carbon transfer reactions involved in thymidylate synthesis, purine nucleotide synthesis, and amino acid metabolism (1). The forms of folate in circulation in mammals have 1 mol of glutamic acid/mol of pteroic acid. These folate monoglutamates, chiefly 5-methyltetrahydrofolate (2), are readily transported into cells by the reduced folate carrier and the proton-coupled folate transporter but can facilely efflux from cells on these same transporters (3–5). To achieve the concentration of folate cofactors required for metabolism, mammalian cells use the enzyme folylpoly-γ-glutamate synthetase (FPGS)[2] (EC 6.3.2.17) for the addition of several glutamate moieties successively linked to the γ-carboxylate group on the glutamic acid intrinsic to all folates (6). The products of this reaction, the folylpoly-γ-glutamates, are thought to be poor substrates for efflux for the reduced folate carrier and proton-coupled folate transporter systems despite the fact that these transporters are clearly bidirectional (5, 7). Using inward transport as an index of acceptance of polyglutamates by transporters, even the diglutamate of methotrexate showed inward transport by the reduced folate carrier at only a few percent of that for methotrexate (8). Transporters of the multidrug-resistance associated protein family have been described as potential efflux mechanisms that accept polyglutamates of classical folate antimetabolites as substrates (9, 10). Nevertheless, the polyglutamate derivatives of methotrexate clearly efflux from the cell much slower than the parent drug, although this persistence also reflects tight binding to dihydrofolate reductase (4, 7). The actual rate of efflux of folylpolyglutamates or polyglutamate derivatives of methotrexate or other antifolates from the cell either intact or after cleavage to monoglutamates has been difficult to directly estimate.

The cellular requirement for FPGS was first demonstrated by the fact that Chinese hamster ovary (CHO)-derived AUXB1 cells that lack FPGS enzyme activity, are auxotrophic for purines, thymidine, and glycine because they are unable to retain folates (11). These cells were isolated by selection for the simultaneous acquisition of auxotropy for all these pathways after ethyl methanesulfonate mutagenesis of CHO cells. Likewise, selection experiments using bromodeoxyuridine and UV

* This work was supported, in whole or in part, by National Institutes of Health Grant R01-CA-39687.
[1] To whom correspondence should be addressed: Massey Cancer Center, 401 College St., Richmond, VA 23298-0035. Tel.: 804-828-5783; E-mail: rmoran@vcu.edu.

[2] The abbreviations used are: FPGS, folylpoly-γ-glutamate synthetase; SHMT, serine hydroxymethyltransferase; VDAC, voltage-dependent anion channel; GCS, glycine cleavage system; MFT, mitochondrial folate transporter; MEM-α, minimal essential medium-α.

light as a mutagen were performed on the hamster fibroblastic V79 cell, yielding the V79 glycine, purine, and thymidine auxotrophic (ght$^-$) cell lines (12). AUXB1 cells stably transfected with cDNA encoding only the cytosolic form of FPGS no longer required purines and thymidine for survival, but still required glycine (13), indicating that purines and thymidine were synthesized using cytosolic folates. In addition, cells with deficiencies in mitochondrial folate metabolism, attributed to either a lack of mitochondrial serine hydroxymethyltransferase (SHMT) activity (glyA cells) (14) or lack of transport of folates through the inner mitochondrial membrane (glyB cells) (15), require glycine (but not thymidine and purine) for survival, demonstrating that glycine metabolism requires a folate-dependent mitochondrial process not complemented by cytosolic metabolism.

The use of glycine to produce 1-carbon units via the folate-dependent glycine cleavage system (GCS) is specific to mitochondria. Glycine metabolism and mitochondrial folate metabolism are key to cancer cell growth with high levels of glycine consumption in cancer cells and knockdown of the GCS impairing tumor cell proliferation (16–19). Thus, it is apparent that cell survival and proliferative programs depend on the intracellular serine and glycine metabolism which, in turn, rely on communication of the cytosolic and mitochondrial folate metabolism.

It has been known for some time that both folate polyglutamates and FPGS are found in both the cytosol and mitochondria (20). The mRNA encoding the human mitochondrial FPGS isoform has a sequence for an additional 42-amino acid N-terminal peptide that serves as a mitochondrial leader targeting sequence; the remaining sequence of the human cytosolic and mitochondrial FPGS isoforms is identical (13). The presence of FPGS in both the cytosolic and mitochondrial compartments would suggest that folylpolyglutamates cannot be transported through the mitochondrial inner membrane. However, there has been controversy about this point for some time (13, 21, 22) and the concept that folylpolyglutamates can efflux from the mitochondria intact appears in influential reviews (*e.g.* Ref. 23). We have questioned the functions for subcellular isoforms of FPGS and have directly studied the flux of folate polyglutamates across mitochondrial membranes in both directions, and the plasma membrane in the efflux direction using a doxycycline-inducible system that generated two cell lines that express the individual isoforms of FPGS at or near endogenous levels. We present evidence that folate polyglutamates are not substrates for transport across the mitochondrial membrane in either direction nor can they efflux from intact cells at measurable rates over several cell generation times. This impermeability of the mitochondrial and plasma membranes to the polyanionic folate polyglutamates explains the cellular requirement for subcellular isoforms of FPGS for optimal growth and survival.

EXPERIMENTAL PROCEDURES

Cultures—CEM cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS), CHO and AUXB1 cells in MEM-α (which contains nucleosides) and 10% FBS. An AUXB1 cell line stably transfected with the full-length *fpgs* cDNA encoding the mitochondrial isoform of FPGS cloned into pcDNA3, M7.2 (13), was cultured in the same media as AUXB1 cells but with 1 mg/ml of G418. The *tet*-inducible cFPGStet and mFPGStet cell lines were grown in MEM-α supplemented with 10% tetracycline-free FBS, 0.5 mg/ml of G418, and 0.2 mg/ml of hygromycin. AUXB1 and AUXB3 cells were kindly supplied by Professor Gordon Whitmore (Ontario Cancer Center), and V79 and V79ght1 cells were a gift from Professor C. C. Chang (Michigan State University).

Sequencing of the Hamster fpgs Gene—In a previous study (13), we had serendipitously isolated the most 5′-region of the hamster FPGS cDNA. The remaining sequence of the CHO-K1 *fpgs* cDNA was PCR amplified as overlapping cDNA clones using hamster primers known from the 5′-region sequence combined with primers chosen from 3′-regions of high identity between mouse and human *fpgs*. Subsequently, two full-length cDNA clones were isolated from a CHO cDNA library (Stratagene) and sequenced. cDNAs for AUXB1, AUXB3, V79, and V79ght1 cells were amplified from reverse transcribed RNA by PCR, and the PCR fragments were sequenced *en masse*. The sequences of the parental CHO and V79 *fpgs* were previously submitted to GenBank^{TM} and can be retrieved under accession numbers AF283646 and AF283647.

Preparation of tet-activated fpgs cDNA Constructs—Full-length cDNAs for the protein coding regions of human cytosolic and mitochondrial FPGS were excised from a pcDNA3-based plasmid (13) with HindIII and XbaI, ligated into the pTRE-Tight vector (BD Biosciences, Clontech) and resequenced.

Generation of a fpgs cDNA Construct with a Mutated Cytosolic Translational Start Site—The downstream ATG start codon in full-length mitochondrial *fpgs* cDNA corresponding to the translational start site for cytosolic FPGS was mutated to GCC (encoding an alanine) using overlap extension PCR to eliminate any generation of cytosolic FPGS from these mitochondrial constructs via any inherent internal ribosome entry site.

Generation of Cells Stably Transfected with cFPGStet and mFPGStet in a Tet-on Expression System—AUXB1 cells were plated at 1×10^5 cells/100-mm dish and triplicate plates were transfected with 5 μg of a Tet-ON vector (BD-Clontech) encoding a *tet*-responsive transcriptional activator, using a calcium phosphate procedure (13). Selective media (MEM-α with nucleosides, 10% tetracycline-free FBS, and 1 mg/ml of G418) was applied 48 h after transfection. Sixty colonies resistant to G418 were transferred to 48-well plates 10 days after transfection and each clonal derivative was seeded in three wells of a 12-well plate. Two of these wells were transiently transfected with pTRE-Tight-Luc (Clontech), a plasmid encoding luciferase under doxycycline control, using Lipofectamine 2000 (Invitrogen), and the third well was treated with Lipofectamine 2000 alone. Doxycycline (1 μg/ml) was added to one of the vector-transfected wells for 24 h, and the cells in all wells were separately harvested and assayed for luciferase expression. A clone, AUXB1tet18, that expressed very low levels of luciferase in the absence of doxycycline and >100-fold induction of luciferase in the presence of doxycycline was expanded and used for subsequent transfections.

AUXB1tet18 cells were co-transfected with a pTRE-Tight plasmid containing a cDNA encoding human mitochondrial

FPGS, human cytosolic FPGS, or human mitochondrial FPGS in which the cytosolic translational start site was mutated and a pTRE-hyg plasmid encoded a hygromycin resistance cassette. Stable transfectants were selected with 1 mg/ml of G418 and 0.2 mg/ml of hygromycin and clones were isolated after 10 days and mass cultured. Clones stably transfected with cytosolic FPGS were screened for growth in media without purines and thymidine (with glycine), and with or without 1 μg/ml of doxycycline. Of the 84 cytosolic clones screened, two clones met the criteria of no growth in the absence of doxycycline, but growth in the presence of doxycycline approached that seen in full media (with purines, thymidine, and glycine). Clones stably transfected with mitochondrial FPGS were screened in a similar fashion, but in media without glycine (with purines and thymidine) with or without 1 μg/ml of doxycycline. Of the 100 mitochondrial clones screened, 11 clones met the criteria of no growth in the absence of doxycycline, and growth in the presence of doxycycline. One of the two cytosolic clones and one of the 11 mitochondrial clones that met the growth criteria were selected to represent the cFPGStet and mFPGStet cell lines used in functional studies.

Preparation of Whole Cell Lysates—Adherent cells were scraped into ice-cold phosphate-buffered saline (PBS) with protease inhibitors (Roche Complete EDTA-free protease inhibitor pellets). Cell pellets were collected, washed with PBS with protease inhibitors, and lysis buffer (62.5 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 5% 2-mercaptoethanol with protease inhibitors) was added. This solution was repetitively passed through a 21-guage needle and centrifuged at 20,000 $\times$ *g* for 5 min at 4 °C. Protein concentrations were determined using the Bradford reagent. FPGS expression in cFPGStet and mFPGStet cells was induced by the addition of 1 μg/ml of doxycycline starting 48 h prior to cell harvest.

Isolation of Cytosolic and Mitochondrial Protein Fractions—Subcellular fractions were isolated from whole cell lysates as previously described (24) using a Dounce homogenizer and differential centrifugation with protease inhibitors present in all solutions. The mitochondrial pellet was resuspended in lysis buffer and sonicated. Ice-cold trichloroacetic acid (TCA) was added to the cytosolic fraction to a final concentration of 6%. This solution was incubated on ice for 30 min and centrifuged at 7800 $\times$ *g* for 15 min at 4 °C. After centrifugation, the TCA-containing supernatant was discarded, an equivalent volume of ice-cold acetone was added, and protein was collected at 7800 $\times$ *g* for 15 min at 4 °C. The acetone wash and centrifugation steps were repeated two additional times. The precipitate was resuspended in lysis buffer and sonicated.

Western Blotting—Previous experiments estimated that 32 μg of cytosolic protein and 16 μg of mitochondrial protein were present in a CHO cell pellet equivalent to 50 μg of whole cell lysate. Hence, any Western blots that compared FPGS levels in cellular compartments from equal numbers of cells used 50 μg of protein for whole cell lysates, 32 μg for cytosolic protein, and 16 μg for mitochondrial protein. Protein samples were brought to boiling in lysis buffer for 5 min, loaded into a SDS-PAGE gel, electrophoresed in 250 mM glycine, 25 mM Tris, and 0.1% SDS, pH 8.3, and transferred to nitrocellulose membrane at 100 V for 45 min in 192 mM glycine, 25 mM Tris, and 0.1% SDS, pH 8.3. Nonspecific protein binding was blocked with 5% nonfat dry milk, 10 mM Tris, pH 7.4, 0.14 M NaCl, and 0.1% Tween 20 for 1 h, followed by three 5-min washes in Tris-buffered saline with 0.05% Tween 20 (TBS-T). All membranes were incubated with the primary antibody diluted in TBS-T and 5% nonfat dry milk at 4 °C for 16 h. The membrane was washed three times in TBS-T and incubated for 1 h at room temperature with a secondary antibody diluted in TBS-T and 5% nonfat dry milk, followed by three washes in TBS-T. Protein complexes were detected using a chemiluminescent substrate kit and the membrane were exposed to x-ray film. Mouse monoclonal antibody against human FPGS (25) was used at a dilution of 1:500, goat antibody against mouse IgG at 1:15,000, mouse antibody against tubulin at 1:10,000, goat antibody against mouse IgG at 1:10,000, rabbit antibody against voltage-dependent anion channel (VDAC) at 1:500, and goat antibody against rabbit IgG at 1:5,000.

Folate Retention in CHO, cFPGStet, and mFPGStet Cells Exposed to (6S)-[^{3}H]5-Formyltetrahydrofolate—HPLC-purified (6*S*)-[3′,5′,7,9-^{3}H]5-formyltetrahydrofolate was obtained from Moravek Radiochemicals and purified before use. The isotope was HPLC purified before use, using a 15-cm $\times$ 3-μm Luna C18 column developed with a 20 to 55% methanol gradient in water containing 5 mM tetrabutylammonium phosphate paired ion (PicA, Waters) run over 30 min. Fractions corresponding to (6*S*)-5-formyltetrahydrofolate were adsorbed onto a 0.25-ml column of DEAE-cellulose, the column was rinsed with low salt and labeled material was eluted with 0.5 ml of 0.25 M ammonium acetate to remove the PicA. Purified labeled (6*S*)-5-formyltetrahydrofolate exactly co-chromatographed with unlabeled standard. Cells were plated in MEM-α with 1 μg/ml of doxycycline at about 5% confluence. After 24 h, culture medium was changed to RPMI 1640 folate-free media containing 0.05 μM (6*S*)-[^{3}H]5-formyltetrahydrofolate at 0.25 μCi/ml, supplemented with 10% tetracycline-free FBS, 1% penicillin/streptomycin, 0.5 mg/ml of G418, 0.2 mg/ml of hygromycin, and 1 μg/ml of doxycycline. Cells were incubated with isotope for 24 h, washed twice with PBS, and fresh MEM-α with 1 μg/ml of doxycycline was added. Cells were harvested immediately and then at 12-h intervals thereafter. At all times during these experiments, the density of the labeled cultures was well below confluence. Cytosolic and mitochondrial protein fractions were isolated as above. Isolated mitochondrial pellets were dissolved in 0.5 ml of 75 mM NaOH, then neutralized with 0.5 ml of 100 mM HCl; radioactivity in mitochondrial and cytosolic fractions was determined by scintillation counting. Cell numbers at the time of removal of isotope were determined on plates that had been identically treated, but not exposed to radioactivity. Total cellular folate contents (as fmol) of (6*S*)-[^{3}H]5-formyltetrahydrofolate and metabolites were obtained by summing those in the cytosolic and mitochondrial compartments.

Colony Formation Assays—Cells were exposed to doxycycline at concentrations of 0–5 μg/ml for 48 h prior to these experiments. Approximately 150 cells from these doxycycline-conditioned cultures were plated in 60-mm dishes and grown in basal media (MEM-α media without purines, thymidine, or glycine, with 10% dialyzed tetracycline-free FBS, 1% penicillin/streptomycin, 0.5 mg/ml of G418, and 0.2 mg/ml of hygromycin) with and without combinations of supplements (100 μM

TABLE 1
Sequence of FPGS cDNAs from hamster cells auxotrophic for thymidine, purine, and glycine

Cell line	Genotype
Parental CHO-K1	Protein coding region was 1761 nucleotides (587 codons), which was 85% identical to human FPGS and 87% identical to mouse FPGS at the amino acid level.[a]
Parental V79	Identical to CHO-K1 except for silent polymorphisms at codons 94 and 187and two differences: Ala to Ser at codon 173 and Gln to Glu at codon 536.[b]
AUXB1	C to T transition at nucleotide 1294 generating a premature stop at codon 432.
AUXB3	G to A transition at nt 416; R139Q mutation.
V79ght1	G103R at one allele; G220E at the other.

[a] Submitted to GenBank accession number AAK69545.
[b] GenBank accession number AAK69546.

inosine, 5.6 μM thymidine, and 50 mg/liter of glycine) and doxycycline concentrations. Fresh media containing these additions was added every other day for 8–10 days, and colonies were then fixed, stained, and manually counted.

Determination of cFPGStet and mFPGStet Cell Growth Rates—The growth rate of mFPGStet and cFPGStet cells was determined over the course of 96 h. Cells were plated at 2.5×10^4 cells/well in basal media with 1 μg/ml of doxycycline and the indicated growth supplements. Cells were detached with trypsin/EDTA with the indicated time thereafter, and counted with an electronic particle counter (Coulter Electronics, model Z1).

RESULTS

FPGS Mutations in ght$^-$ Hamster Cells—Two sets of somatic cell mutants have been previously selected for simultaneous acquisition of auxotrophy for thymidine, preformed purines, and glycine, following chemical and UV-induced mutagenesis (11, 12). We obtained these cell lines and found that all of these somatic cell mutant lines had point mutations in the *fpgs* open reading frame by sequencing RT-PCR-derived fragments and comparison with the sequence of parental CHO-K1 and V79 hamster lung cells (Table 1). The hamster *fpgs* gene had an open reading frame corresponding to 587 amino acids, the same length as the human FPGS, with 85% identity to the human protein sequence (Fig. 1); the hamster FPGS sequence contained start codons at positions 1 and 42, again identical to positions of the translational start sites for mitochondrial and cytosolic FPGS in *Homo sapiens.* The AUXB1 cell, which is devoid of FPGS enzyme activity (11), had a premature stop codon at residue 432; this stop codon truncates two-thirds of the predicted C-terminal domain of the human protein, including the extreme C-terminal peptide that is conserved from bacteria to man (Fig. 1). The AUXB3 cells had a single point mutation, R139Q, in the FPGS coding region responsible for the diminished catalytic activity of the FPGS expressed in this cell; this mutation modifies an arginine that is invariant in the sequences of FPGS in eukaryotes, placed in the critical Ω loop of this protein (Fig. 1). Both cell lines appeared to be hemizygous at the FPGS locus, based on the fact that selection for revertants to prototrophy in either cell usually resulted in reversion of the parental CHO *fpgs* sequence (data not shown). In addition, sequencing of a genomic fragment amplified from AUXB1 DNA demonstrated a single mutant sequence at *fpgs* nucleotide 1294, indicating that AUXB1 cells contained only a single *fpgs* allele. The V79 hamster fibroblasts selected for ght auxotrophy by Chang and colleagues (12) had two clear nucleotide peaks at codons 103 and 220 not present in V79 cell cDNA. Sequencing of individual cDNA clones spanning this region demonstrated two alleles of the *fpgs* gene in V79ght1 cells, one with the G103R mutation and one with the G220E (Table 1); Gly-103 is invariant across species and Gly-220 is highly conserved (Fig. 1). Interestingly, the G220E mutation was previously reported to account for inactivation of one allele of the *fpgs* gene in mouse leukemia cells selected for resistance to the antifolate lometrexol (26). In the experiments of this study, the AUXB1 cell was used for complementation with human *fpgs* cDNAs.

FPGS Expression in CHO, AUXB1, and AUXB1 Cells Stably Transfected with cDNA for fpgs under a Viral Promoter—Two previous studies examined the cellular roles for FPGS isoforms using constructs containing mitochondrial leader sequences from either a heterologous gene (21) or the endogenous *fpgs* mitochondrial leader sequence (13). The constructs used strong viral promoters that would have resulted in high level of expression of FPGS. For instance, the M7.2 cell line is an AUXB1-derived cell line from one of those studies (13) stably transfected with a cDNA encoding the mitochondrial isoform of human FPGS constructed in pcDNA3 under a CMV promoter; this cell line no longer required supplementation with purines, thymidine, or glycine for survival. Such results have previously been interpreted to imply that folylpolyglutamates can be made in the mitochondria and exported to the cytosol intact (21, 22), although this conclusion was dependent on whether cells complemented with cDNAs for mitochondrial FPGS delivered enzyme only to mitochondria. In the interim since those studies, robust antibodies have become available for human FPGS that would allow this question to be addressed. Subcellular protein fractions from parental CHO cells, AUXB1, and M7.2 cells were probed by immunoblotting, using a peptide antibody raised against human FPGS residues 531–541 (25). This antibody was also able to detect hamster FPGS (Fig. 2, *A* and *B*). FPGS was found in the cytosol and mitochondria of CHO cells, but was not detected in either compartment of AUXB1 cells (Fig. 2, *A* and *B*). The M7.2 cell line had superphysiologic levels of FPGS protein in both the mitochondria and cytosol. We concluded that M7.2 cells, and probably other transfected cell lines that expressed FPGS using strong viral promoters, were not suitable for the study of the role of FPGS in subcellular fractions and to overall folate metabolism. The concept that folylpolyglutamates effluxed intact from the mitochondria to the cytosol of mammalian cells intact also appeared to require re-investigation.

Inducible Expression of FPGS Isoforms in Stably Transfected cFPGStet and mFPGStet Cells—The expression of human FPGS isoforms at endogenous levels from cDNA constructs encoding the endogenous mitochondrial leader peptide or missing this sequence should allow proper subcellular trafficking. We created such a set of cell lines using a doxycycline-inducible Tet-ON expression system; one cell line, denoted mFPGStet, contained the full-length *fpgs* cDNA previously demonstrated (13) to target the protein to mitochondria, whereas the cFPGStet cell line was made by transfecting the *fpgs* cDNA that produced only cytosolic FPGS protein under

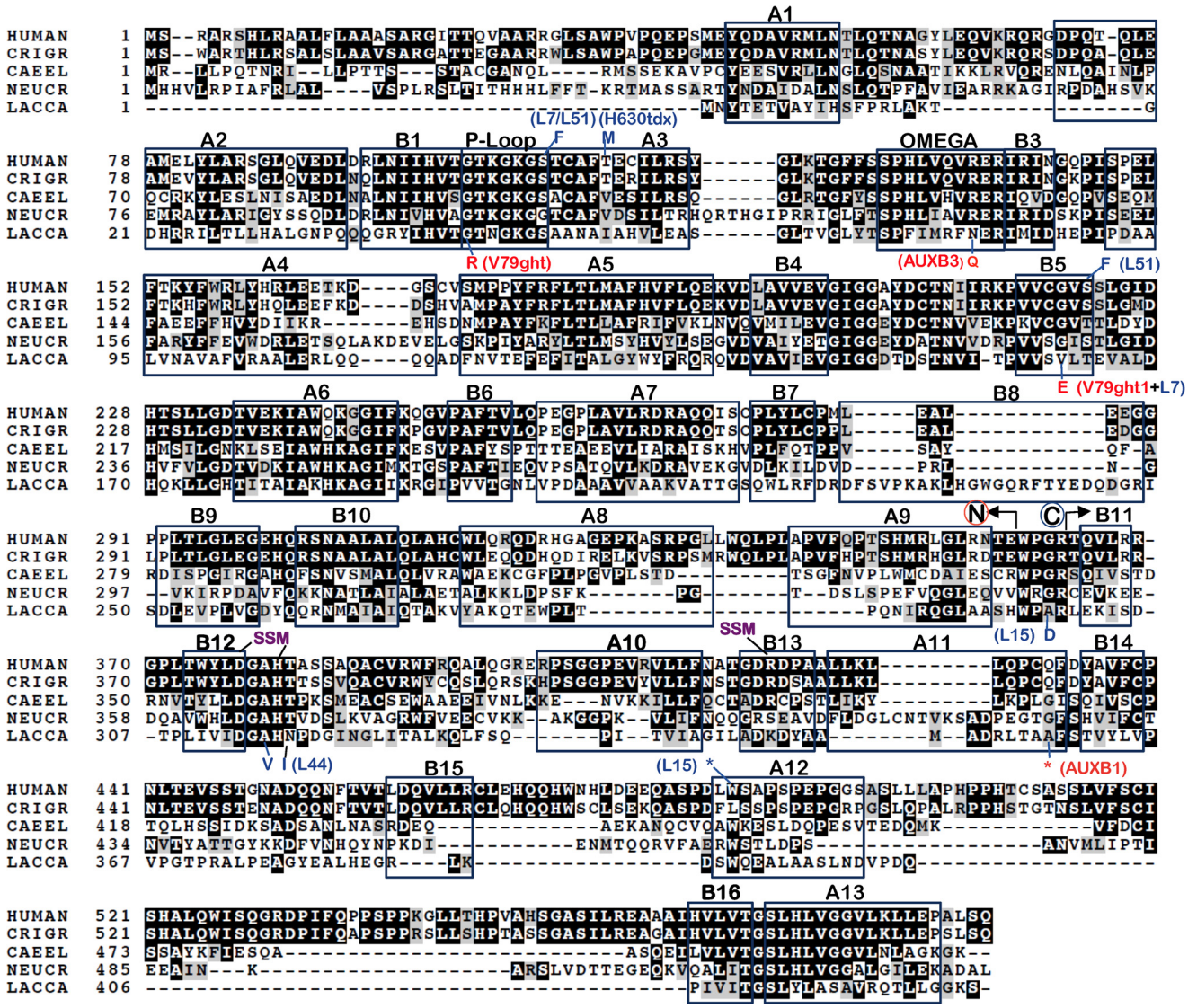

FIGURE 1. **Sequence alignment of FPGS from human, hamster, *Caenorhabditis* elegans, *Neurospora crassa,* and *Lactobacillus casei*.** The position of every mutation known that diminishes the enzyme activity of mammalian FPGS is shown, as well as the positions of point mutations in AUXB1, AUXB3, and V79ght1 cell lines. The mutations denoted as L7, L15, L44, and L51 are mutations found in L1210 cell lines selected for resistance to lometrexol (26), *SSM* marks three residues involved in substrate binding and catalysis with human FPGS by site-directed mutagenesis (27), *H460tdx* indicates a mutation found in a non-small cell lung cancer cell selected for resistant to tomudex (28). The positions of the mutations found in AUXB1, AUXB3, and V79ght1 cells (Table 1) are noted, as is the peptide marking the transition from the amino-terminal domain (*circled N*) and carboxyl-terminal domain (*circled C*) from the crystal structure of the *L. casei* FPGS (29, 30). The A and B regions represent α helices and β strands in that structure.

doxycycline control. Lysates from these cell lines, as well as from untransfected hamster (CHO) and human (CEM) cell lines were probed for FPGS expression in the presence and absence of doxycycline (Fig. 2*B*). Neither cFPGStet nor mFPGStet cells expressed detectable amounts of FPGS in the absence of doxycycline. However, in the presence of 1 μg/ml of doxycycline, FPGS protein was observed in both stably transfected cell lines at or near the levels detected in human and hamster cells (Fig. 2*B*).

The subcellular expression of FPGS isoforms in cFPGStet and mFPGStet cells was examined. The cell lines were exposed to a range of doxycycline concentrations and subcellular protein fractions were isolated and probed for FPGS expression by immunoblotting. Although cFPGStet cells contained FPGS in the cytosol, no FPGS protein was detected in mitochondria (Fig. 3*A*) even after extended development of the blots. In the mFPGStet cells, FPGS was detected in mitochondria at levels similar to the expression of cFPGStet in the cytosol at equivalent doxycycline concentrations (Fig. 3, *A* and *B*). However, very low levels of enzyme were also detected in the cytosol of mFPGStet cells upon longer exposure of film (Fig. 3*B*); we estimated that the FPGS remaining in the cytosol of mFPGStet cells was about 3–6% of the total cellular FPGS. The purity of subcellular fractions probed for FPGS expression was clear from immunoblots with antibodies against a mitochondrial-specific protein, VDAC, and against a cytosolic protein not found in mitochondria, tubulin (Fig. 3, *A* and *B*). Hence, it was unlikely that the FPGS detected in the cytosol of mFPGStet cells resulted from cross-contamination with the mitochondrial protein fraction. We considered two possibilities that could explain why cells transfected with a cDNA encoding a mitochondrially targeted FPGS protein also had low levels of cytosolic FPGS protein: 1) cells used an internal ribosomal entry site to access the in-frame

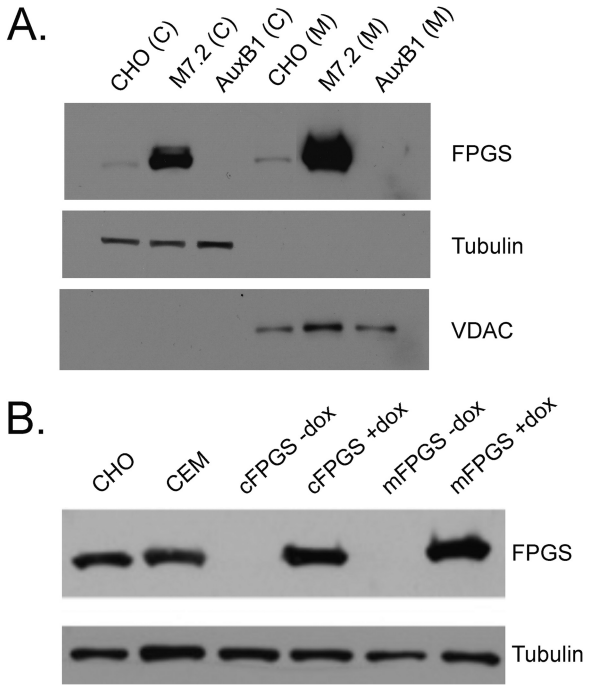

FIGURE 2. *A*, subcellular expression of FPGS in CHO, M7.2, and AUXB1 cells. Cytosolic (C)(32 μg) and mitochondrial (M)(16 μg) protein fractions from CHO, M7.2, and AUXB1 cells were probed for FPGS expression by immunoblotting. These protein fractions were also probed for cytosol-specific tubulin and mitochondria-specific VDAC expression to estimate protein loading and subcellular fraction purity. *B*, FPGS expression in CHO, CEM, and doxycycline-inducible cFPGStet and mFPGStet cells. Total cellular protein (50 μg) from CHO and CEM cells, and from cFPGStet and mFPGStet cells, grown in the presence (+*dox*, 1 μg/ml) and absence (−*dox*) of doxycycline for 48 h, was probed for FPGS expression by immunoblotting. These protein fractions were also immunoblotted for tubulin to estimate equivalent protein loading.

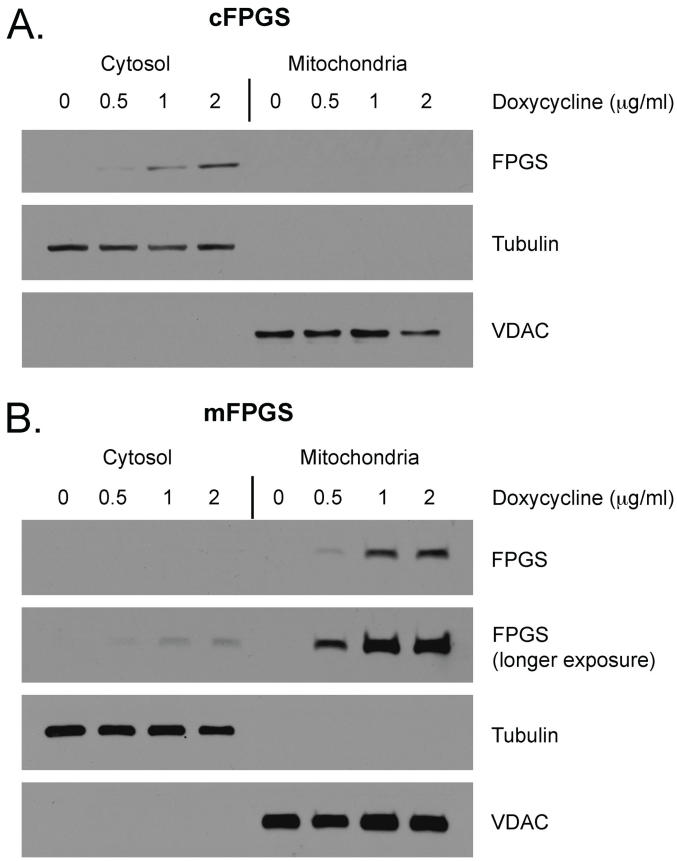

FIGURE 3. **Subcellular expression of FPGS in cFPGStet and mFPGStet cells.** Cytosolic (32 μg) and mitochondrial (16 μg) protein fractions from cFPGStet (*A*) and mFPGStet (*B*) cells exposed to various doxycycline concentrations for 48 h were probed for FPGS expression by immunoblotting. These protein fractions were also probed for tubulin and VDAC expression to estimate protein loading and subcellular fraction purity.

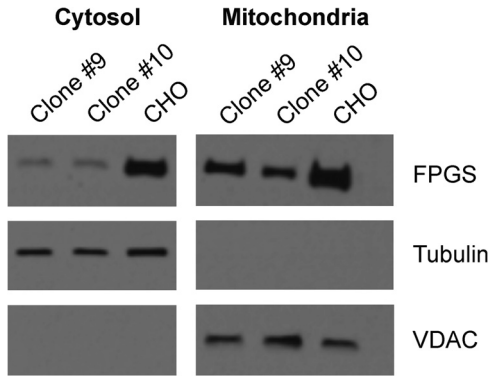

FIGURE 4. **Subcellular expression of FPGS in AUXB1 cells transfected with a mutated, full-length fpgs cDNA.** Cytosolic (32 μg) and mitochondrial (16 μg) protein from CHO cells and two clones of AUXB1 cells stably transfected with a *fpgs* cDNA, in which the translational start codon corresponding to the cytosolic protein was mutated, were probed for FPGS expression by immunoblotting. These protein fractions were also probed for tubulin and VDAC expression to estimate equivalent protein loading and subcellular fraction purity.

downstream ATG site used to generate cytosolic protein, or 2) the rate of mitochondrial import of the expressed FPGS protein was slow enough that protein translation in the cytosol resulted in low but detectable levels of cytosolic protein in transit to the mitochondria.

Subcellular Expression of FPGS from a Full-length cDNA That Lacks the Downstream Cytosolic Start Site—The full-length *fpgs* cDNA that encodes the mitochondrial FPGS isoform also contains the translational start site for the cytosolic FPGS isoform in-frame. If this start site was being accessed by an internal ribosomal entry site to translate cytosolic FPGS protein from the full-length *fpgs* cDNA transfected into mFPGStet cells, then mutation of the downstream ATG codon should prevent any internal ribosomal entry site activity.[3] The downstream ATG codon in the full-length *fpgs* cDNA was mutated to encode an alanine residue; this cDNA was stably transfected into AUXB1 cells and 10 clonal isolates were selected and expanded, and the cytosolic and mitochondrial protein fractions from these clones were probed for FPGS expression; surprisingly, all clones contained detectable amounts of FPGS in the cytosol. Of the 10 clones, only four clones expressed FPGS in mitochondria at amounts equivalent to or less than parental hamster cells; two of these clones are shown in Fig. 4. VDAC and tubulin antibodies indicated that neither subcellular fraction was contaminated with protein from the other compartment (Fig. 4). These data suggests that use of an internal ribosomal entry site was not responsible for producing cytosolic FPGS in cells transfected with mitochondrially targeted *fpgs* cDNA and it appears that a fraction of the mitochondrially targeted FPGS expressed in these cells is detectable in the cytosol while in transit to the mitochondria following translation from a cDNA-encoded mRNA. Within the limitations of this

[3] A similar approach was described briefly in a prior symposium paper (22).

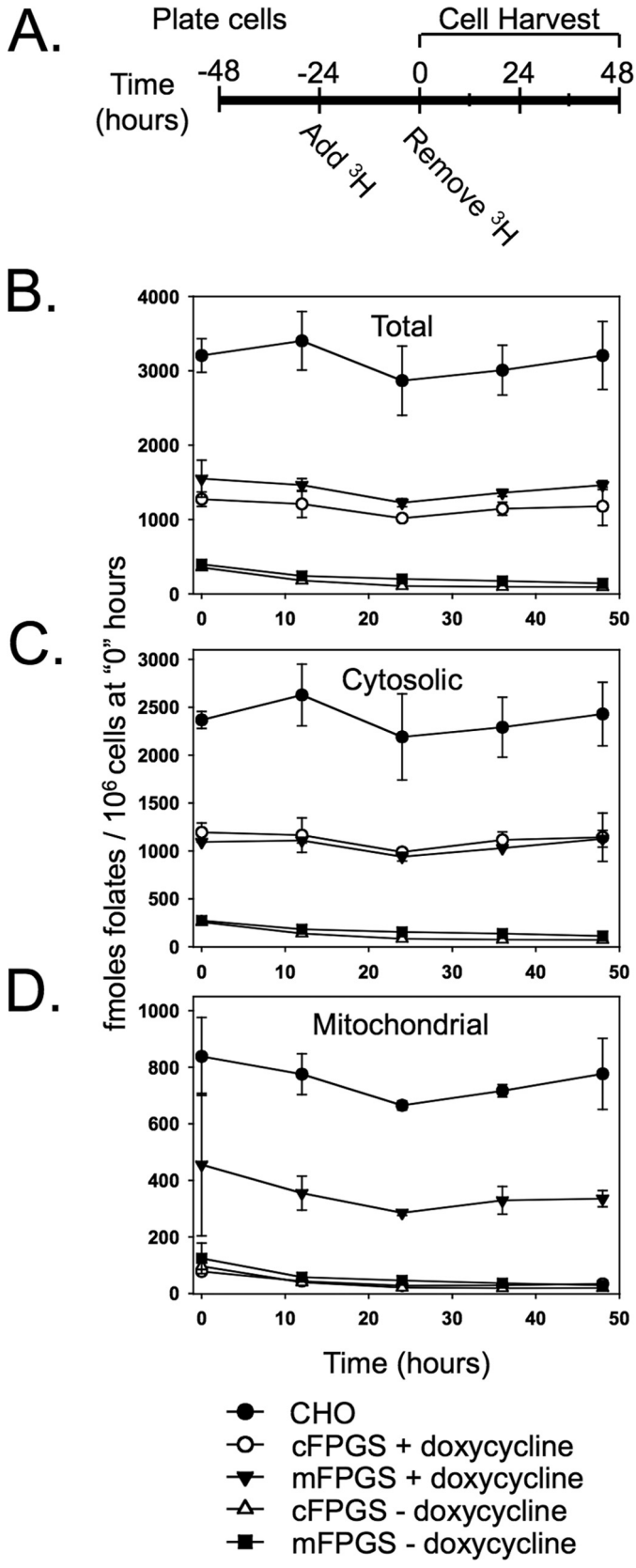

FIGURE 5. **Subcellular accumulation of folate polyglutamates in CHO, cFPGStet, and mFPGStet cells.** *A*, schematic of experimental design. All cells were plated 48 h prior to the "0 h" time point in *B–D*, and cFPGStet and mFPGStet cells were exposed to 1 μg/ml of doxycycline, where indicated. Twenty-four h prior to 0 h, cells were incubated with 0.05 μM (6*S*)-[^{3}H]5-formyltetrahydrofolate as the only source of folate in the media. After 24 h exposure to isotope, labeled medium was replaced with isotope-free RPMI 1640, cells were harvested, and subcellular fractions were isolated at the indicated time after removal of isotope. Radioactivity in the entire cell population was determined at each time point and expressed relative to cells present at removal of isotope so that the intracellular retention of isotope is indicated, not the labeled folate content per million cells. During the period shown in *panels B–D*, total cell populations expanded by 4 – 8-fold.

subcellular distribution, we used cFPGStet and mFPGStet cell lines to study folate distribution across plasma and mitochondrial membranes and the metabolic role of each FPGS isoform.

Subcellular Retention of Folate Polyglutamates in cFPGStet and mFPGStet Cells—To directly investigate the movement of folate polyglutamates across the mitochondrial membranes, cells were exposed to (6*S*)-[^{3}H]5-formyltetrahydrofolate for 24 h, after which the isotope was removed and cells were allowed to grow for up to an additional 48 h in isotope-free media (Fig. 5*A*); all of the cells in each culture were harvested as a function of time after removal of label from the medium and ^{3}H content of the entire intracellular compartment was determined. In these experiments, radiolabeled folates that were not polyglutamated by FPGS would presumably efflux from cells and the retention of radiolabeled folates would only occur through FPGS-mediated polyglutamation. Indeed, cFPGStet and mFPGStet cells did not retain any radiolabel in the absence of doxycycline and only accumulated significant levels of isotope in the presence of doxycycline (Fig. 5, *B–D*). The low levels of radiolabeled folates present in cells in the absence of FPGS expression effluxed efficiently when transferred to label-free medium (Fig. 5). Remarkably, CHO, cFPGStet, and mFPGStet cells contained the same amount of labeled folates after a 48-h isotope-free period as they did immediately after isotope exposure ("0 h") (Fig. 5); during this period of time, these CHO-derived cell lines doubled three times. To our knowledge, this is the most direct evidence presented to date that cellular folylpolyglutamates are not permeable to the plasma membrane and do not efflux once made. Folates were not detected in mitochondria of cFPGStet cells despite the presence of a cytosolic folate pool (Fig. 5, *C* and *D*), proving that transport of intact cytosolic folate polyglutamates into mitochondria does not occur. Only the mFPGStet cells (and CHO cells) contained folates in mitochondria, establishing the idea that the role of mitochondrial FPGS was to trap folate in the mitochondrial matrix. Most interestingly, the level of mitochondrial folates in wild-type CHO cells and mFPGStet cells was stable for the duration of the experiment; hence, efflux of folate polyglutamates from mitochondria was not apparent (Fig. 5*D*) under conditions in which we can safely say, from the lack of folates in mitochondria in cFPGStet cells, that folate polyglutamates cannot pass into the mitochondria. Cytosolic folates were, somewhat surprisingly, observed in both cFPGStet and mFPGStet cells in the presence of doxycycline (Fig. 5*C*). However, this is consistent with the presence of detectable FPGS in this compartment in both cell lines (Fig. 3, *A* and *B*). Nevertheless, we can say that the flux of folate polyglutamates across the mitochondrial membrane in either direction was not detectable over a time interval equivalent to three CHO cell generations: had mitochondrial folates transited intact to the cytosol but had not been capable of re-entry into the mitochondria, labeled mito-

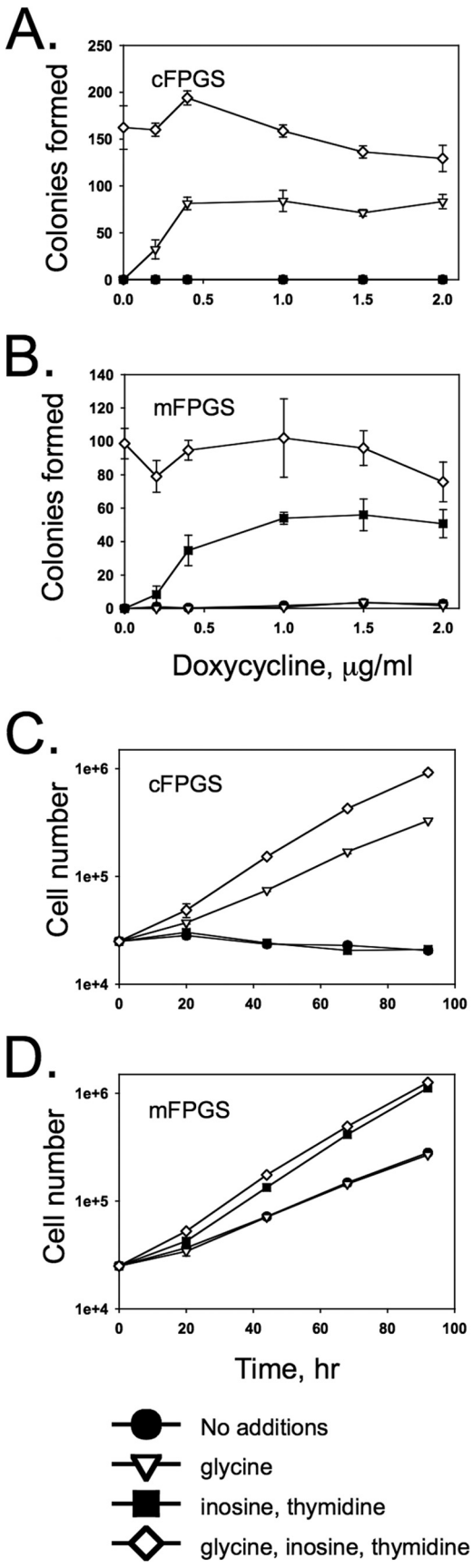

FIGURE 6. **Growth and survival of doxycycline-inducible cFPGStet and mFPGStet cells.** *A* and *B*, cells were grown in MEM-α supplemented with tetracycline-free dialyzed serum and the indicated concentrations of doxycycline for 48 h prior to use. Thereafter, ~150 cells were plated in basal media with tetracycline-free dialyzed serum, with combinations of glycine, thymidine, and inosine or in basal media lacking purines, thymidine, and glycine and with the indicated concentrations of doxycycline. For colony formation, cFPGStet (*A*) and mFPGStet (*B*) cells received fresh experimental media containing identical supplementation and doxycycline every other day for 8–10 days, after which colonies were fixed, stained, and counted. For growth rate studies, cFPGStet (*C*) and mFPGStet (*D*) cells were grown in basal media supplemented with 1 μg/ml of doxycycline and combinations of thymidine, inosine, and glycine. Every 24 h, cells were counted and split to a density of 25,000 cells/well in fresh experimental media containing identical supplementation and doxycycline. The cumulative increase in cell number is plotted *versus* time.

chondrial folates would have diminished over the time course shown in Fig. 5*D* for CHO and mFPGStet cells, and labeled cytosolic folates would have increased. The data of Fig. 5 were fit to a linear regression model to test the significance of the slopes observed. The mean slopes of the change in total folate content of CHO, cFPGS with doxycycline, and mFPGS with doxycycline was 0.15 $\pm$ 0.04% per h or a loss of 2.3% of the total pool in one 16-h generation time. The change in cytosolic folate content was 0.039 $\pm$ 0.05% per h, or less than 1% in one generation time; the rate of loss estimated from mitochondrial fractions for CHO and mFPGS cells was 0.37% per h or 5.9% in one generation time. These rates of change were not significantly different from 0 with p values ranging from 0.44 to 0.95, so we concluded that there is no evidence for loss of folates over 3 generations times.

Role of Cytosolic and Mitochondrial FPGS in Cell Growth and Survival—Folate polyglutamates do not appear to be substrates for transport across the mitochondrial membranes, suggesting that the cell requires FPGS in the cytosol and mitochondria to establish and maintain separate pools of folate polyglutamates for subcellular metabolism. The effect of individual isoforms of FPGS on cell growth rates and clonogenic survival was examined (Fig. 6). In the absence of doxycycline, cFPGStet and mFPGStet cells required purines (inosine), thymidine, and glycine for survival (Fig. 6, *A* and *B*), reinforcing the requirement for FPGS in this model system. In the presence of sufficient doxycycline to drive expression of cytosolic FPGS (Fig. 3*A*), supplementation with glycine alone was sufficient to permit survival of cFPGStet cells; microscopic examination of plates supplemented with inosine and thymidine or with no supplementation indicated that they were completely free of colonies or residual adherent cells. This supports the concept that the glycine requirement of mammalian cells is due to a folate-dependent mitochondrial process (Fig. 6*A*). In the presence of sufficient doxycycline to drive mitochondrial FPGS production (Fig. 3*B*), inosine and thymidine but not glycine were required to permit survival of mFPGStet cells (Fig. 6*B*). However, microscopic colonies were evident on plates treated with 1–2 μg/ml of doxycycline with mFPGStet cells exposed to media supplemented with only glycine or even with no additions. This suggested that sufficient cytosolic folates were present in the mFPGStet cells to permit some growth, although it was slow enough to result in an apparent lower rate of colony formation. Therefore, the growth rates of cFPGStet and mFPGStet cells were determined in media supplemented with combinations of thymidine, purines, and glycine.

cFPGStet and mFPGStet cells treated with 1 μg/ml of doxycycline grew optimally in media supplemented with purines,

thymidine, and glycine (Fig. 6, *C* and *D*). cFPGStet cells treated with doxycycline grew somewhat slower when supplemented only with glycine, and were unable to grow at all in media without any additions or in media supplemented only with inosine and thymidine (Fig. 6*C*). This agreed with the behavior of cFPGStet cells in clonogenic assays, and reinforced the idea that mitochondrial folate metabolism was carrying out a glycine-dependent step not supplied by cytosolic metabolism. mFPGStet cells treated with doxycycline grew normally in media supplemented with inosine and thymidine (Fig. 6*D*), as expected from the concept that thymidylate and *de novo* purine syntheses were cytosolic processes and were not complemented adequately by FPGS directed to the mitochondria. However, mFPGStet cells also exhibited growth, albeit distinctly slower, in media without any additions or in media supplemented only with glycine; this was in agreement with the presence of low levels of FPGS in the cytosol of mFPGStet cells (Fig. 3*B*) and the persistence of cells on the doxycycline-treated mFPGStet plates in the clonogenic experiments (see above). Overall, we concluded that FPGS was required in both cytosolic and mitochondrial compartments to stimulate folate metabolism sufficiently for optimal cell growth and survival and that this requirement reflected the fact that folylpolyglutamates do not pass through the plasma or mitochondrial membranes intact.

DISCUSSION

These studies were ultimately conducted to determine whether or not intact folate polyglutamates could cross the mitochondrial membrane. We directly examined the flux of folate polyglutamates across the mitochondrial membrane by incubating cells that had endogenous levels of FPGS only in the cytosol or chiefly in mitochondria using a modified pulse-chase experimental design with radiolabeled folate. To our knowledge, this was the first time that the flux of folate polyglutamates between compartments was followed in this manner and certainly the first time it was followed over the time scale of multiple cell generations. We definitively showed that cytosolic folate polyglutamates were unable to be transported into mitochondria of cFPGStet cells (Fig. 5). Therefore, using the experimental design of Fig. 5, which captured all cytosolic folates and all mitochondrial folates despite the expanding cell mass after removal of isotope, a decrease in mitochondrial folate polyglutamates would have been observed in mFPGStet cells or CHO cells if folate polyglutamates were transported out of mitochondria intact. However, no significant drop in mitochondrial folates was seen over 48 h. Furthermore, a concomitant increase in labeled cytosolic folate polyglutamates would have been observed if mitochondrial folates were transported into the cytosol. This was not observed. This evidence strongly suggests that folate polyglutamates are not substrates for transport across mitochondrial membranes in either direction, answering a fundamental question that has been difficult to approach.

A transporter is present in the inner mitochondrial membrane, the mitochondrial folate transporter (MFT, SLC25A32), which facilitates the entry of folates into the mitochondrial matrix (15, 24, 31). From the data of Fig. 5, it appears that the MFT does not accept folylpolyglutamates as substrates either in or out of the mitochondrial matrix. Folate monoglutamates clearly can both enter and exit mitochondria, presumably on the MFT. This is consistent with the report that AUXB1 cells require mitochondrial FPGS to establish mitochondrial folate pools (13)(also in the data of Fig. 5). If folate monoglutamates were unable to exit mitochondria, then transport of folates into mitochondria would be sufficient for retention of mitochondrial folates and mitochondrial FPGS would not be required. Yet, we clearly demonstrate that CHO-derived cells require mitochondrial FPGS to establish mitochondrial folate pools.

The most challenging aspect of these studies was to fastidiously create a cell line that contained FPGS in mitochondria but not in the cytosol. Every cell line that we designed to contain FPGS in mitochondria ultimately was found to have some detectable FPGS in the cytosolic compartment using a highly specific antibody (25) unavailable during previous studies on these metabolic fluxes. We showed that the low levels of cytosolic FPGS protein in mFPGStet was most likely not translated from the cytosolic *fpgs* start codon in mFPGStet cDNA but rather represented functional FPGS protein in the cytosol awaiting import into mitochondria. Although this finding is confounding, previous studies have demonstrated survival of cells with mutant FPGS with only 2–5% FPGS activity (32, 33). Perhaps this observation reflects that hamster cells do not efficiently translocate the human FPGS protein into mitochondria or, more likely, that the mRNA untranslated regions absent from cDNA play some role in directing mitochondrial import. The endogenous mitochondrial FPGS mRNA may well represent one of the nuclear-encoded mRNAs whose protein product is imported into mitochondrial co-translationally (34), an effect that is probably not faithfully mimicked with transfected cDNAs.

Serendipitously, we observed that total cellular folate polyglutamates, whether distributed between cytosolic and mitochondrial compartments or restricted just to the cytosol, were maintained in cells for remarkably long periods. There seems to be little other feasible explanation than the efflux mechanisms described for antifolates in previous studies do not, in fact, operate on folate polyglutamates, at least in the CHO cells used in this study.

The transfer of folates across the mitochondrial membrane would appear to only require a transport rate needed by the expansion of the mitochondrial mass during growth and proliferation. In contrast, the flux of 1-carbon units between the cytosol and mitochondria appears to be driven by the transport of serine, glycine, and formate across the mitochondrial membrane, consistent with the model of compartmentalized 1-carbon metabolism offered by Appling and colleagues (23, 35, 36). The complementation of deficiencies in mitochondrial FPGS and cytosolic FPGS by metabolites shown in Fig. 6 reflect the subcellular compartments' requirement of thymidine, purine synthesis, and glycine metabolism, but also reflect the role of the two compartments in flux of 1-carbon units. Most studies based on NMR of purines and thymidylate made from stable isotopes of serine and glycine in *Saccharomyces cerevisiae*, and in human cancer cells have indicated that the 1-carbon units used for purine synthesis in the cytosol stem largely from formate generated from serine and glycine by mitochondrial SHMT and the mitochondrial GCS (reviewed in Ref. 23). However, a recent metabolomics study indicates that cytosolic purine synthesis incorporate carbon 13-labeled glycine only by

direct incorporation by the glycinamide ribonucleotide synthetase reaction and not by 1-carbon transfers of C2 and C8 of the purine ring, and that the majority of C5-methyl groups of thymidylate come from serine and not mitochondrial glycine (37). Studies in intact mouse embryos and whole body studies in humans suggest that the majority of the methyl groups donated to *S*-adenosylhomocysteine are derived from mitochondrial folate metabolism and that the mitochondrial GCS generates as much as 20 times more 1-carbon units than needed for all methylation reactions (38, 39). Recent studies have broadened our perspectives on mitochondrial 1-carbon metabolism with the observation that the large percentage of mitochondrial glycine-derived 1-carbon units in mammalian-transformed cells are oxidized completely, generating a major proportion of cellular NADPH (37). The expression of SHMT isoforms in the cytosol and mitochondria have complicated the interpretation of the role of cytosolic serine in 1-carbon supply, but studies in yeast demonstrate that mitochondrial or cytosolic sources of 1-carbon units can be used to make purines, depending on the metabolic conditions (40). This flexibility in the use of mitochondrial and cytosolic sources of 1-carbon fragments may explain the fact that glycine supplementation alone allows robust growth and survival of CHO cells with FPGS only in the cytosol; it implies that, in the absence of mitochondrial folate metabolism, cytosolic folate metabolism can supply 1-carbon units for thymidine and purine synthesis.

It is a perplexing question why the glycine produced by cytosolic SHMT cannot enter the mitochondria and prevent cytotoxicity in cells with a mitochondrial deficiency of folate metabolism, whereas extracellular glycine can do so. Yet glyB cells, which lack the MFT (15), glyA cells, which have a mutation in the mitochondrial isoform of SHMT (14), or AUXB1 cells transfected with only cytosolic FPGS require only glycine for growth and survival (see Ref. 13 and Fig. 6). In addition, how glycine in the medium can complement a deficiency in mitochondrial folate metabolism is still not understood, in light of the fact that mitochondrial GCS is folate dependent. It is possible that in cFPGS cells, the exogenous glycine is used only for protein synthesis, whereas mitochondrial GCS is inactive, and mitochondrial protein synthesis initiation proceeds without *N*-formylmethionine (41).

Finally, the subcellular partitioning of folates suggests that cytosolic polyglutamation is likely in competition with mitochondrial import for a pool of folate monoglutamates. Indeed, Lin and Shane (20, 21) reference unpublished studies where high expression of cytosolic FPGS diminished mitochondrial folate accumulation. Thus, the cell must be able to control and coordinate the level of expression of subcellular FPGS isoforms, or possibly the relative expression of cytosolic FPGS and the MFT, to allow folate polyglutamates to accumulate in the cytosolic and mitochondrial compartments; the mechanism of this control is unknown.

Acknowledgments—We thank Professor Andrew Bognar for critical review of this manuscript. We also thank Dr. Kellie Archer for assistance with statistical analysis supported by the VCU Massey Cancer Center Biostatistics Shared Resource with funding from National Institutes of Health-NCI Cancer Center Grant P30 CA016059.

REFERENCES

1. Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland, Amsterdam
2. Ratanasthien, K., Blair, J. A., Leeming, R. J., Cooke, W. T., and Melikian, V. (1974) Folates in human serum. *J. Clin. Pathol.* **27,** 875–879
3. Zhao, R., Diop-Bove, N., Visentin, M., and Goldman, I. D. (2011) Mechanisms of membrane transport of folates into cells and across epithelia. *Annu. Rev. Nutr.* **31,** 177–201
4. Goldman, I. D., Lichtenstein, N. S., and Oliverio, V. T. (1968) Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. *J. Biol. Chem.* **243,** 5007–5017
5. Visentin, M., Diop-Bove, N., Zhao, R., and Goldman, I. D. (2014) The intestinal absorption of folates. *Annu. Rev. Physiol.* **76,** 251–274
6. Shane, B. (1989) Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vitam. Horm.* **45,** 263–335
7. Fry, D. W., Yalowich, J. C., and Goldman, I. D. (1982) Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high pressure liquid chromatography in the Ehrlich ascites tumor cell *in vitro*. *J. Biol. Chem.* **257,** 1890–1896
8. Sirotnak, F. M., Chello, P. L., Piper, J. R., and Montgomery, J. A. (1978) Growth inhibitory, transport and biochemical properties of the γ-glutamyl and γ-aspartyl peptides of methotrexate in L1210 leukemia cells *in vitro*. *Biochem. Pharmacol.* **27,** 1821–1825
9. Chen, Z. S., Robey, R. W., Belinsky, M. G., Shchaveleva, I., Ren, X. Q., Sugimoto, Y., Ross, D. D., Bates, S. E., and Kruh, G. D. (2003) Transport of methotrexate, methotrexate polyglutamates, and 17β-estradiol 17-(β-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. *Cancer Res.* **63,** 4048–4054
10. Kruh, G. D., and Belinsky, M. G. (2003) The MRP family of drug efflux pumps. *Oncogene* **22,** 7537–7552
11. McBurney, M. W., and Whitmore, G. F. (1974) Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells. *Cell.* **2,** 173–182
12. Chu, E. H., Sun, N. C., and Chang, C. C. (1972) Induction of auxotrophic mutations by treatment of Chinese hamster cells with 5-bromodeoxyuridine and black light. *Proc. Natl. Acad. Sci. U.S.A.* **69,** 3459–3463
13. Freemantle, S. J., Taylor, S. M., Krystal, G., and Moran, R. G. (1995) Upstream organization of and multiple transcripts from the human folylpoly-γ-glutamate synthetase gene. *J. Biol. Chem.* **270,** 9579–9584
14. Taylor, R. T., and Hanna, M. L. (1982) Folate-dependent enzymes in cultured Chinese hamster ovary cells: impaired mitochondrial serine hydroxymethyltransferase activity in two additional glycine-auxotroph complementation classes. *Arch. Biochem. Biophys.* **217,** 609–623
15. Titus, S. A., and Moran, R. G. (2000) Retrovirally mediated complementation of the glyB phenotype: cloning of a human gene encoding the carrier for entry of folates into mitochondria. *J. Biol. Chem.* **275,** 36811–36817
16. Moran, D. M., Trusk, P. B., Pry, K., Paz, K., Sidransky, D., and Bacus, S. S. (2014) KRAS mutation status is associated with enhanced dependency on folate metabolism pathways in non-small cell lung cancer cells. *Mol. Cancer Ther.* **13,** 1611–1624
17. Tedeschi, P. M., Markert, E. K., Gounder, M., Lin, H., Dvorzhinski, D., Dolfi, S. C., Chan, L. L., Qiu, J., DiPaola, R. S., Hirshfield, K. M., Boros, L. G., Bertino, J. R., Oltvai, Z. N., and Vazquez, A. (2013) Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. *Cell Death Dis.* **4,** e877
18. Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A. L., Kafri, R., Kirschner, M. W., Clish, C. B., and Mootha, V. K. (2012) Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science* **336,** 1040–1044
19. Zhang, W. C., Shyh-Chang, N., Yang, H., Rai, A., Umashankar, S., Ma, S., Soh, B. S., Sun, L. L., Tai, B. C., Nga, M. E., Bhakoo, K. K., Jayapal, S. R., Nichane, M., Yu, Q., Ahmed, D. A., Tan, C., Sing, W. P., Tam, J., Thiruganamam, A., Noghabi, M. S., Pang, Y. H., Ang, H. S., Mitchell, W., Robson, P., Kaldis, P., Soo, R. A., Swarup, S., Lim, E. H., and Lim, B. (2012) Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell* **148,** 259–272

20. Lin, B. F., Huang, R. F., and Shane, B. (1993) Regulation of folate and one-carbon metabolism in mammalian cells: III. role of mitochondrial folylpoly-γ-glutamate synthetase. *J. Biol. Chem.* **268,** 21674–21679
21. Lin, B. F., and Shane, B. (1994) Expression of Escherichia coli folylpolyglutamate synthetase in the Chinese hamster ovary cell mitochondrion. *J. Biol. Chem.* **269,** 9705–9713
22. Qi, H., Atkinson, I., Xiao, S., Choi, Y. J., Tobimatsu, T., and Shane, B. (1999) Folylpoly-γ-glutamate synthetase: generation of isozymes and the role in one carbon metabolism and antifolate cytotoxicity. *Adv. Enzyme Regul.* **39,** 263–273
23. Tibbetts, A. S, and Appling, D. R. (2010) Compartmentalization of Mammalian folate-mediated one-carbon metabolism. *Annu. Rev. Nutr.* **30,** 57–81
24. Perchiniak, E., Lawrence, S. A., Kasten, S., Woodard, B. A., Taylor, S. M., and Moran, R. G. (2007) Probing the mechanism of the hamster mitochondrial folate transporter by mutagenesis and homology modeling. *Biochemistry* **46,** 1557–1567
25. Dotzlaf, J., Carpenter, J., Luo, S., Miles, R. R., Fisher, D., Qian, Y. W., Ehsani, M., Wang, X., Lin, A., McClure, D. B., Chen, V. J., and Zuckerman, S. H. (2007) Derivation and characterization of monoclonal antibodies against human folypolyglutamate synthetase. *Hybridoma* **26,** 155–161
26. Zhao, R., Titus, S., Gao, F., Moran, R. G., and Goldman, I. D. (2000) Molecular analysis of murine leukemia cell lines resistant to 5,10-dideazatetrahydrofolate identifies several amino acids critical to the function of folylpolyglutamate synthetase. *J. Biol. Chem.* **275,** 26599–26606
27. Sanghani, S. P., Sanghani, P. C., and Moran, R. G. (1999) Identification of the three key residues in the C-terminal domain of human folylpolyglutamate synthetase by site-directed mutagenesis. *J. Biol. Chem.* **274,** 27018–27027
28. Drake, J. C., Allegra, C. J., Moran, R. G., and Johnston, P.G. (1996) Resistance to tomudex (D1694): multifactorial in human breast and colon cell lines. *Biochem. Pharmacol.* **51,** 1349–1355
29. Sun, X., Bognar, A. L., Baker, E. N., and Smith, C. A. (1998) Structural homologies with ATP- and folate-binding enzymes in the crystal structure of folylpolyglutamate synthetase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6647–6652
30. Sun, X., Cross, J. A., Bognar, A. L., Baker, E. N., and Smith, C. A. (2001) Folate-binding triggers the activation of folylpolyglutamate synthetase. *J. Mol. Biol.* **310,** 1067–1078
31. Lawrence, S. A., Hackett, J. C., and Moran, R. G. (2011) Tetrahydrofolate recognition by the mitochondrial folate transporter. *J. Biol. Chem.* **286,** 31480–31489
32. Lowe, K. E., Osborne, C. B., Lin, B. F., Kim, J. S., Hsu, J. C., and Shane, B. (1993) Regulation of folate and one-carbon metabolism in mammalian cells: II. Effect of folylpoly-gamma-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-gamma-glutamate specificity of metabolic cycles of one-carbon metabolism. *J. Biol. Chem.* **268,** 21665–21673
33. McCloskey, D. E., McGuire, J. J., Russell, C. A., Rowan, B. G., Bertino, J. R., Pizzorno, G., and Mini, E. (1991) Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. *J. Biol. Chem.* **266,** 6181–6187
34. Ahmed, A. U., and Fisher, P. R. (2009) Import of nuclear-encoded mitochondrial proteins: a cotranslational perspective. *Int. Rev. Cell Mol Biol* **273,** 49–68
35. Barlowe, C. K., and Appling, D. R. (1988) *In vivo* evidence for the involvement of mitochondrial folate metabolism in the supply of cytoplasmic one-carbon units. *Biofactors* **1,** 171–176
36. Appling, D. R. (1991) Compartmentation of folate-mediated one carbon metabolism in eukaryotes. *FASEB J.* **5,** 2645–2651
37. Fan, J., Ye, J., Kamphorst, J. J., Shlomi, T., Thompson, C. B., and Rabinowitz, J. D. (2014) Quantitative flux analysis reveals folate-dependent NADPH production. *Nature* **510,** 298–302
38. Lamers, Y., Williamson, J., Theriaque, D. W., Shuster, J. J., Gilbert, L. R., Keeling, C., Stacpoole, P. W., and Gregory, J. F., 3rd. (2009) Production of 1-carbon units from glycine is extensive in healthy men and women. *J. Nutr.* **139,** 666–671
39. Pike, S. T., Rajendra, R., Artzt, K., and Appling, D. R. (2010) Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitocndrial one-carbon units into the methyl cycle in embryos. *J. Biol. Chem.* **285,** 4612–4620
40. Kastanos, E. K., Woldman, Y. Y., and Appling, D. R. (1997) Role of mitochondrial and cytoplasmic serine hydroxymethyltransferase isozymes in *de novo* purine synthesis in *Saccharomyces cerevisiae*. *Biochemistry* **36,** 14956–14964
41. Li, Y., Holmes, W. B., Appling, D. R., and RajBhandary, U. L. (2000) Initiation of protein synthesis in mitochondria without formylation of the initiator tRNA. *J. Bacteriol.* **182,** 2886–2892