Retrovirally Mediated Complementation of the glyB Phenotype

CLONING OF A HUMAN GENE ENCODING THE CARRIER FOR ENTRY OF FOLATES INTO MITOCHONDRIA*

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The transduction of a human placental cDNA retroviral library into glyB cells, a Chinese hamster ovary K1 subline that is deficient in the transport of folates into mitochondria, resulted in the complementation of glycine auxotrophy of these cells. A 2.6-kilobase pair cDNA insert flanked by retroviral sequences had integrated into genomic DNA in rescued cells. An open reading frame in this cDNA encoded a 35-kDa protein homologous to several inner mitochondrial wall transporters for intermediate metabolites. The subcloned cDNA complemented the glycine auxotrophy of glyB cells and reinstated folate accumulation in the mitochondria of transfected cells. The human origin, chromosomal location, and intron-exon organization of the isolated mitochondrial folate transporter gene were deduced from the expressed sequence tag database and human genome project data.

In mammalian cells, the processes of folate metabolism are distributed between the cytosolic and mitochondrial compartments (1). Mitochondrial folates amount to about 35% of the total cellular pool (2, 3) and are used as cofactors for a mitochondrial serine hydroxymethyltransferase (SHMT)1 by the glycine cleavage system and for the synthesis of the formylmethionine initiator of mitochondrial protein synthesis.

The transport of folates through the plasma membrane into the cytosol has been extensively studied (4–6), and two of the transport systems have been cloned (7, 8). In contrast, the mechanism of the transfer of folates into mitochondria, presumably from the cytosol, is largely unknown as is the release of folates back into the cytosol from the mitochondria. Once the folate monoglutamates, the form of folate found in the circulation, enter the mammalian cells they are quickly metabolized to poly-γ-glutamate derivatives by cytosolic folylpoly-γ-glutamate synthetase (FPGS), a process needed to promote the retention of folate cofactors in cells (9). FPGS is also present in the mitochondrial compartment of mammalian cells (10) translated from transcripts from the FPGS gene, which add a mitochondrial leader sequence to the coding region of the protein found in the cytosol (11, 12). Two studies (13, 14) have demonstrated the penetration into isolated mitochondria by folates in a process that was saturable and temperature-dependent. These studies would support the existence of a transporter responsible for the entry of folates into the mitochondria as does the fact that cells that either lack mitochondrial FPGS (11) or are incapable of accumulation of mitochondrial folates (15) are glycine auxotrophs.

Early studies by Puck and coworkers (16, 17) selected somatic cells that were auxotrophic for glycine and demonstrated that these cells fell into four complementation groups named glyA, glyB, glyC, and glyD. glyA was found to be attributed to a deficiency in mitochondrial SHMT (17, 18). The glyC and glyD mutations have not to our knowledge been assigned to any functions. glyB cells had normal cytosolic folate metabolism and enzymes and had the same mitochondrial SHMT and FPGS as CHO cells, but they lacked mitochondrial folates (15). The most likely candidate for the function missing in glyB cells was a transport protein that facilitated the entry of folates into the mitochondria.

We have transferred a library of human cDNAs in a retroviral vector into glyB cells and isolated a transduced cell line that was no longer auxotrophic for glycine. These cells contained a human cDNA that, when rescued by PCR and recloned into a mammalian expression plasmid, complemented the auxotrophy of glyB cells at high frequency and also reinstated folate entry into the mitochondria. We conclude that we have isolated the human gene encoding the inner membrane protein that is responsible for the entry of folates into the mitochondria.

MATERIALS AND METHODS

Cell Culture—Cells were grown at 37 °C in 5% CO2. The glyB cell line, which was derived from CHO-K1 cells (16, 17), was a generous gift from Dr. Lawrence Chasin (Columbia University). HEK293gp-packaging cells (a gift from Dr. Oliver Bogler of Medical College of Virginia) were grown in Dulbecco’s modified Eagle’s medium, glyB and CHO-K1 were grown in MEM, and CEM was grown in RPMI 1640 medium, all containing 10% fetal calf serum. For virus production, HEK293gp cells were plated at 5 × 10⁶ cells on each of twelve 100-mm dishes. 48 h later, 15 μg of plasmid library DNA, 15 μg of pSVSG plasmid (a gift from Dr. Oliver Bogler), which allows pseudotyping of the viral envelope proteins (19, 20), and 55 μg of LipofectAMINE-2000 (Life Technologies, Inc.) were added to each dish of Dulbecco’s modified Eagle’s medium. After 20 min at room temperature, the medium was replaced with 25 μl chloroquine in Dulbecco’s modified Eagle’s medium, and 1.5 ml of the lipid-DNA complex was added to each plate. Media were changed to complete Dulbecco’s modified Eagle’s medium after 5 h at 37 °C; the supernatant containing the collection of viruses from the library was harvested after 36 and 72 h and filtered. Transduction efficiency and viral titer were determined by monitoring fluorescent cells on control plates transduced with a virus expressing the enhanced green fluores-

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cent protein (CLONTECH). gylB cells (2 × 10⁶) were suspended in 9 ml of MEM and 7 ml of viral supernatant and plated on each of ten 150-mm dishes; after 5 h the medium was replaced with complete MEM. After 48 h at 37 °C, the selection in glycine-free MEM containing dialyzed serum was applied. After 10–14 days, colonies were expanded in glycine-free medium.

**PCR Analysis and Rescue of Transductants**—The presence of a 200-bp pair fragment of the retroviral ψ-packing signal in genomic DNA was verified by PCR (forward primer (5′A), 5′-CCGATGGACACCCA-GACGAC-3′ and reverse primer (3′A), 5′-CTAGAGAAGGAGGTGGG-GCTGG-3′). The PCR conditions were 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s for 35 cycles. Subsequent PCR amplification of the virally encoded insert in genomic DNA from transductant cell line 10–15 used primers that spanned the multiple cloning site of the retroviral vector (forward primer (5′B), 5′-CCGCCCTGCTTCCTC-CCTTCT-3′ and reverse primer (3′B), 5′-CTAGAGTGGGCTGTTT-GTCATT-3′). The product was only observed with the following conditions: 200 ng 5′ and 3′ primers, 1× PCR enhancer (Life Technologies, Inc.), 1.8 mM MgCl₂, 100 μM dNTPs, 250 ng of genomic DNA, 1× Elongase (Life Technologies, Inc.).

**Sequence Homology with Mitochondrial Carrier Proteins**—Pioneering studies (18) established four complementation groups among CHO mutants that were auxotrophic for glycine. One of these complementation groups, defined by the mutation responsible for the glycine auxotrophy of gylB Chinese hamster cells, had a defect in its ability to accumulate folates in mitochondria (15), a process required for the activity of mammalian mitochondrial SHMT (23). We used the gylB cell line as a recipient for transduction of a human cDNA library constructed in the pLIB retroviral vector (CLONTECH). After transduction of 2 × 10⁷ gylB cells with this human placental cDNA library, an isolated colony of rapidly and progressively growing glycine prototrophic cells was cloned. Attempts to isolate revertants from gylB cells had been unsuccessful with similar levels of cells. Likewise, the control plates transduced with a retrovirus encoding green fluorescent protein did not yield colonies in the absence of glycine. The gylB transductant was expanded, and the presence of a DNA sequence of retroviral origin was sought by PCR against genomic DNA. The ψ packaging signal between the viral long terminal repeats flanking the cloning site of this vector was easily detected in DNA from the designated cell line 10–15 but not in control gylB cells (Fig. 1). Further PCR using primers, designed against the upstream flanking ψ sequence and the downstream multiple cloning site cassette, indicated the presence of a 2.6-kbp DNA sequence (Fig. 1) of human origin (see below).

**Sequence Homology with Mitochondrial Carrier Proteins**—The integrated cDNA flanked by viral sequences, which was...
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The predicted amino acid sequence of the cDNA rescued by PCR from cell line 10–15. The predicted transmembrane domains (TM) are indicated in roman numerals. In this figure, the codon number (abscissa) is plotted against the hydrophobicity (positive values) or hydrophilicity (negative values) of a sliding window of nine. To address this possibility, 5’-rapid amplification of cDNA ends (RACE) (29) was applied to cDNA prepared from CEM cell RNA. As many as 54 additional codons of upstream sequence were detected in these RACE products, but a translationally start codon was not found in any frame. Overall, the primary sequence of the protein encoded by this cDNA, which had a molecular mass of 35,387 daltons, suggested that it was an inner mitochondrial membrane transporter that did not require ATP for function.

A GenBankTM search revealed a similarity between the protein encoded by the open reading frame we now report, which we named hMFT, and the sequence of several carriers involved in the transport of metabolic intermediates across the inner mitochondrial membrane. The highest homologies were with the yeast flx1 mitochondrial carrier protein for flavin nucleotides (GenBankTM accession number NP_012132.1) (Fig. 3), carrier proteins involved in ATP/ADP exchange in rodent and human mitochondria (GenBankTM accession numbers P12236, P51881, and Q09073) (Fig. 3), the yeast mitochondrial oxalacetyl-transport carrier (GenBankTM accession number NP_012802.1), and the mitochondrial uncoupling protein from various mammalian species (e.g. GenBankTM accession numbers O97649, BAA28832.1, and BAA90458.1) (see “Discussion”). When the sequence of this 2.6-kbp cDNA was used to search the expressed sequence tag (dbEST) database, several partial sequences of human origin identical to the portions of our clone were identified (Fig. 4A); the dbEST sequences stemmed from libraries constructed from several different tissue types (see legend to Fig. 4) indicating a broad pattern of tissue distribution of the corresponding mRNA. When a 1.1-kbp EcoRI fragment of this cDNA was used to probe poly(A)+ RNA from CEM human leukemia cells on Northern blots, a 1.5-kbp mRNA species was detected, as well as diffuse hybridization between 2.4 and 2.6 kb (Fig. 4B). Likewise, the matching dbEST entries were initiated either at the 3’-end of the cloned sequence or at 1500 bases into the sequence (Fig. 4A) corresponding to the pattern seen in the Northern blot analysis of expressed mRNA transcripts. Neither glyB cell nor CHO cell poly(A)+ RNA hybridized well with this probe. Hence, the library transduction experiment appears to have identified a full-length functional mRNA sequence expressed in human cells.

Human Genome Project Entries Corresponding to This cDNA—A search of the human genome project revealed that the gene corresponding to the hmfT sequence was found on human chromosome 8 on contig RP11-1CS (GenBankTM accession number AC0112213) corresponding to position 8q21.2. The available chromosome 8 sequence allowed an assignment of the corresponding mRNA. When a 1.1-kbp EcoRI fragment of this cDNA was used to probe poly(A)+ RNA from CEM human leukemia cells on Northern blots, a 1.5-kbp mRNA species was detected, as well as diffuse hybridization between 2.4 and 2.6 kb (Fig. 4B). Likewise, the matching dbEST entries were initiated either at the 3’-end of the cloned sequence or at 1500 bases into the sequence (Fig. 4A) corresponding to the pattern seen in the Northern blot analysis of expressed mRNA transcripts. Neither glyB cell nor CHO cell poly(A)+ RNA hybridized well with this probe. Hence, the library transduction experiment appears to have identified a full-length functional mRNA sequence expressed in human cells.

Functional Complementation of the glyB Defect by hmfT cDNA—The 2.6-kbp hmfT cDNA was recloned into the mammalian expression vector pcDNA3 and transfected into glyB cells. Transfectants were screened for neomycin resistance and glycine phototrophy. About 30% of the transfectants, which expressed G418 resistance, demonstrated phototrophy for glycine (Fig. 5). In contrast, no colonies were found on the minus glycine plates transfected with the pcDNA3 vector alone. The retroviral transducant cell line 10–15, in which the glyB phenotype was rescued, also had reacquired the ability to translocate folates into mitochondria as did the cell lines cloned from isolated colonies on plates of glyB cells transfected with the hmfT cDNA in pcDNA3 (Table I). Hence, the small protein

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2 The sequence has been submitted to GenBankTM and can be retrieved under accession number AF283645.
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encoded by the single open reading frame in the hmfT cDNA complemented the mitochondrial transport function that was deficient in glyB cells and simultaneously relieved the prototrophy of these mutants.

**DISCUSSION**

We hereby report the isolation of a novel gene encoding a protein that facilitates the translocation of folates from the cytosol into the mitochondrial matrix of mammalian cells. The cDNA encoding this protein was isolated from a human library by complementation of a mutant cell line, which lacked this function (15). The cDNA complemented both the cellular phenotype, converting transfectants to prototrophy for glycine (Fig. 4), and the biochemical deficiency, allowing folate transport into mitochondria in transfectants (Table I). This finding appears to rule out complementation of the auxotrophy by an indirect effect of the expressed gene. Overall, this manuscript describes the first human protein identified to transport folates and the residues identical to consensus are shaded in black.

![Sequence alignment of hMFt with the human mitochondrial ATP/ADP exchanger (hAAC), the uncharacterized yeast mitochondrial protein (yi1006w), and the yeast inner mitochondrial membrane flavin transporter (fX1). The boxed peptides represent the energy signal motifs. Sequences were aligned using the Clustal V alignment routine (49) of the DNA* program. The initial 28–60 codons, which showed low homology, are not shown. Dashes indicate gaps in the alignment, and the residues identical to consensus are shaded in black.](image)

The several previously reported inner mitochondrial membrane transporters (28–34 kDa) (24, 25, 33–37) and has a predicted primary structure suggestive of six TM domains. Inner mitochondrial membrane transporters can be divided into three repeated segments, each of about 100 amino acids in length. The repeats consist of two TM domains and a mitochondrial ES motif, PX(D/E)X(I/V/A/T)(R/K)XX(L/I/V/M)/XX(X/D/E)XX(X/L/R/H)(L/I/V/M/A/T)(Q/G/A/I/V/M) (24, 26, 42), which is found immediately after TM domains 1, 3, and 5. These signals are thought to be required for mitochondrial targeting, translocation across the outer mitochondrial membrane, and insertion into the inner membrane (43, 44). The hMFt carrier contains three repeat segments, each containing a consensus ES motif located precisely after TM domains 1, 3, and 5 (Fig. 2). Interestingly, the first ES motif located after TM1 is a perfect match to the consensus, whereas the second and third motifs do not have an Asp or Glu at position 3. The second motif has a Trp at position 3, and the third motif has a Gln at position 3; these same deviations from the overall consensus are seen for the flx-related family members (Fig. 3).

Given that the function of the second and third ES signals is thought to involve binding to inner membrane proteins necessary for tracking and insertion into the inner mitochondrial membrane (44), this structural difference appears unique to the flx-related subfamily. A recent comparison of sequence homologies among 200 recognizable mitochondrial carrier open reading frames (42) identified a small group of proteins, which have PIW or PLW in the second energy signature motif, hMFt clearly belongs to this subgroup. Alignment of these repeats in hMFt with other members of the PIW-PLW subfamily revealed regions of high homology and several residues that are absolutely conserved among the family members (Fig. 3). Thus, in addition to the PX(D/E)XX(I/V/A/T)(R/K)XX energy signature immediately after TM1, 3, and 5, there are two other recognizable conserved regions in this limited series: an ES-related weaker consensus of Y(D/E)XX(I/K/R) is located after TM2, 4, and 6, and a (D/E)XX(R/K)G-(L/F)(Y/K/R)/G motif is located immediately before TM2.
4, and 6. These homologies help the assignment of TM domains within the sequence and presumably involve interactions that are essential to transporter membrane positioning and function, such as the charge interactions previously described (42).

Open reading frames closely related to hMFT were easily identified by computer searches against those genomes sequenced to date. A systematic search of the yeast database (42) identified 35 inner mitochondrial membrane transporters based on homology to known family members of which the function of eight can currently be assigned. BLAST searches of the yeast genome revealed several proteins homologous to hMFT. The most closely related yeast putative transporters were YEL006w and YIL006w (Fig. 3), which were each 27% identical to hMFT at the amino acid level. However, when we cloned these genes from yeast DNA by PCR and transfected them individually and together into glyB cells in the pcDNA3 vector, they were unable to support the growth of glyB cells under selective conditions (data not shown). A BLAST search performed against the Caenorhabditis elegans and Drosophila genomes using the hMFT protein sequence as probe, identified at least 25 related members in each genome based on homology in the placement of ES motifs in a tripartite protein and the position of critical amino acids.

It is interesting to note that glyB cells do not accumulate folates in the cytosol to the same extent as either the wild type CHO-K1 cells from which they were derived or the CHO-de-

![Fig. 4. Transcripts from and intron-exon structure of the hmf gene. A.](image)

**FIG. 5.** Complementation of the glycine auxotrophy of glyB cells by transfection of the hmf cDNA. After transfection with either pcDNA3 or the hMFT cDNA cloned into the pcDNA3 vector, plates were cultured either in medium containing G418 (-Glycine) or glycine plus G418 (+Glycine), and colonies were fixed and stained after 14 days. The plates shown are representative of triplicate dishes from each of the two experiments.
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The indicated cell lines were grown for 48 h in MEM containing 0.3 μCi/ml purified [3H]folic acid. A fraction of the cells were harvested and washed, and total radioactive folates were determined by liquid scintillation counting ("Total"). The remaining cells were homogenized, a "Nuclear and unbroken cell" fraction was separated, and the supernatant fraction was further fractionated into "Mitochondria" and "Cytosol" components as described under "Materials and Methods." Three cultures were used for each cell line in each of two experiments, and 15–30 million cells were in the cultures harvested for analysis.

| Cell line          | Description          | Nuclear + unbroken cells | Cytosol    | Mitochondria | Total      |
|--------------------|----------------------|--------------------------|------------|--------------|------------|
| CHO-K1             | Wild type            | 0.75 ± 0.29              | 9.1 ± 0.57 (100%) | 3.29 ± 0.77 (100%) | 15.2 ± 0.56 (100%) |
| glyB                | Mutant               | 1.35 ± 0.24              | 9.5 ± 0.45 (50%)  | 0.06 ± 0.01 (1.8%)  | 5.5 ± 0.52 (36%)   |
| 10–15               | Viral transfectant   | 2.26 ± 0.30              | 8.9 ± 0.46 (98%)  | 1.59 ± 0.20 (48%)  | 12.2 ± 0.12 (80%)  |
| glyB/hmft-01        | cloned hMFT transfectant | 1.54 ± 0.13          | 8.9 ± 0.46 (97%)  | 1.42 ± 0.16 (43%)  | 12.1 ± 0.08 (80%)  |

*The radioactivity found in this fraction amounted to 10 cpn/10⁶ cells above background.

rived viral transductant or plasmid transfectants with the hmft gene (Table I). This observation would appear to support the concept that folate polyglutamates made in the mitochondria can support folate metabolism in the cytosol of mammalian cells apparently by direct transfer of folate polyglutamates that are intact from the mitochondria. This transfer had been hypothesized (45) based on the fact that the naturally occurring mitochondrial folate polyglutamates use this transporter for efflux to the cytosol, and the cytosolic isoform of FPGS; these transfectants have intact cytosolic folate metabolism but are unable to accumulate folates in the mitochondria and are simple glycine auxotrophs (11, 12). Apparently, exogenous glycine can be delivered to the mitochondria, presumably through the cytosol, and can easily penetrate the outer and inner mitochondrial membranes. Hence, the glycine auxotrophy of glyB cells indicates that cytosolic SHMT cannot supply glycine for mitochondrial metabolism at any appreciable concentration. This finding is in accordance with the current thought that cytosolic SHMT is kinetically controlled and funnels serine to glycine for the production of 5,10-methylenetetrahydrofolate for cytosolic 1-carbon metabolism (47); however, other reactions, e.g. de novo purine synthesis and cytosolic protein synthesis, must consume cytosolic glycine as soon as it is produced. It should be noted that the experiments of Taylor and Hanna (15) showed that the glycine auxotrophy of the glyB cells can be reversed by high concentrations of 5-formyltetrahydrofolate, a fact that has never been explained. Thus, the operation of mitochondrial folate metabolism and the related interconversions of glycine and serine have yet to be well understood despite decades of experimental work.

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