Disruption of the \textit{Mthfd1} Gene Reveals a Monofunctional 10-Formyltetrahydrofolate Synthetase in Mammalian Mitochondria*

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The \textit{Mthfd1} gene encoding the cytoplasmic methyleneturahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase enzyme (DCS) was inactivated in embryonic stem cells. The null embryonic stem cells were used to generate spontaneously immortalized fibroblast cell lines that exhibited the expected auxotrophy. Elimination of these cytoplasmic activities allowed for the accurate assessment of similar activities encoded by other genes in these cells. A low level of 10-formyltetrahydrofolate synthetase was detected and was shown to be localized to mitochondria. However, NADP-dependent methenyltetrahydrofolate dehydrogenase activity was not detected. Northern blot analysis suggests that a recently identified mitochondrial DCS (Prasanan, P., Pike, S., Peng, K., Shane, B., and Appling, D. R. (2003) \textit{J. Biol. Chem.} 278, 43178–43187) is responsible for the synthetase activity. The lack of NADP-dependent dehydrogenase activity suggests that this RNA may encode a monofunctional synthetase. Moreover, examination of the primary structure of this novel protein revealed mutations in key residues required for dehydrogenase and cyclohydrolase activities. This monofunctional synthetase completes the pathway for the production of formate from formyltetrahydrofolate in the mitochondria in our model of mammalian one-carbon folate metabolism in embryonic and transformed cells.

Folate is a B vitamin that plays important roles in cellular growth and division. The one-carbon substituted forms of tetrahydrofolate (THF)\(^1\) are involved in the \textit{de novo} synthesis of purines and thymidylate and support cellular methylation reactions through the regeneration of methionine from homocysteine. In eukaryotes folate metabolism is separated into two compartments, the cytoplasm and the mitochondria. Some enzymes, such as serine hydroxymethyltransferase, are found in both compartments, usually encoded by separate nuclear genes that encode enzymes involved in folate metabolism. The cytoplasmic DCS has been identified and cloned in several organisms (2, 6–8). The genes encode a single polypeptide possessing the three DCS activities. The 100-kDa monomer dimerizes to form the active enzyme. However, until recently the only mitochondrial homologue of \textit{Saccharomyces cerevisiae} mitochondrial DCS to be identified in mammals was the NAD-dependent methyleneTHF dehydrogenase-methenylTHF cyclohydrolase (NMDMC) (9). Metabolic studies of transformed fibroblasts derived from murine NMDMC knockouts revealed that they are glycine auxotrophs (10). The glycine auxotrophy is most likely because of the formation of a type of folate trap; the available THF in the mitochondria is trapped as methyleneTHF. Moreover, the glycine auxotrophy indicates that NMDMC is the only mitochondrial methyleneTHF dehydrogenase expressed in transformed cells. These studies also supported the concept that mammalian mitochondria generate formate to supply the cytoplasmic DCSs with one-carbon units during periods of rapid growth, such as embryogenesis. It was suggested that a yet to be identified 10-formylTHF hydrolase or a monofunctional 10-formylTHF synthetase might provide the missing link between the 10-formylTHF produced by NMDMC and the formate released to the cytoplasm.

Barlowe and Appling (11) reported the detection of all three of the DCS activities in purified adult rat liver mitochondria preparations. However, because NMDMC null cell lines appear not to express another mitochondrial methyleneTHF dehydrogenase, it is unlikely that a trifunctional DCS is expressed in the mitochondria of transformed and ES cell lines. The detection of the DCS activities in mitochondrial preparations is complicated by contamination by the high activities of the cytosolic protein. To determine whether either of the dehydrogenase or synthetase activities exist in the mitochondria of immortalized cell lines we undertook to inactivate the cytoplasmic DCS gene.

Here we report the production of DCS null cell lines that we used to detect mitochondrial synthetase activity. We propose that the detected activity is due to a recently identified mitochondrial DCS protein in which the dehydrogenase and cyclohydrolase activities have been inactivated, resulting in a monofunctional synthetase.

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† The abbreviations used are: THF, tetrahydrofolate; DCS, methyleneTHF dehydrogenase-methenylTHF cyclohydrolase-formylTHF synthetase; NMDMC, mitochondrial NAD-dependent methyleneTHF dehydrogenase-methenylTHF cyclohydrolase; mtDCS, mitochondrial methyleneTHF dehydrogenase-methenylTHF cyclohydrolase-formylTHF synthetase; ES, embryonic stem; DC, methyleneTHF dehydrogenase-methenylTHF cyclohydrolase.
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**FIG. 1.** Schematic diagram of the homologous recombination strategy used to knock out DCS. A, shows the region of the DCS gene that was targeted. PH 1.65 and PH 1.5 are the probes used for genotyping the ES cell lines. B, shows the targeting vector used in this experiment. The white box represents the mouse DCS cDNA, from which the first 106 nucleotides of the coding sequence were removed and to which the K386E mutation has been added. A poly(A) tail signal/intron cassette was attached to the end of the cDNA sequence. C, shows the targeted gene after homologous recombination. Letters N, E, and H, representNeo, EcoRI, and HindIII restriction sites.

### EXPERIMENTAL PROCEDURES

**Knock-out of DCS in Embryonic Stem Cells**—Thegene and cDNA for DCS were isolated as described previously (8) (GenBank™ accession numbers AF364580 to AF364592).2 The targeting vector used for homologous recombination is shown in Fig. 1. The vector is designed to insert the cDNA of DCS into the first exon of the nuclear gene; a rabbit β-globin intron 2SV40 polyadenylation signal cassette obtained from the pSG5 vector (Stratagene) was attached to the cDNA to provide processing signals. The 5′ arm is a 1.5-kb HindIII Neo fragment containing exon 1 up to the start codon, and the 3′ arm is a 2.3-kb SacI fragment containing exon 2. The arms and cDNA were inserted into the pMC1neoA vector (Stratagene) to which a herpes simplex virus thymidine kinase cassette had been added. To knock out the gene encoding DCS with this vector the first 106 nucleotides of the coding sequence were removed, altering the reading frame of the remaining sequence. The cDNA also contained a K386E mutation that abolishes synthetase function (data not shown). Generation of heterozygous and homozygous embryonic stem cells was done as described previously (12). Clones were screened by Southern blot (12); HindIII-digested genomic DNA was probed with a 1.65-kb HindIII PstI fragment containing exon 1 and a 1.5-kb PstI HindIII fragment of intron 2 as shown in Fig. 2.

**Generation of Null Mutant Fibroblasts**—The homozygous null DCS (−/−) ES cell lines were injected into blastocysts as described previously (12). The E11.5 chimeric embryos were used to generate primary embryonic fibroblast cell lines as described previously (10). Disrupted embryos were incubated in Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum supplemented with 1× non-essential amino acids, 1× glutamine, 1× penicillin/streptomycin (all Invitrogen), 30 μM thymidine, and 30 μM hypoxanthine (both Sigma) with 0.3 mg/ml G418 (Genetecin, Invitrogen) to select for null mutant cells. All cell lines described in this work were routinely cultured in this medium, unless otherwise noted. The embryonic fibroblasts were spontaneously immortalized by continuous passage in culture.

**Purine Auxotrophy of Null Mutant Fibroblasts**—Cells lacking the cytoplasmic DCS cannot produce the 10-formylTHF required for the synthesis of purines from either formate or methylenTHF. To functionally test the knock-out, the growth of the cells with and without supplementation by the purine hypoxanthine was evaluated. SF6 (+/+), IF74 (-/−), and SF66 (DCS (-/−)) cell lines grown to confluence in a T175 flask using the Qiagen RNeasy kit. 20 μg of each RNA sample was electrophoresed on a 1% formaldehyde gel in quadruplicate and transferred to a Hybond-N membrane (Amersham Biosciences) by vacuum transfer in 10× SSC for 2 h. The RNA was cross-linked to the membrane under ultraviolet light for 5 min. Each subset of membrane was probed with one of: full-length NMDMC cDNA (2-kb, GenBank™ accession number NM_006636), full-length DCS cDNA (3-kb, GenBank™ accession number AF364580), a 1.3-kb Neo fragment of the clone 6311761 (GenBank™ accession number NM_008638), which codes for the 3′ end of the mDCS, and a 1.7-kb PstI fragment of the clone 5374413 (GenBank™ accession number BC030437), which codes for the 3′ end of the mtDCS. Clones 6311761 and 5374413 were obtained from Open Biosystems. Each probe was labeled using the Rediprime II random prime labeling system (Amerham Biosciences) Hybridization, washing, and exposure of the blots were performed according to standard protocols.

**Multiple Sequence Alignment**—A multiple sequence alignment was constructed using ClustalW 1.82 at the European Bioinformatics Institute website (www.ebi.ac.uk/clustalw) (16) using standard defaults and the entire sequence of each protein. The following protein sequences were used in the alignment: human mtDCS, human NMDMC, human DCS, mouse mtDCS, mouse NMDMC, mouse DCS, Drosophila NMDMC, Drosophila DCS, S. cerevisiae mitochondrial DCS, S. cerevisiae cytoplasmic DCS, and Escherichia coli DCS (GenBank™ accession numbers: AY374130, NM_006636, NM_005956, NM_172308, NM_008638, AF364579, L07958, AF082097, J03724, M12875, M74789).3

RESULTS

**DCS Null Fibroblasts**—DCS heterozygous and null ES cell lines were established as described, and their genotypes were verified by Southern blot analysis (Fig. 2). Null ES cells were injected into blastocysts, and DCS null fibroblast cell lines were established from chimera using high G418 medium to select against wild type cells. Fig. 3 shows that the DCS null cell lines are purine auxotrophs; they have no means of producing the 10-formylTHF required for purine synthesis in the cytoplasm from either serine or formate and are therefore dependent on

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2 GenBank™ accession numbers: AF364580, AF364581, AF364582, AF364583, AF364584, AF364585, AF364586, AF364587, AF364588, AF364589, AF364590, AF364591, AF364592.

3 GenBank™ accession numbers: AY374130, NM_006636, NM_005956, NM_172308, NM_008638, AF364579, L07958, AF082097, J03724, M12875, M74789.
exogenous sources of purines. This phenotype confirms the knock-out of the cytoplasmic methylene\(\text{THF}\) dehydrogenase and 10-formyl\(\text{THF}\) synthetase activities.

**Enzyme Assays**—The dehydrogenase and synthetase activities of wild type and DCS null mutant cell lines are shown in Fig. 4 and Table I. These results show low but significant synthetase activity in cell extracts in the absence of the usually confounding cytoplasmic DCS activities. The synthetase activity of mtDCS is about 6% of the total synthetase activity in the wild type cells.

Although the synthetase activity can be detected in whole cell extracts, assays of mitochondrial extracts were required to confirm the subcellular localization and to allow the assay of more concentrated protein samples to confirm the lack of NADP-dependent dehydrogenase activity. The results of these assays are shown in Fig. 5 and Table I. The NAD-dependent dehydrogenase activity serves as a marker for the enrichment of mitochondriomal proteins. Although there is significant synthetase activity, confirming the mitochondrial location of mtDCS, there is no detectable NADP-dependent dehydrogenase activity. To confirm that the NAD-dependent dehydrogenase activity observed is NMDMC, the assay was repeated without added magnesium and with EDTA added to the assay mix to chelate magnesium present in the protein sample. In the absence of magnesium ions, which are uniquely required by NMDMC for dehydrogenase activity (17), no NAD-dependent dehydrogenase activity is observed.

**Northern Analysis**—Two separate laboratories have recently published the discovery of mitochondrial methylene\(\text{THF}\) dehydrogenase-cyclohydrolase-synthetase cDNAs in mouse and human (18, 19). The encoded protein conforms to known DCS activity, but no activity was detected.4

Despite the relatively high activity of the cytoplasmic DCS, attempting to detect a mitochondrial synthetase from whole cell extracts, even if overexpressed, presents a technical challenge. Even when subcellularly fractionated, very small amounts of contamination from the cytoplasmic enzyme could have a significant effect on the results of enzyme assays. Therefore, a knock-out of DCS provides the best opportunity to examine mitochondria for additional dehydrogenase and synthetase activities.

In the DCS null cells 10-formyl\(\text{THF}\) synthetase activity is observed both in whole cell and mitochondria extracts. Even when the sample is concentrated for mitochondrial proteins, as in Fig. 5, there is no detectable dehydrogenase activity that is not attributable to NMDMC. These assays can detect NADP-dependent dehydrogenase activity down to 1 milliunit/ml, which would permit the detection of as little as 1% of the observed NAD-dependent dehydrogenase activity. The mtDCS described by Prasannan et al. (18) and Sugiuera et al. (19) may provide the identity of the observed synthetase. Northern blots of total RNA extracted from wild type, NMDMC null, and DCS null transformed cell lines show that the mtDCS is expressed in these cell lines (Fig. 6). Recent work by Di Pietro et al. (12) and Patel et al. (10) has shown that there is only one methylene\(\text{THF}\) dehydrogenase expressed in embryonic and transformed cells. This observation is in agreement with the failure of both groups to detect dehydrogenase activity both in yeast

4 N. R. Mejia, unpublished observation.
overexpressing the mtDCS cDNA (18) and in purified preparations of the DC domain (19).

To determine whether there is a structural basis for the lack of detectable dehydrogenase activity a multiple sequence alignment of known mtDCS, NMDMC, and DCS proteins was constructed. Fig. 7 shows regions of the lineup of particular interest. In the human DCS, Lys56 is a residue crucial to cyclohydrolase activity (23); mutation to asparagine, as in mt-DCS, will abrogate cyclohydrolase activity. Residues in the positions of human DCS Arg173, Ser174, Gly178, and Ser197 are important in binding the adenine and diphosphate moieties of NAD(P) (24, 25). The amino acid changes in mtDCS observed at these positions introduce oppositely charged, bulky, and hydrophobic side chains into the cofactor binding site. These mutations, both individually and collectively, will compromise NAD(P) binding. In addition, strictly conserved residues identified as interacting with the nicotinamide moiety of NADP (Thr148, Val177, Ile218, Gly276, and Thr279) (24) are not conserved in mt-DCS. Residue Asp208 of the yeast monofunctional NAD-dependent methyleneTHF dehydrogenase, which is involved in NAD binding (26), is also not found in mtDCS. Based on this comparison, mtDCS is most likely a monofunctional synthetase.
These activities are found in separate DC and synthetase proteins. In the mitochondria of embryonic and transformed cells (adapted from Di Pietro et al. (12)). In the cytoplasm these activities are found in a trifunctional methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, and formyltetrahydrofolate synthetase activities, respectively. In the cytoplasm these activities are found in a trifunctional DCS protein. In the mitochondria of embryonic and transformed cells these activities are found in separate DC and synthetase proteins.

In most cases, monofunctional 10-formylTHF synthetases are found to be tetramers (27). The trifunctional proteins, however, are generally dimers that interact such that the synthetase domains of each monomer would be directed away from each other (23, 24), precluding a strong interaction of the synthetase domains. When separated from the DC domain through proteolytic cleavage or protein mutagenesis, the synthetase domain has been found to be unstable (28, 29). Although there are some significant deletions of amino acids within the DC domain, all of the structural elements involved in DC dimerization appear to be intact. Therefore, the non-functional DC domain has been found to be unstable (28, 29). Although there are some significant deletions of amino acids within the DC domain, all of the structural elements involved in DC dimerization appear to be intact. Therefore, the non-functional DC domain may serve to stabilize the synthetase domain by allowing for dimerization.

It is likely that mtDCS, encoding a monofunctional synthetase, provides the missing metabolic reaction required to link the mitochondria and the cytoplasm in the mammalian model of one-carbon folate metabolism in embryonic and transformed cells (Fig. 8). However, firmly establishing the role of mtDCS in this system will await the development of a knock-out cell line. Together with NMDMC, the mitochondrial synthetase provides an example of divergent evolution to optimize the enzyme activities in the cell. The effects of the cofactor substitution to NAD and the embryonic expression pattern of NMDMC, suggest that its role is to supply one-carbon units to the cytoplasm during periods of rapid growth such as embryogenesis and tumorgenesis. The up-regulation of mtDCS in transformed cells (19) fits with the proposed role of NMDMC. The separation of the two genes allows for a more precise control of the expression of these proteins; NMDMC is expressed only during periods of rapid growth, during which the mitochondrial synthetase is up-regulated. Under other conditions, varying amounts of mitochondrial synthetase are expressed independently of NMDMC (18), perhaps to ensure a pool of formylTHF to supply the formylmethionyl-tRNA used in the initiation of mitochondrial protein synthesis.

It is not clear how this model of one-carbon flux in embryonic tissues and tumor cells can be reconciled with the observations of formate generation by adult liver mitochondria (11). The mRNA for NMDMC has been detected at quite low levels in adult liver (30), although NAD-dependent dehydrogenase activity was not detectable by enzyme assay of liver extracts (9). It is possible that extremely low levels of NMDMC expression, in cooperation with mtDCS, are adequate for the generation of formate necessary for one-carbon flux. It is possible that extremely low levels of NMDMC expression, in cooperation with mtDCS, are adequate for the generation of formate necessary for one-carbon flux. It is possible that extremely low levels of NMDMC expression, in cooperation with mtDCS, are adequate for the generation of formate necessary for one-carbon flux.

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